An analysis of polyphenolic blackcurrant (*Ribes nigrum*) extracts for the potential to modulate allergic airway inflammation

A thesis presented in partial fulfilment
of the requirements for the degree of

Master of Science
in
Nutritional Science

at Massey University, Palmerston North,
New Zealand.

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2009

Statement of originality

'I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made in the acknowledgements'.

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Date.....

Abstract

The allergic disease of asthma is characterized by an infiltration of inflammatory cells to the lung, a process co-ordinated by T-helper (TH) cells. The TH_2 cytokine Interleukin (IL)-4 promotes infiltration of eosinophils to sites of inflammation. Eosinophil-selective chemoattractant cytokines (eg. eotaxins) are synthesized by lung epithelial cells. Eotaxin-3 is expressed at high levels in the asthmatic lung, predominantly after IL-4 stimulation. Eotaxin-3 is therefore a marker of inappropriate airway inflammation.

Polyphenolic (PP) compounds found in high concentrations in berries may have beneficial effects in inflammatory conditions. Plant and Food Research produced high-PP extracts of blackcurrant (BC) cultivars that were tested for inflammation modulating effects.

Since high doses of PPs have been shown to cause cell death, we tested two BC cultivars at a range of concentrations in a cell viability (WST-1) assay. While no toxic effects were attributable to the BC extracts (1-50µg/ml), a dose-related trend in cell death was observed and therefore 10µg/ml was chosen for further experiments.

Ten BC cultivars were compared for efficacy by measuring eotaxin-3 production in IL-4 stimulated human lung epithelial (A549) cells *in vitro*. Cells were incubated with BC extracts (10µg/ml) and IL-4 (10ng/ml) for 24 hours. The supernatants were then quantified for eotaxin-3 levels by an enzyme-linked immunosorbent assay (ELISA). All ten BC extracts reduced eotaxin-3 levels after stimulation with IL-4, and six BC extracts were effective by statistically significant levels ($P<0.05$), (BC cultivars -01, -02, -03, -05, -09 & -10). Of those, BC extracts of four cultivars demonstrated a reduction of more than 65% from the IL-4 stimulated control. In addition, a positive trend in inflammation modulation vs. one anthocyanin (ACN) in the BC extracts was shown.

This study has demonstrated the beneficial inflammation modulatory effects of polyphenolic BC extracts, which could be related to cyanidin 3-*O*-rutinoside content. These results may have therapeutic potential for asthma.

Acknowledgements

For their invaluable guidance and encouragement, my thanks to my supervisors, Marlena Kruger and Roger Hurst, and to Birgit Schrage. I am especially grateful to Roger Hurst for the generous use of his time, and for the opportunity to undertake this project in his laboratory at Plant and Food Research. In addition, my thanks to Robyn Wells for her kind assistance with cell culture and laboratory techniques.

For their support, I am indebted to Plant and Food Research, and to the Institute of Food, Nutrition and Human Health, Massey University. Assistance was also provided by the Foundation for Research, Science and Technology, New Zealand government.

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Abbreviations

ACN	Anthocyanins
APC	Antigen-presenting cells
BALF	Bronchoalveolar lavage fluid
BC	Blackcurrant
BSA	Bovine serum albumin
CCR	CC chemokine receptor
CVD	Cardiovascular disease
Cy-glu	Cyanidin 3-*O*-glucoside
Cy-rut	Cyanidin 3-*O*-rutinoside
DMSO	Dimethyl sulfoxide
Dp-glu	Delphinidin 3-*O*-glucoside
Dp-rut	Delphinidin 3-*O*-rutinoside
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked Immunosorbent assay
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible NO synthase
LPS	Lipopolysaccharide
NO	Nitric oxide
$OONO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PP	Polyphenolic
RONS	Reactive oxygen and nitrogen species
RDA	Recommended daily allowance
STAT	Signal transducer activator of transcription
TH	Thymus helper (cell)
TLR	Toll-like receptor
TMB	Tetramethylbenzidine

Chapter 1

Introduction

1.1 Biochemical and physiological requirements of food

Population health targets to reduce chronic disease risks include high intakes of fruit and vegetables as a natural way to increase levels of essential nutrients. (New Zealand Ministry of Health, 2007). Much of the protective effect of fruit has been attributed to phytochemicals (Boyer & Liu, 2004), and of these, a wide range of biological effects including anti-inflammatory properties have been attributed to plant polyphenolics (PPs) (Philpott & Ferguson, 2004). Berries are a rich source of PPs, and this study intends to evaluate the effectiveness of selected Plant and Food Research blackcurrant (*Ribes nigrum*) (BC) cultivars as inflammation modulators.

Asthma is an allergic disease that triggers a cascade of events leading to cellular damage, airway constriction and difficulty in breathing. This response is largely orchestrated by the Thymus helper (TH) cells that recruit inflammatory cells, including eosinophils, to the site of allergen challenge via chemoattraction by inflammatory cytokines (chemokines) (Verstraelen et al., 2008).

By measuring levels of specific chemokines (eg. eotaxin-3) produced by human lung epithelial cells after stimulated TH cell challenge *in vitro*, this study aims to assess selected BC cultivars for their beneficial effects for asthma.

Dietary recommendations focus on vitamins and minerals provided by whole foods, but fruit and vegetables contain many other biologically active phytochemicals, especially polyphenols such as flavonoids. These constituents have a beneficial role in nutrition, but are not considered essential for human health. A wide range of biological effects including antioxidant, anti-mutagenic and anti-inflammatory properties have been attributed to plant polyphenols (Philpott & Ferguson, 2004).

Beneficial effects of fruit and vegetable intake on markers of inappropriate inflammation and oxidative stress have been demonstrated in a cohort of young adults, and provide support for dietary guidelines (Holt et al., 2009). These foods are rich in flavonoids and antioxidants, and population studies have associated fruit and vegetable intake with a lower risk of coronary heart disease (Arts & Hollman, 2005).

The role of nutrients and dietary components in regulating genome structure, expression and stability, and the regulation of metabolic pathways and networks is an emerging field in science. The chemical properties of dietary constituents and their biochemical, metabolic, physiological and epigenetic functions is increasingly becoming the basis for nutrient requirements. Nutrition-based interventions have the potential to improve the quality of life for individuals (Philpott & Ferguson, 2004).

1.1.1 Metabolic burden

Humans are constantly exposed to an array of chemicals that are toxic to the body, including atmospheric pollutants and allergens that are inhaled. In addition, endogenously produced reactive molecules add to this burden. Some of these chemicals may be biologically transformed within cells and tissues into reactive species that can interact with molecules or structures to cause cell and tissue damage.

The metabolic processes of growth and repair of cells involve electron transport systems using oxygen as an electron acceptor. Within cellular organelles, large enzyme complexes, which contain multiple reduction-oxidation (redox) centres, convert electron-motive force into the high energy phosphate bonds of protein complexes. While severe redox imbalance may result in cell death following widespread macromolecule oxidation, more subtle changes play a role in modulating a range of signal transduction pathways (Suzuki, Forman & Sevanian, 1997).

Formation of reactive oxygen and nitrogen species (RONS) occurs as a by-product of normal metabolism, but oxidative burden also increases as a result of many disease processes. Although short-lived, RONS leave a detectable trace of modified products, including oxidised proteins, lipids, carbohydrates, nucleic acid bases and enzymatic markers of oxidative stress. RONS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH) and the peroxynitrite radical ($OONO^-$). Over-production of nitric oxide (NO) in chronic inflammatory conditions leads to the generation of $OONO^-$, which is a

source of oxidative DNA damage, lipid peroxidation, and protein nitration (Nevin & Broadley, 2002).

The immune system is the body's primary defence against invading pathogens, non-self components and cancer cells. Inflammatory processes, including the release of pro-inflammatory cytokines and formation of RONS, are an essential part of these defences (Philpott & Ferguson, 2004). Various steps involved in this defence can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals such as vitamins and flavonoids (Middleton, Kandaswami & Theoharides, 2000).

The co-ordination of signals involved in the inflammation reaction cascade normally involves appropriate anti-inflammatory effects, but excessive production of pro-inflammatory cytokines leads to chronic inflammation.

1.1.2 Immune system signalling

Immune signalling involves communication between many cell types via signal transduction pathways that lead to the induction of gene expression and cell growth. Receptor-mediated events at the plasma cell membrane initiate sequences of intracellular biochemical reactions involving receptor-ligand binding, electron couplers, protein phosphorylation and activation by second messengers (Figure 1.1).

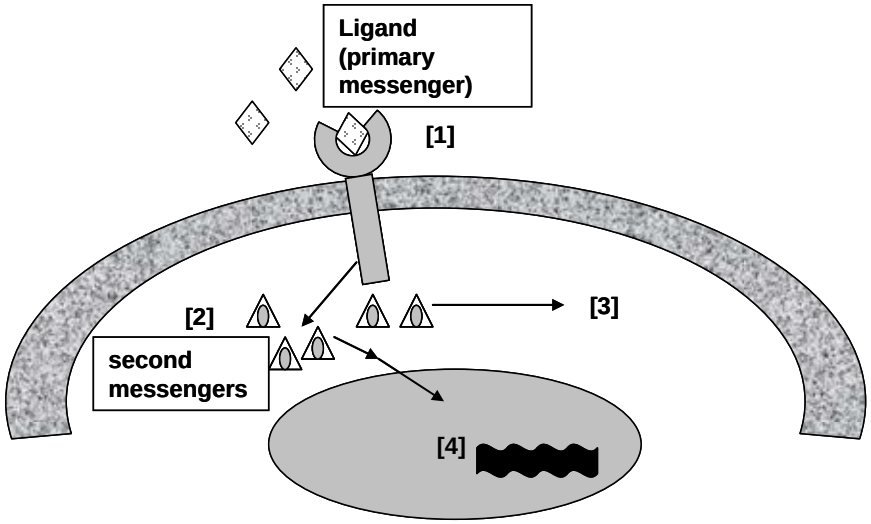


Figure 1.1. Signal transduction processes. Receptor-ligand binding [1] on the outside of the cell transduces a signal via second messengers [2] in the cell cytosol to effect cellular responses [3] and/or changes in gene expression in the nucleus [4]. Modified from Phillips, Murray & Kirk, (2001).

Transcription factors within the cell nucleus may be activated as a result of various enzyme activities at the cytokine receptor complex. Cytokine receptor molecules include kinase and phosphatase enzymes, which contain thiol (sulfhydryl) regulatory domains that are particularly responsive to modification by oxidants, and produce RONS as a result of receptor interactions (Suzuki et al., 1997).

Oxidative stress results from an imbalance between oxidant production and antioxidant defences. Antioxidants within cells, cell membranes and extracellular fluids may be up-regulated and mobilized to neutralise excessive and inappropriate RONS formation (Stephens, Khanolkar & Bain, 2007).

1.1.3 Response to metabolic burden

The human body has developed defences involving detoxification enzymes and antioxidant systems to reduce RONS and maintain the redox state of cells and tissues. But some essential antioxidants must be provided in the diet, as humans lack the ability to synthesize all nutrients required for biochemical processes. The antioxidant status of blood plasma is directly linked to diet and is defined by components such as vitamins and flavonoids (Cemeli, Baumgartner & Anderson, 2009).

Flavonoids fulfil most of the criteria of antioxidants; inhibiting the enzymes responsible for superoxide production, and with low redox potentials that allow them to reduce highly oxidising free radicals. Besides scavenging, they may stabilise RONS by complexing with them, and there is clear evidence that radical scavenging activity depends on the structure of the flavonoids (Cemeli et al., 2009). The redox state of cells modulates the efficiency of receptor activation (Suzuki et al., 1997).

Antioxidants within cells, cell membranes and extracellular fluids may be up-regulated and mobilized to neutralise excessive and inappropriate RONS formation (Stephens et al., 2007), but while dietary micronutrients can enhance genetic transcription of detoxification enzymes, inductive or signalling effects may occur at concentrations much lower than that required for effective antioxidant activity (Virgili & Marino, 2008).

1.1.4 Health benefits of fruit consumption

The rationale of the current New Zealand population health target to improve nutrition is based on a review of epidemiological evidence relating to diet in the 1990s, which found a protective effect of fruit and vegetable intake against cardiovascular disease (CVD) and some common cancers (New Zealand Ministry of Health, 2007). Although much of the protective effect of fruit and vegetables is attributed to phytochemicals (Boyer & Liu, 2004), recommended daily intakes (RDA's) are set for whole foods. A high fruit diet accordingly provides a greater intake of phytochemicals.

A European lifestyle study (n>10,000) aimed to search for chronic disease links to dietary components. Associations were found between apple consumption and asthma in this cohort (Knekt et al., 2002), which may have indicated the protective effect of flavonoids since apples are a significant source of these phytochemicals in the diet. The antioxidant activity of apples was shown to be due to the synergistic combination of phytochemicals, not from vitamin C content (Liu, 2003).

A nutritional (physiologic) dose to improve or maintain optimal health is differentiated from a pharmacologic dose. Antioxidant nutrients in some cases can be toxic. For instance, vitamin C at high doses (500mg/day) may act as a pro-oxidant in the body (Liu, 2003). While phytochemical-rich food may reduce the risk of chronic diseases, high doses of purified polyphenolics may also not be without risk (Mennen, Walker, Bennetau-Pelissero & Scalbert, 2005).

For whole foods, population-based studies have indicated a protective effect of apples on asthma prevalence (Shaheen et al., 2001), and these commonly eaten fruit have also been linked in a preventive capacity to healthy lung function in children (Okoko, Burney, Newson, Potts & Shaheen, 2007).

1.1.5 Flavonoid phytochemicals in fruit

Flavonoids are a major class of phytochemical in fruit and vegetables. More than 6000 of these micronutrients have been described in plants. Flavonoid molecules contain six-carbon rings (phenols) derived from aromatic amino acids. The flavonoid structure is a 2-phenylbenzopyrylium cation which undergoes various modifications to form polyphenolic (PP) compounds. Major properties of flavonoids, such as the ability to complex with proteins and antioxidant free-radical scavenging capacity, are primarily related to the number and accessibility of the phenol moieties (Stevenson & Hurst, 2007). They can be grouped into several classes on the basis of their general chemical structures (Figure 1.2). Higher molecular weight procyanidins (tannins) precipitate proteins and chelate metal ions, whereas anthocyanins are various glycosylated forms of flavonoids.

Plants contain polyphenol oxidase enzymes which polymerise PPs into secondary metabolites (Stevenson & Hurst, 2007). Flavonoids are involved in photosensitization and energy transfer, the actions of plant growth hormones, the control of respiration, photosynthesis, and morphogenesis, as well as defence against infection (Middleton, Kandaswami & Theoharides, 2000).

Plant flavonoids activate bacterial genes involved in nitrogen fixation, which suggests important relationships between particular flavonoids and the activation and expression of mammalian genes (Middleton et al., 2000).

The flavonoids are phenolic compounds, and therefore, act as metal chelators and free radical scavengers due to chain-breaking antioxidant properties. However, both antioxidant and pro-oxidant effects of polyphenols have been described (Scalbert, Johnson & Saltmarsh, 2005).

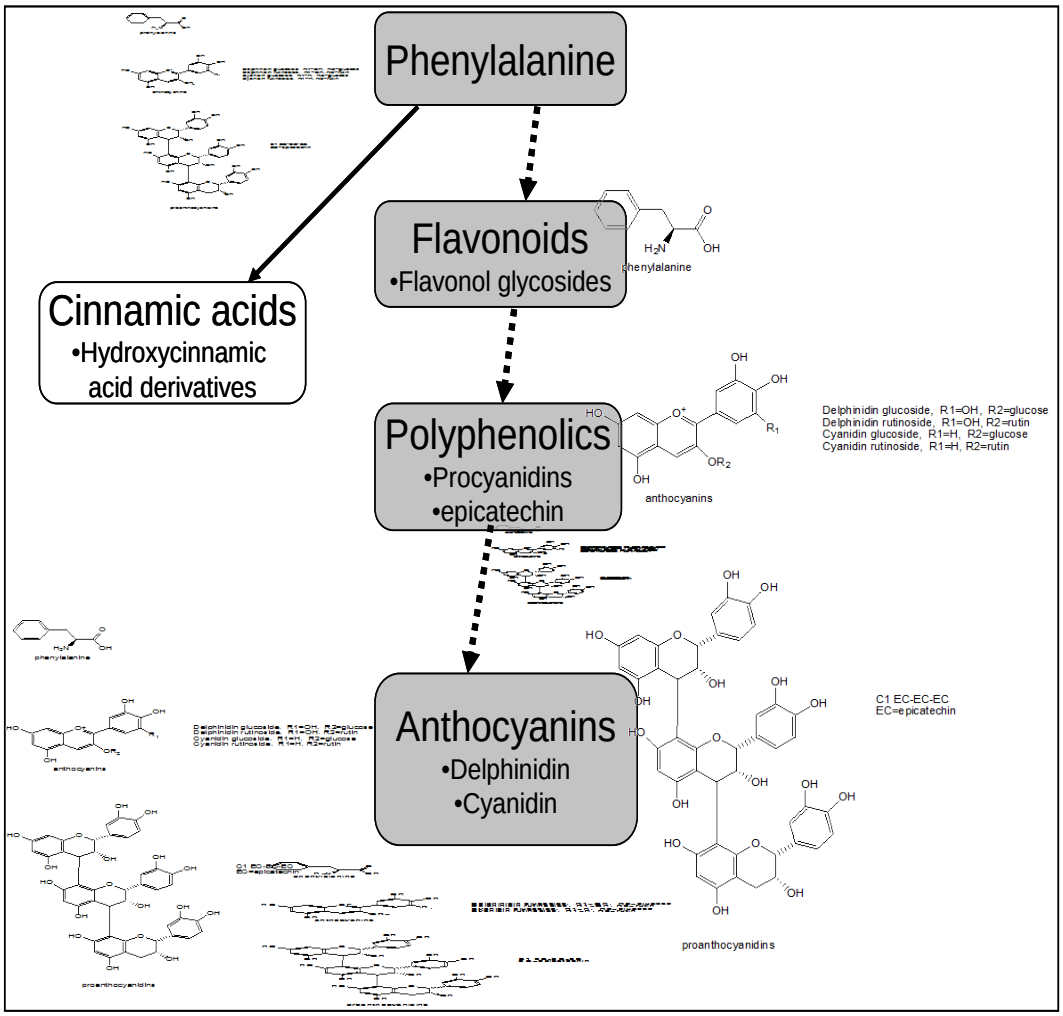


Figure 1.2. Synthesis of polyphenolics. Scheme of polyphenolic structural origins from the aromatic amino acid phenylalanine. Modified from Stevenson & Hurst, (2007).

The elevation of mammalian phase II detoxification and antioxidant enzymes, such as quinone reductase (QR), by phytochemicals is recognized as one of the mechanisms by which fruit and vegetables exert their chemoprotective effects. High doses of grape polyphenols were shown to be effective anti-carcinogenic agents by inducing QR in murine hepatoma cells (Yang & Liu, 2008).

While research indicates that flavonoids have important roles in cancer chemoprevention, biological activities include modulation of cellular pathways in addition to free radical scavenging effects. Degradation of damaged or obsolete proteins within the ubiquitin-proteasome pathway is an important, and highly conserved, cellular maintenance function. Unmethylated flavonoids were demonstrated to inhibit proteasomal activity in cultured human leukaemia cells (Landis-Piwowar, Milacic & Dou, 2008).

1.1.6 Polyphenolic flavonoids and anthocyanins

Polyphenolics (PPs) constitute one of the largest groups of flavonoids, and their signature attachment to phenol ring structures are two or more hydroxyl (OH^-) groups. Anthocyanins (ACNs) are highly reactive water-soluble glycoside pigments derived from flavonoids that confer dark red and blue colours to fruits.

PPs in fruit have a role in regulating oxidative stress in CVD and metabolic diseases of aging by creating a strong antioxidant cellular environment (Weaver et al., 2008). Markers of inflammation such as elevated eosinophil counts often characterize chronic age-related diseases.

Apple juice extracts were demonstrated to reduce oxidative cell damage in human colon cell lines (Schaefer et al., 2006), but high concentrations of purified apple PP extracts caused a re-increase of DNA damage (Schaefer, Baum, Eisenbrand & Janzowski, 2006).

In cell culture, low doses of ACNs and PP fractions of blackcurrants and boysenberries protected the DNA of human leukaemia cells from damage (Ghosh, McGhie, Zhang, Adaim & Skinner, 2006). Both animal trials and cell culture studies confirm an association between PP compounds and a wide variety of effects that may help prevent chronic disease. But *in vivo*, an isolated pure compound may lose or change its bioactivity.

Epigallocatechin gallate (EGCG), a major tea polyphenol with demonstrated health benefits, was administered to mice in physiologically relevant doses over an extended period. Degraded EGCG, rather than the intact molecules, was shown to be effective in inhibiting the cancer-associated enzyme telomerase, in human tumor xenografts on mice (Naasani et al., 2003).

In a human nutrition intervention trial, measurable differences in vascular CVD risk-factors, such as blood pressure and serum lipids, were found after an 8-week dietary intervention with berryfruit (Erlund et al., 2008). Plasma concentrations of PPs were significantly greater than controls, and these effects were attributed to PPs in the berryfruit, not other nutritional markers such as vitamin C or folate.

1.1.7 Bioavailability of polyphenolics

Micronutrients must be effectively absorbed, transported into the circulatory system, and delivered to target tissues in order to become bioavailable. While many flavonoid compounds have been shown to affect signal transduction pathways, the concentrations used in cell culture experiments is generally much higher than those found *in vivo* (Lambert, Hong, Yang, Liao & Yang, 2005). The bioavailability, that is, the *in vivo* tissue and cellular concentrations of dietary PPs, is a determining factor in their effectiveness. While polyphenolic compounds are effective antioxidants *in vitro,* their *in vivo* bioabsorption generally is low (Matsumoto et al., 2001), and therefore antioxidant activity may not be a relevant function. The amount of flavonols excreted as a proportion of intake, is ~ 1% (Aherne,& O'Brien, 2002).

Phenolic compounds are highly unstable and are rapidly transformed. This may occur at all stages from the moment plant cells are damaged, through processing and storage, to cellular influences on absorption of individual PPs. Post-harvest enzymatic and chemical reactions during food storage, processing and with constituents in the food matrix greatly affect the properties of PPs. Interaction with food proteins and digestive enzymes alter PP bioavailability (Cheynier, 2005).

During the passage through the gastrointestinal tract, ACNs are exposed to different pH environments. The flavylium cation exists only in the lumen of the stomach due to low pH, while other forms will predominate lower down the tract where pH is more alkaline. Differences in ACN absorption in various intestinal

segments from mice indicated that the jejunum is a major site of absorption (Matuschek, Hendriks, McGhie & Reynolds, 2006).

In a berry feeding trial, both the nature of the sugar conjugate and the phenol structure of PPs were found to be important determinants of absorption and excretion (McGhie, Ainge, Barnett, Cooney & Jensen, 2003). Substantial variation between individual human responses in ACN excretion were recorded, and maximum levels were detected in the urine 120 minutes after dosing.

Berry consumption by weanling pigs confirmed that the aglycone and sugar moieties altered the absorption and metabolism of specific ACNs (Wu, Pittman, McKay & Prior, 2005). Interestingly, 80-90% of the excreted ACN compounds from blackcurrant and other berries that existed as rutinoside, were shown to be absorbed as the intact molecule.

Anthocyanins undergo enzymatic conversions within organs of the mammalian digestive system, particularly methylation and glucurono-conjugation (Talavéra et al., 2005). Intestinal mucosal cells contain various enzymes that modify flavonoids. PPs undergo further metabolic processes, including modification by colonic microflora, before becoming available to cellular mechanisms.

Flavonoids that are not absorbed in the small intestine can be metabolized by colonic microflora into aglycones and phenolic acids, which in turn, may be absorbed from the colon. An array of flavonoid glycoside-hydrolyzing enzymes have been identified in anaerobic fecal floral cultures (Aherne,& O'Brien, 2002).

However, large doses of berry PPs (1 – 5mg/ml) selectively inhibited the growth of pathogenic intestinal bacteria (Puupponen-Pimiā et al., 2005). Differences shown were between Gram-positive and Gram-negative strains, and between host-resident bacterial species in response to the PPs.

The incorporation of PPs into cells has been reported through interaction with certain transporters, and ACNs were demonstrated to be more effective than other flavonoid derivatives (Youdim, McDonald, Kalt & Joseph, 2002). This may be due to more stereologically favourable interactions with cell surface receptor molecules, including the nature of the sugar moieties attached to the PP structure of anthocyanins.

PPs are highly reactive and good substrates for various enzymes involved in metabolic processes. The epicatechin PPs of green tea are able to bind to DNA (Kuzuhara, Sei, Yamaguchi, Suganuma & Fujiki, 2006). Other flavonoids inhibit gene transcription (Kanazawa, Uehara, Yanagitani & Hashimoto, 2006), or act at the post-translational level via enzymatic modifications (Hwang et al., 2008). But whether these data are relevant for human disease outcomes is dependent on bioavailability, metabolism, and gut host/microbe interactions.

When excreted in bile, the flavonoids are passed into the duodenum and metabolized by intestinal bacteria, and the resulting metabolites may be reabsorbed to enter an entero-hepatic cycle. Substitution on the flavonoid molecule, degree of polarity, and molecular weight determine the extent of

biliary excretion. Flavonoids are also eliminated by renal excretion after conjugation in the liver (Aherne & O'Brien, 2002).

1.1.8 Modulation of inflammatory conditions

The protective effects of flavonoids towards oxidative stress-responsive signalling pathways has been examined. The actions of quercetin and catechin on cultured human monocyte cells suggested that these flavonoids may be of benefit in chronic diseases where inflammation is implicated (Huang, Wu & Yen, 2006). In an animal model, high doses of purified anthocyanins down-regulated pro-inflammatory cytokines produced in murine lung tissue after stimulation (Park, Shin, Seo & Kim, 2007).

Many PPs affect signal transduction pathways, leading to inhibition of cell growth, enhanced controlled cell death (apoptosis), reduced inflammatory marker recruitment, and ultimately slower disease progression (Virgili et al., 2008).

Kinase enzymes involved in the regulation of cell transformations, gene expression and cell adhesion interactions have been shown to be inhibited by flavonoids. Each of the steps involved in trans-membrane signalling and second messenger signal relays, whereby activation of cells is accompanied by translocation of kinases, can be flavonoid-sensitive (Middleton et al., 2000).

Cell membranes may contain micro-domains (lipid rafts) with anchored proteins that co-operate with transmembrane receptors in signal transduction.

Inflammatory stimulation of immune signals has been shown to induce the translocation of co-receptor subunits and kinases (Olsson & Sundler, 2005). One such receptor is a toll-like receptor (TLR), and some flavonoids have been shown to inhibit the association of TLR subunits, which suppresses its activation (Youn et al., 2008). This indicates that one mechanism of action of PPs may be upstream of ligand-receptor complexes.

Dysregulation of TLR-mediated immune responses is closely linked to many chronic diseases, and culminates in the expression of inflammatory gene products, including cytokines and chemokines. Some flavonoids have been shown to modulate the TLR-mediated inflammatory response (Lee et al., 2009).

The regulation of cellular signals by phytochemicals, especially direct interaction with nuclear receptors and the ability to modulate the activity of key enzymes involved in cell signalling, implies potential benefits of physiological doses of bioactive flavonoids for inflammatory diseases.

1.2 Berryfruit

Berries are a rich source of polyphenols, as well as other bioactive substances such as vitamin C. Berryfruit may contribute 100-300mg of ACNs to the diet in a single serving (Matuschek, Hendriks, McGhie & Reynolds, 2006). Polyphenol-rich food may induce beneficial changes in pathways related to inflammatory diseases, but the role of different micronutrients in this protection is not clear.

In consideration of potential harmful effects of high doses of purified PPs, and losses and modifications during food processing, it is realistic to consider the functionality of whole foods. A typical compositional analysis of blackcurrants indicates that anthocyanins constitute most of the phenolic compounds, and levels up to 1500 mg/kg fresh weight have been recorded in berries (Määtä, Kamal-Eldin & Törrönen, 2001).

1.2.1 Berries and health

Berries exhibit a wide range of PP bioactivities *in vitro*, including powerful antioxidation, and synergistic effects between berryfruit components (Halliwell, Rafter & Jenner, 2005).

In a rodent feeding trial, a 5% berry diet effectively inhibited chemically induced oesophageal cancer (Stoner et al., 2007). Since even the low-anthocyanin berry extract in this study induced chemoprotective protein expression, components other than the anthocyanins contributed to reduced levels of DNA damage.

Berry and grape juices demonstrated cytoprotective activities at sub-toxic concentrations of phenolics (3 – 30µg/ml) *in vitro* by increasing cell survival and decreasing lipid peroxidation (Garcia-Alonso, Ros, Vidal-Guevara & Periago, 2006).

Short-term human intervention studies report rapid but poor absorption of anthocyanins in the diet. Nevertheless, the intake of phenolic-rich juice was

shown to improve the antioxidant status in healthy subjects (Garcia-Alonso, Ros & Periago, 2006).

1.2.2 Polyphenolic berryfruit extracts

PP extracts of boysenberries and blackcurrants were tested in an *in vitro* cell assay measuring oxidative stress. These extracts contained complex mixtures of PPs, and both types of berry significantly reduced the oxidative stress imposed on cell cultures at low concentrations. Among fractions tested, the phenolic extract of blackcurrant showed the highest degree of protection, and this was significantly higher than the same dose of the phenolic extract of boysenberry (Ghosh et al., 2006). An important consideration was to keep the concentrations of PPs within the human physiological bioabsorption range.

An animal model was designed to clarify physiological effects of different PP fractions of raspberries. A cardioprotective effect measured by vasodilation, correlated with antioxidant activity, and was attributed to the phenolic, but not anthocyanin, fractions of raspberry PPs. (Mullen et al., 2002).

1.2.3 Blackcurrant cultivars

Extensive breeding programmes at Plant and Food Research have selected for berry crops with enhanced levels of nutrients. With the aim of including fruit in healthful food formulations, there is interest in identifying blackcurrant cultivars that attenuate cellular responses to inflammation challenge. The polyphenolic BC extracts in this study contained anthocyanins (≈30%), and an HPLC profile of one Plant and Food Research cultivar is shown (Figure 1.3), identifying four

major blackcurrant anthocyanins (delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside).

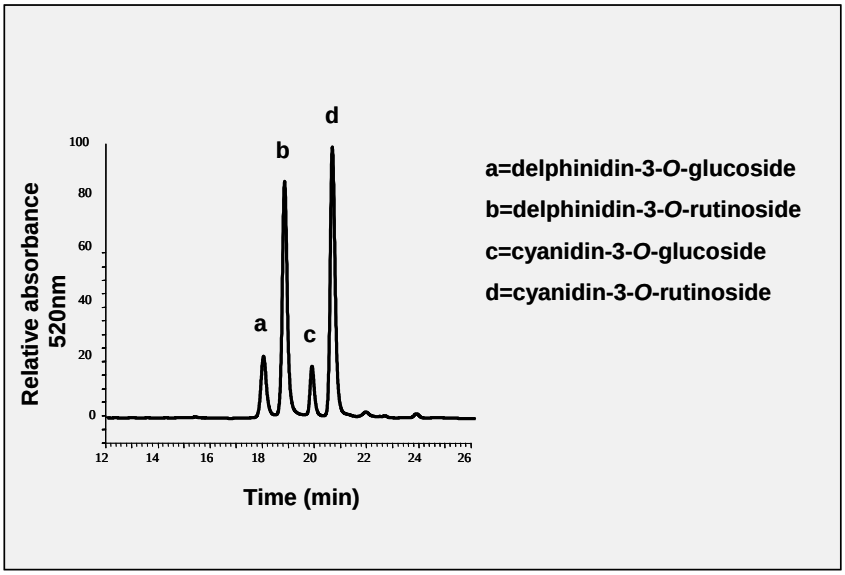


Figure 1.3. HPLC of blackcurrant polyphenolic extract. Peaks identified for four major anthocyanins. Modified from McGhie et al., (2003).

1.3 Physiology of asthma

1.3.1 Lung physiology

The bronchi formed by division of the trachea warms and cleanses incoming air to the lungs, and saturates it with water. The tissue composition of the walls of the primary bronchi mimic the trachea, but as the conducting tubes become smaller, support structures change and epithelial cell types differ.

The gas-filled airspaces are termed alveoli, and the respiratory membrane consists of the alveolar epithelium fused with the blood capillary endothelium.

The narrow width of this wall and the large surface area generated by the presence of millions of sacs support gas exchange.

The single layer of pneumocytes lining the alveolar sacs in the terminal bronchioles are primarily squamous epithelial cells (type I cells). Type I cells are fragile and cannot divide, but scattered amid them are cuboid type II cells that divide and elongate to replace damaged type I cells of the epithelial lining (Malkinson, Dwyer-Nield, Rice & Dinsdale, 1997). Type II cells synthesize and secrete a surfactant which coats the gas-exposed alveolar surfaces to reduce the surface tension of alveolar fluid (Burgoyne & Morgan, 2003).

Surfactant components in type II alveolar epithelial cells are stored in distinct membrane-bound lamellar bodies (Chander, Sen & Spitzer, 2001). Many proteins, such as cytokines, are imported into cells by receptor-mediated endocytosis and stored in granules. Secretory proteins produced by the cell, and released by a stimulus-induced exocytosis, are also stored in granules (Burgoyne & Morgan, 2002).

1.3.2 Asthma

Asthma is a chronic inflammatory lung disease characterized by symptoms of cough, wheeze, shortness of breath and chest tightness due to obstructions of the smaller bronchioles during respiration (Rao, 2005).

The pathogenesis of asthma is dependent on gene/environment interactions modulated by the immune system. Substances that induce an immune

response (antigens) may elicit a hypersensitive response (allergens), where the immune system reacts excessively to antigens (Phillips, Murray & Kirk, 2001).

Allergens stimulate the humoral immune response that results in production of antibody-secreting plasma cells and memory cells. The plasma cells excrete excessive amounts of Immunoglobulin E (IgE), an antibody which attracts inflammatory cell types to sites of inflammation in order to counteract damage by foreign cell invasion. Tissue resident cells also secrete cytokines that alter the local microenvironment, leading to further recruitment of inflammatory cells (Rao, 2005).

The presence of an inflammatory cell infiltrate at the site of allergen challenge characterizes asthma. This response is largely orchestrated by the Thymus helper (TH) cells (Croker, Kirtibala, Townley & Khan 1998). Thymus helper type-II (TH_2) cells are a sub-group of lymphocytes. TH_2 cells can both directly recognise allergen peptides via the T cell receptor, and release Interleukins (ILs) (Romagnani, 2001). ILs are cell signalling cytokines that regulate the proliferation and post-mitotic differentiation of inflammatory cells such as eosinophils in both allergic and non-allergic asthma (Walker, Virchow, Bruijnzeel & Blaser, 1991). Both these types of asthma are characterized by chronic inflammation and local tissue eosinophilia (Ying et al., 1999).

Twelve hours or more after allergen exposure (late response), cell-mediated hypersensitivity reactions occur in the lung tissue predominantly involving T lymphocytes, especially the TH lymphocytes and antigen-presenting cells

(APC's) (Phillips, et al., 2001). Airway inflammation, mediated by eosinophils, causes damage to the epithelial airway tissue (Smit & Lukacs, 2006). These events are schematically represented (Figure 1.4).

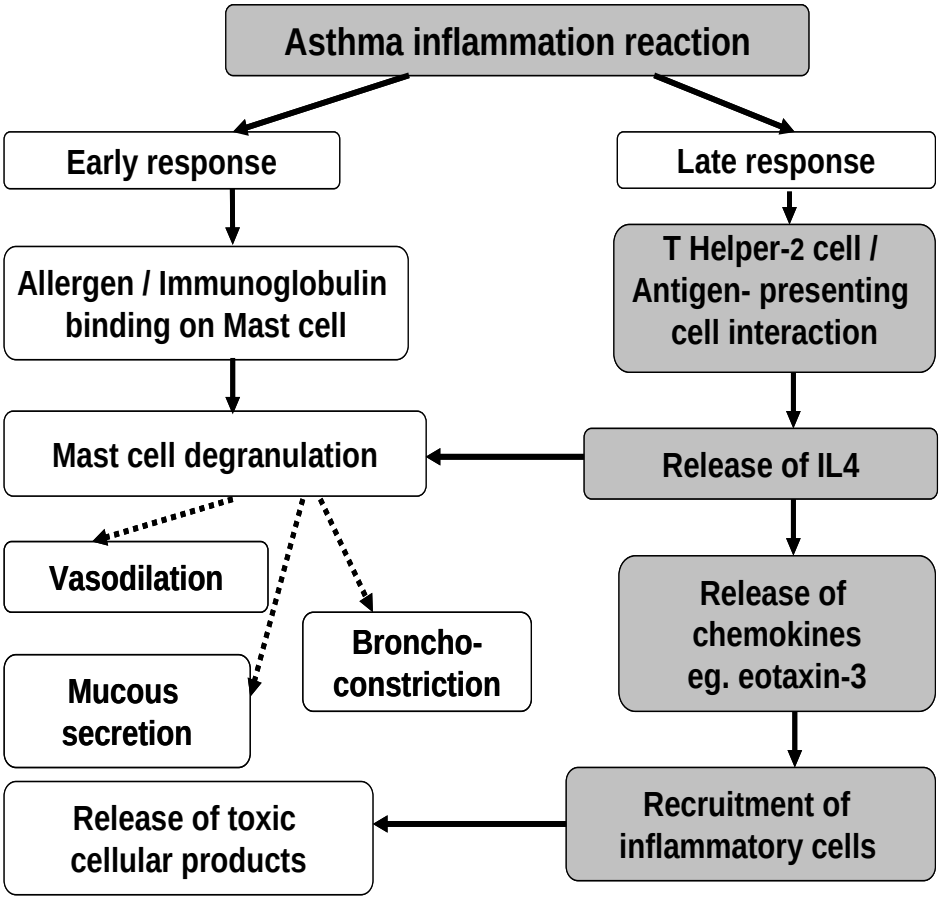


Figure 1.4. Overview the asthma inflammation reaction. Modified from Rao, (2005).

1.4 Biochemical and cellular responses during asthma

The process of respiratory sensitization in response to inhaled allergens involves the orchestrated interaction between a variety of cells and molecules in ways that are tightly regulated.

1.4.1 Pulmonary alveolar signalling

Most of the T cells are small, non-dividing cells circulating through the thymus-dependent tissues. In order for the resting T cell to be activated, it must receive a second signal (Rao, 2005). The allergic inflammatory state requires the production of an array of soluble cytokine mediators. Of the TH_2 cytokines, Interleukin-4 (IL-4) is important for the initiation of allergic immune responses because it induces activation, proliferation, and differentiation of a variety of haematopoietic cells, including B cells, T cells and mast cells (Knisz & Rothman, 2007).

The magnitude and duration of cytokine signalling are controlled through several check-points in the inflammatory reaction cascade. Regulation of receptor expression, and intracellular signalling steps within the membrane, cytoplasm and nucleus all contribute to moderation of the biological effects of cytokines (Knisz & Rothman, 2007).

1.4.2 Inflammatory cytokines and chemokines

Once activated, TH cells differentiate into TH_1 or TH_2 subsets and secrete various cytokines, depending on the signals they receive.

Since IgE production and T cell activation are dependent on IL-4, then inhibiting the production of IL-4 may lead to attenuation of the allergic response related to asthma. In a murine model of asthma, a significant reduction in serum IgE was demonstrated after anti-IL-4 was administered as a pre-treatment to antigen challenged mice (Zhou, Croker, Koenig, Romero & Townley, 1997).

IL-4 directs the maturation of TH_2 cells from naïve TH cells, and is able to induce adhesion of circulating eosinophils onto endothelial cells (Romagnani, 2001). TH_2 cells and their cytokines are crucial for promoting hypersensitivity responses, and also for maintaining eosinophilia that is characteristic of chronic allergic inflammation (Verstraelen et al., 2008).

An increase in bronchial hyperresponsiveness of asthmatics was associated with eosinophil chemotactic cytokines (chemokines) and their receptor expression in bronchial biopsies (Ying et al., 1999). A correlation was found between the presence of eosinophils and chemokine over-expression in the bronchial mucosa from asthmatics. This represented the late-phase allergic reaction from both atopic and non-atopic asthmatics.

Like many other cytokines, IL-4 affects a variety of target cells in multiple ways, including transendothelial migration (extravasation) of leukocytes at sites of inflammation.

In order to identify molecules involved in the IL-4 dependent eosinophil extravasation, a cultured human vascular endothelial cell-line was studied. It was revealed that IL-4 up-regulated the protein expression of a specific chemokine for eosinophils (eotaxin-3) (Shinkai et al., 1999).

1.4.3 Regulation of cytokine signalling

T lymphocytes and eosinophils are important sources of IL-4, which is produced in response to antigen-receptor binding. Released by TH2 cells, IL-4 induces the expression of chemokines to attract eosinophils into inflamed airways.

A variety of tissue-specific cells also have receptors for IL-4, and are able to respond to its presence in the microenvironment. The mRNA and protein expression of IL-4 receptor subunits have been detected in cultured bronchial epithelial cells (van der Velden et al., 1998), and intestinal epithelial cells (Blanchard et al., 2005).

Of particular interest in the context of asthma is the role of CC chemokines, which are potent chemoattractants for eosinophils (Verstraelen et al., 2008).

Endogenous eosinophil chemoattractant (eotaxin) activity in the lungs was detected in bronchoalveolar lavage fluid (BALF) of sensitized guinea pigs (Jose et al., 1994). These results elucidated a number of key properties of the eotaxins; that sharing of receptors between CC chemokines occurs, that eotaxin is potent *in vivo*, and that eotaxin exhibits selectivity, inducing eosinophil but not neutrophil accumulation.

In a study of human asthmatic BALF, it was demonstrated that eotaxin-3, and not other eotaxin chemokines, accounted for the ongoing eosinophil recruitment to asthmatic airways 24 hours after allergen challenge (Berkman, Ohnona, Chung & Breuer, 2001). While the other eotaxins were expressed in the early

stages, only eotaxin-3 mRNA levels dramatically increased after 24 hours. Bronchial biopsy specimens from asthmatics were analysed for eotaxin levels 48 hours after allergen challenge (Ravensberg et al., 2005). It was shown that eotaxin-3 protein expression was increased after allergen challenge, and the association was positively correlated with the late asthmatic response.

The eotaxins promote the initiation and maintenance of an allergic reaction by ensuring the presence of antigen-specific TH_2 cells. Since eosinophils can release granules and membrane products that cause tissue damage characteristic of asthma, then the eotaxins play a role in the development of tissue damage by activating the pro-inflammatory effector functions of the eosinophils (Gutierrez-Ramos, Lloyd & Gonzalo, 1999). These events are represented diagramatically (Figure 1.5).

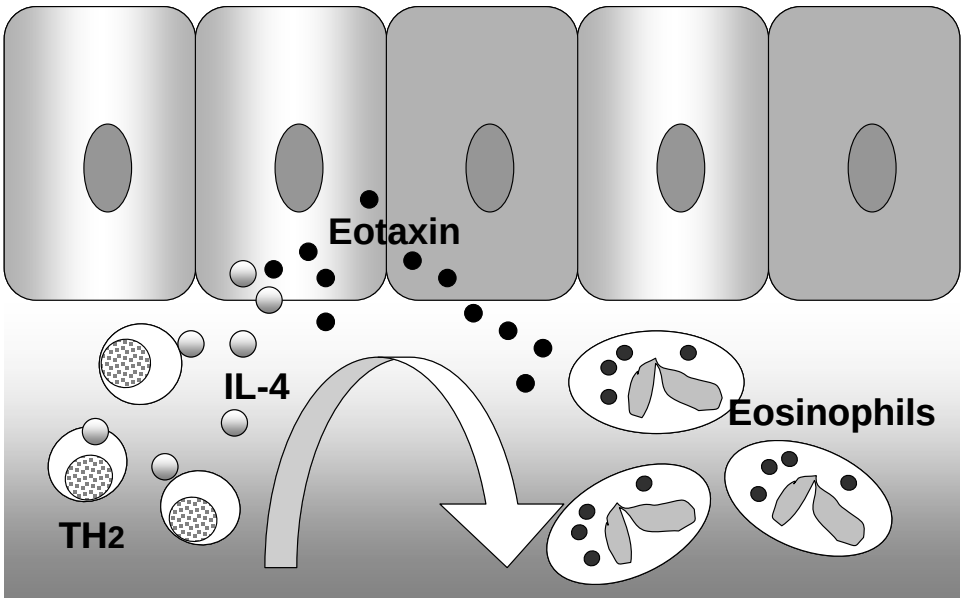


Figure 1.5. Key events in the cellular inflammatory response. T-Helper type-2 (TH_2) immune effector cells secrete Interleukin-4 (IL-4) cytokine which is uptaken by receptors on lung epithelial cells. In response, these cells produce and secrete eotaxin chemokines. The eotaxin receptors are highly expressed on eosinophils, which are attracted to the site of inflammation.

Eosinophils also generate membrane-derived lipid mediators and RONS that may aid their pro-inflammatory effects (Menzies-Gow & Robinson, 2001). Polycationic proteins released from eosinophils have been shown to damage the epithelium, potentiating airway reactivity (Nevin & Broadley, 2002).

Helper T cells are capable of influencing a variety of immune cells via proliferation, production and secretion of cytokines, and commitment to a differentiated phenotype to co-ordinate a host response to allergen challenge. Major biochemical pathways in the cytosol of the TH cell are activated by kinase enzymes present on the T cell receptor, which are activated via phosphorylation. IL-4 drives TH_2 cell production, but also carries out many other functions of the immune response, and possesses intracellular controls similar to TH cells and other cytokines.

When IL-4 docks to its receptor complex, signal transducer and activator of transcription (STAT) signalling cascades are activated via phosphorylation (Pernis & Rothman, 2002). STATs are a family of transcription factors common to cytokine-mediated signal transduction, and are recruited to the cytoplasmic region of cell surface receptors. Once activated, STATs dimerize, and translocate to the cell nucleus where they influence gene expression. Control of gene expression involves inhibition by suppressor proteins. These events are outlined (Figure1.6). STATs play a role in regulating cell growth processes and cell differentiation.

A STAT-6 binding site was shown to be necessary for full eotaxin-3 promoter activity in intestinal epithelial cells. (Blanchard et al., 2005) These key intracellular regulatory mechanisms responsible for the TH_2 profile are activated by IL-4 ligand-receptor engagement (Figure 1.6).

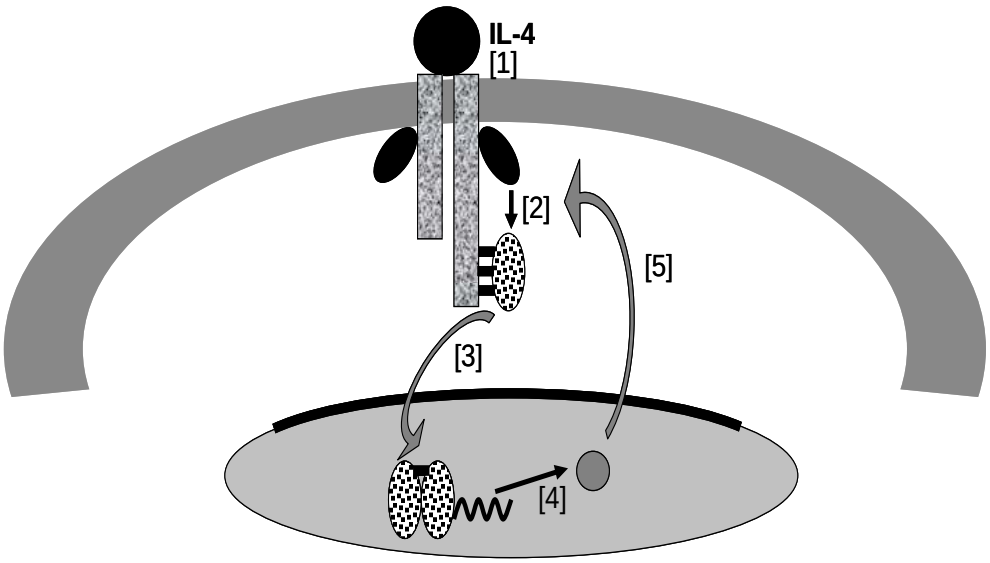


Figure 1.6. Intracellular cytokine regulation. IL-4 docks to its receptor complex at the cell plasma membrane [1], and activates a STAT-6 transcription factor [2], which translocates to the nucleus as a dimer [3] to activate transcription of cytokine responsive element genes [4]. Regulation of STATs [5] influences cell growth and differentiation. The figure has been modified from Knisz & Rothman, (2007)

Activation and expression of the STAT-6 transcription factor in bronchial epithelial cells was also demonstrated (Mullings et al., 2001). In oesophageal tissue, gene expression profiling revealed that the most highly induced transcript was eotaxin-3, and a single nucleotide polymorphism in the eotaxin-3 gene was strongly correlated with oesophageal eosinophilia disease severity (Blanchard et al., 2006).

1.4.4 Chemokines

In humans, chemokines constitute a family of approximately 50 small peptides that coordinate leukocyte trafficking and recruitment to sites of inflammation (Pease & Williams, 2006). Chemokines are stored in secretory granules within cells and are released in a rapid, controlled response (Sallusto & Baggiolini, 2008).

Four chemokine groups have been defined according to the position of cysteine amino acid disulphide bridges, which are the primary structural features in tertiary folding of the protein (Baggiolini, 1998). The disulphide bonds keep functional domains in close proximity, and are essential for biological activity. All chemokines share the same protein fold, in which three anti-parallel β-pleated sheets are overlaid by a C-terminal α-helix. (Pease & Williams, 2006) (Figure 1.7).

The position of the first two sulphur-containing cysteines forming the intermolecular bonds are either adjacent (CC) or separated by amino acids (CXC). These structural variations effect diverse physiological outcomes. For instance, CXC chemokines will recruit neutrophils in the early phase of an inflammatory response, while CC chemokines support mainly eosinophil recruitment in the late phase (Pease & Williams, 2006).

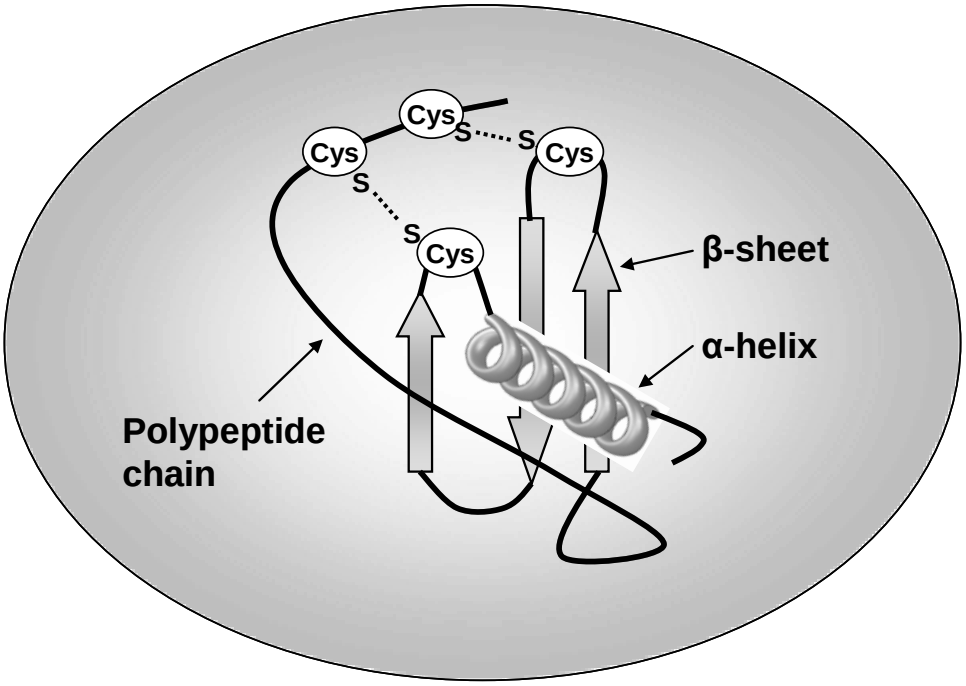


Figure 1.7. Structure of chemokines. Disulfide bridges (S-S) between Cysteine (Cys) residues are the main structural features, which keep functional domains in close proximity and are essential for biological activity. Modified from Sallusto & Baggiolini, (2008).

1.4.5 Eotaxins

Eotaxins are CC chemokines. The eotaxin receptor (CCR3) has been described on many cell types, including alveolar type II epithelial cells (Abonyo, Alexander & Heiman, 2005). IL-4 regulates the expression of CCR3 receptors, and eotaxin-3 transcription and synthesis occurs predominantly after IL-4 or IL-13 stimulation (Abonyo et al., 2006). CCR3 is the major chemokine receptor on eosinophils (Zimmerman, Conkright & Rothenberg, 1999). Docking and binding of the eotaxins to CCR3 induces biochemical changes in eosinophils, rapidly mobilizing their stores of IL-4.

As with many biological ligands, the initial step of chemokine-induced signalling is mediated through Guanine (G) -protein cell-surface receptor coupling. Eotaxins dock onto externally exposed loops of the helical receptor structure, and a short amino acid sequence fits into the central binding pocket to initiate a signal-transduction cascade. (Sallusto & Baggiolini, 2008).

The CC eotaxins (eotaxin-1, -2, & -3) signal via a single receptor, but exhibit different potencies due to differences in primary structures. CCR3 can bind more than 10 different ligands, but the three eotaxin molecules compete exclusively at CCR3 (Duchesnes, Murphy, Williams & Pease, 2006).

While chemokines are produced in high amounts by inflammatory cells, they can also be produced in substantial amounts by structural cells. (Smitt & Lukacs, 2006). Eotaxin-3 was shown to be the most dynamically expressed peptide from the eotaxin family in airway epithelial cells after IL-4 stimulation (Blanchard et al., 2005).

IL-4 and IL-13 released by TH_2 cells induce the expression of eotaxins that attract eosinophils into the pulmonary airway (Abonyo et al., 2005).

While all the eotaxins are expressed in A549 cells, only eotaxin-3 reduced expression of CCR3 receptors (Duchesnes et al., 2006). In addition to its receptor ligands, airway epithelial cells express functional CCR3 (Banwell, Tolley, Williams & Mitchell, 2002). This co-expression of both ligand and receptor suggests auto-regulation. Eotaxin-3 not only tightly regulates its own

transcription and hence translation during an allergic response, but also regulates expression of the CCR3 receptor in the A549 cell culture model (Abonyo et al., 2005).

Since eotaxin-3 is expressed in alveolar type II cells, and is dependent on activation of the IL4 binding site for gene expression, then TH_2- mediated inflammation in asthma is regulated in part by lung epithelial cells.

1.5 Potential beneficial effects of fruit and polyphenolics on asthma

In section 1.1.4 it was established that fruit consumption is beneficial for health, and apples have a protective effect against disease risk.

Links between low fruit intakes and adult-onset asthma incidence were made in a prospective study of lifestyle diseases in the 1990s (n=68,535) (Romieu et al., 2006), and an analysis of dietary antioxidant intake linked a low fruit intake with symptomatic asthma (Patel et al., 2006).

A cross-sectional analysis of children's diet (n=18,737) found that the consumption of fruit was associated with reduced wheezing, a symptom of airway obstruction (Forastiere et al., 2000). The intake of one to two citrus fruit or kiwifruit gave a protective effect, but the association was stronger in those with a history of asthma. A population-based survey of children (n=2640) found an association between those who regularly included apple juice in their diet, and a lower prevalence of asthmatic symptoms (Okoko et al., 2007).

The consumption of apples and pears, but not specific nutrients, was associated with a range of asthma definitions used in a clinical study, including bronchial hyperreactivity (Woods et al., 2003). No associations between intakes of B-carotene, retinol, vitamin C, vitamin E and asthma were demonstrated in this analysis. Low measures of pulmonary function inversely correlated with increased plasma vitamin C in the Scottish Health Survey, but not to the levels attained by increasing amounts of whole fruit in the diet (Kelly, Sacker & Marmot, 2003). Therefore the intake of whole fruit, in the form of apples and pears, is positively associated with pulmonary function, and this effect is stronger than that shown for individual antioxidant nutrients.

1.5.1 Antioxidant and inflammation modulatory properties

Although a protective role for dietary antioxidants in asthma has been proposed, epidemiological evidence implicating antioxidant vitamins appears weak. Declining dietary intakes of fruit and vegetables in the UK has been linked with increased cases of asthma (Devereux & Seaton, 2005). It is possible that the low antioxidant levels of these diets could lead to an increased vulnerability of the pulmonary airways to RONS. Many age-related diseases have an underlying aetiology of oxidative damage (Weaver et al, 2008).

Since the vitamin C in apples with skin accounted for only 0.4% of total antioxidant activity (Liu, 2003), much of the protective effect of apples may come from phenolics and flavonoids.

A dietary survey (n=9709) of flavonoid-rich foods, antioxidant vitamins (C,E, and carotene), indicated an association between apple and red wine consumption and asthma (Shaheen et al., 2001). While two or more apples a week in the diet was beneficial, intakes of tea, onions and red wine were not related to asthma. There was no evidence from this study of an association between asthma and dietary intake of vitamin C and vitamin E.

Therefore not all antioxidants are associated with benefits to asthmatics.
In section 1.1.6 *in vitro* assays demonstrated antioxidant functions of anthocyanins and flavonoids. However, *in vivo*, anthocyanin absorption levels appear to be too low to enter cells and affect metabolic processes in an antioxidant capacity (Prior, 2003).

In section 1.2.1 *In vitro* studies of a phenolic-rich juice at cellular sub-toxic doses showed antioxidant protection, but it was unclear whether this was due to a radical scavenging effect or not (Garcia-Alonso,Ros & Periago, 2006).

While some anthocyanins can be absorbed intact, the proportion metabolized and excreted in the urine was found to be less than 0.1% of total intake (Wu et al., 2004). No significant changes were detected in plasma antioxidant levels in humans fed high anthocyanin-berry diets (Matsumoto et al., 2001). High toxicity of purified PPs has been demonstrated (Zhang, Stanley, Adaim, Melton & Skinner, 2006).

The antioxidant properties of PPs have been widely studied, but it has become clear that their mechanisms of action go beyond the modulation of oxidative stress. Absorption by cells may not be required for PPs to protect them from oxidative damage (Deng et al., 2006).

The role of PPs in cellular responses and in preventing pathologies that involve chronic inflammation includes direct interaction with ligand-activated transcription factors (nuclear receptors), and the ability to modulate the activity of key enzymes directing cell signalling (Virgili & Marino 2008).

Mechanisms regulating the oxidative response were investigated in an *in vitro* system of endothelial cells, and PPs isolated from berryfruit were added. It was found that the secretion of chemoattractants by the cells altered their redox state, and PPs isolated from blueberry and cranberry protected cells against oxidative and inflammatory stressors (Youdim et al., 2002). The anthocyanin PPs were found to be the most biologically active fraction.

Anthocyanins were investigated for their ability to reduce markers of airway inflammation in a murine asthma model (Park et al., 2007). The induced asthmatic response was characterised by eosinophilia. The anthocyanins in the mouse diet were shown to down-regulate TH_2 cytokines and reduce inflammatory cell recruitment. In the lung tissue, cyclooxygenase enzyme, key in the formation of crucial mediators of inflammation, was found in the bronchial epithelial cells. Anthocyanins reduced cyclooxygenase protein expression.

Glycation end-product accumulation associated with diabetic inflammation and the associated influence of pro-inflammatory cytokines (TNFα & IL-1β) was assessed. A ligand for the receptor of these end-products was added to an *in vitro* cell line of human monocytes, which significantly increased gene expression of the cytokines, associated chemokines and adhesion factors (Huang et al., 2008). The flavonoids quercetin and catechin inhibited the expression of chemokine and adhesion genes, demonstrating that flavonoids have the potential to regulate an inflammatory condition.

Extracts of green kiwifruit (*Actinidia polygama*) were fed to allergen-sensitized mice over a 12 week period to assess effects on chronic airway inflammation and hyper-responsiveness associated with asthma (Lee, Kim, Seo, Roh & Lee, 2006). A reduction in eosinophil accumulation into the airways, and reduced levels of TH_2 cytokines and IgE in the bronchoalveolar lavage fluid of mice fed kiwifruit extract were shown. These effects were linked to reduction of the eotaxin receptor (CCR3) on the surfaces of cells in the fluid, especially eosinophils.

Symptoms of asthma include wheezing, and shortness of breath. Antioxidant-rich passionfruit peel extract (150mg/d) was administered as a dietary supplement to asthmatics in a randomized cohort of human subjects (Watson et al., 2008). It was found that the fruit extract treatment significantly lowered the prevalence of shortness of breath compared to placebo, and that symptoms of asthma improved after four weeks of treatment.

Epidemiological, animal and *in vitro* evidence supports the concept of asthma health benefits from specific fruit consumption. More specifically, attenuation of cellular markers of inflammation known to participate in the late-phase inflammatory reaction cascade would provide direct evidence of being beneficial in asthmatic conditions.

Berries are a natural food source high in polyphenolic compounds shown to be preventative for chronic disease states. Beneficial effects of specific anthocyanins contained in blackcurrants have been demonstrated to protect cells *in vitro* at high doses. PP extracts of blackcurrant cultivars may exhibit differences in response to inflammatory challenge in lung epithelial cells at bioavailable doses. Particular BC cultivars may be identified for their asthma preventative potential.

1.6 Objectives

The general aim of this thesis was to identify blackcurrant (*Ribes nigrum*) cultivars that may modulate airway inflammation associated with asthma by measuring lung cell response after stimulation with a TH_2 cytokine.

For this purpose, BC extracts were analysed in an *in vitro* system using human lung epithelial cells stimulated with IL-4 to produce eotaxin-3.

Results from this study may identify BC cultivars of benefit in a natural food formulation designed to reduce symptoms of asthma.

Key question asked in this study:

1) Are any of the blackcurrant cultivars effective as inflammation modulators in an *in vitro* asthma model?

2) Is there a positive association between any one anthocyanin in the Plant and Food Research polyphenolic BC extracts and reduction of eotaxin-3 levels after IL-4 challenge to lung cells?

The hypothesis in this study is that some blackcurrant cultivars will be identified as being effective at attenuating an inflammation marker in a lung epithelial cell system.

Chapter 2

Methods and materials

2.1 Cell culture

A continuous cell line from a human alveolar cell carcinoma with properties of type II alveolar cells was used for this *in vitro* study. Aseptic tissue culture techniques were employed, with all cell work carried out in a Laminar Flow Biosafety level 2 hood to contain aerosols. Human A549 alveolar type II epithelial-like cells were purchased from American Type Culture Collection (ATCC CCL-185), and grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Auckland, New Zealand) nutrient mix F-12 supplemented with 10% foetal bovine serum (Invitrogen, Auckland, New Zealand), and a 1% antibiotic/antimycotic mix (containing 10,000U penicillin, 10,000U streptomycin & 25 µg/ml amphotericin B) (Invitrogen, Auckland, New Zealand).

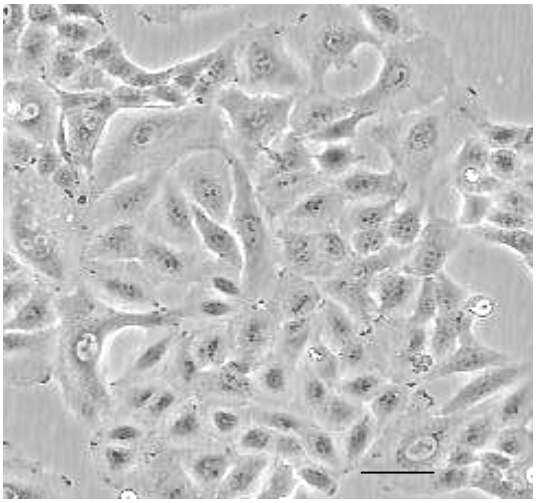
Cells were incubated in a 100% humidified atmosphere in 5% carbon dioxide/air at 37°C.

2.1.1 Routine cell-line maintenance

Human A549 cells are adherent cells and were grown and maintained in 75cm^2 sterile flasks (Corning, Hallam, Australia) in 10ml culture medium DMEM, and passaged every 5-8 days after sub-cultured cells had reached > 80% confluency.

Passaging of the cells required washing the cells and then releasing them from attachment to the plastic flasks. In passaging of cells, the culture media was discarded, cells were then washed twice with 10ml Phosphate Buffered Saline (PBS) (Invitrogen, Auckland, New Zealand). The PBS did not contain calcium or magnesium salts to aid detatchment.

The cells were dislodged from the flasks by the addition of 1ml of the dispersing agent TrpLE Select (Invitrogen, Auckland, New Zealand), incubation for 2-4 minutes, with observation under an inverted light microscope (x 10 magnification) until the cell layer was observed to be completely dispersed. 5ml media was then added to inactivate the dispersal agent, and 0.5 ml of the cell suspension was then added to a new sterile flask, along with 10 ml culture medium. Cells were rocked gently to evenly disperse them across the surface of the flask, which was placed in an incubator to grow cells to confluency. Fresh media was added every 2-3 days to replace nutrients. All experiments were performed after sub-cultured cells had reached > 80% confluency.



Scale = 50μm

Figure 2.1. Phase contrast Light Micrograph of adherent A549 cells grown in a tissue culture flask. Nuclei and cytoplasmic granules are visible.

2.1.2 Experimental conditions

Cells were removed from flasks as described in 2.1.2, and counted under the microscope using an hematocytometer to determine the cell density.
The average of two cell counts was taken (cell count/2 x 10^4 = cells/ml) and fold dilution in fresh medium to obtain the appropriate cell number for experiments was calculated.

All biological experiments were carried out in 12-well tissue culture-treated plates (Corning, Hallam, Australia) at a plating cell density of 6 x 10^5 cells/well except for the validation of cell number and cytotoxicity experiments. 1ml of cells containing the appropriate number of cells was aliquoted into the wells and 2ml of culture medium added. The cells were then incubated for 24 hours in which they formed a uniform confluent layer. After incubation the culture media was replaced for experiments, and experimental test solutions applied as described later. In the case of pre-incubation with BC extracts, cells were washed twice with culture medium between fruit extract and addition of IL-4.

2.2 Interleukin-4 (IL-4)

IL-4 was used as a cytokine inflammatory challenge in many of the experiments of this study. Lyophilized Recombinant human IL-4 prepared in *E coli* (PeproTech, Rocky Hill, U.S.A.) was used for this purpose. IL-4 was reconstituted in sterile water to a concentration of 100µg/ml, and frozen until used. Fresh dilutions to a working concentration of 1000ng/ml in PBS were made, and the final concentration of IL-4 in culture media for experiments was 10ng/ml.

Supplier (PeproTech) information for this IL-4 product stated that the endotoxin level was less than 0.1ng/µg. This meant that the contribution to IL-4 experimental reactivity due to the bacterial lipopolysaccharides in the outer cell membrane of the bacterial host *E. coli* was negligible.

Aliquots of IL-4 were mixed with cell growth medium, or medium plus BC extracts, then 2mls of media were added to each well of A549 cells. The 12-well plates were then incubated for 24 hrs, and aliquots of sample supernatant were collected and frozen for later quantification of eotaxin-3.

2.3 Cytotoxicity

To determine the toxicity of BC extracts to A549 cells, a cell viability assay (WST-1) was used. The assumption that 100 minus percent metabolically active cells equals percent cell death, cannot be made due to some proportion of damaged cells with low metabolic activity. However, the aim was to find a BC concentration range that was non-toxic to cells, and for this purpose the assay was adequate.

2.3.1 Cell toxicity assay

The colourimetric assay WST-1(Roche, Mannheim, Germany) contained a tetrazolium salt and an electron coupling reagent. Cleavage of WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to the yellow indicator formazan occurs after incubation with cells. The use of tetrazolium salts such as WST-1 to measure cell viability has been shown to

depend on metabolic activity (Tan & Berridge, 2000), in particular, cellular production of reduced pyridine nucleotides. These cofactors act as substrates for cellular dehydrogenase enzymes that reduce tetrazolium salts to soluble formazan end-products.

A549 cells were seeded into sterile 96 well plates at 5×10^4 cells/well, and 100µL growth medium added prior to incubation with BC extracts. Cells were then checked for confluency and media was aspirated out. 100µL of sample or control were then added and plates were incubated for a further 24 hrs. H_2O_2 as positive control (5mM), and a range of BC extracts (0 – 50µg/ml) were tested.

After incubation, 10µL of WST-1 was added per 100 µL of culture medium as per manufacturer's instructions, and cells were incubated for 4 hours. A fluorescent spectrophotometer (FLUOstar OPTIMA) (Alphatech Systems, Auckland, New Zealand), was used to detect the formazan product, measured at 450nm absorbance, minus the reference wavelength of 620nm.

Percent cell viability was calculated by subtracting the blanks (no WST-1) from the samples, dividing by average negative control (cells + WST-1) x 100 and then averaged from triplicate samples (see Appendix 2).

2.4 Co-incubation of berryfruit extracts and IL-4

2.4.1 Blackcurrant extracts

PP extracts were prepared from blackcurrants by homogenisation with acetone, undergoing a series of extractions and partitionings on a resin column. Lipids were removed, and the BC polyphenolics were isolated by adsorbance to resin beads, then eluted from the column and dried to form a friable dark red powder.

The phenolic composition of the BC extracts were determined by Liquid chromatography-electro-spray ionization-mass spectroscopy (LC-ESI-MS) combined with Photo diode array (PDA) detection. Anthocyanins were the major class of phenolic compound present in the BC extracts, accounting for ≈30% of the total phenolic content.

Plant and Food Research BC extracts were supplied as friable dark red powders kept frozen at -4°C, and in the dark. They were reconstituted in DMSO (to a final concentration of 0.01%) and sterile PBS, and stored frozen at 10mg/ml in 10µl aliquots. For biological assays the BC extracts were reconstituted in culture medium and added to cells with IL-4, to a final concentration of 10µg/ml.

2.4.2 Dimethyl sulfoxide (DMSO)

The BC extracts were reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, U.S.A.). Successive dilutions of the BC extracts that the cells were exposed to contained a range of DMSO concentrations to determine the dose of DMSO that was non-toxic to the cells. Finally the BC extracts were reconstituted with 0.01% DMSO.

2.4.3 Co-incubation of blackcurrant extracts with IL-4

Polyphenolic BC extracts were reconstituted in DMSO (0.01%) and sterile PBS to 10 mg/ml and frozen. Cells were grown to confluency in 12-well plates (6 x 10^5 cells/ml) and 2ml of media/extract/IL-4 added and incubated for 24 hours. Final concentrations used in cell assays were: BC cultivars (10µg/ml) in cell culture medium and IL-4 (10 ng/ml). Supernatant containing the eotaxin-3 cell product was then collected and frozen until ELISA detection.

2.5 Pre-incubation

Pre-incubation experiments involved pre-treating the cells (6 x 10^5 cells/ml) with BC extracts (final concentration 10µg/ml) for 24 hours, followed by 2 x washing in fresh culture medium, then stimulating with IL-4 (10ng/ml) in culture media for 24 hours. The supernatant was then collected and frozen until the ELISA detection assay.

2.6 Eotaxin-3 detection by specific ELISA

Eotaxin-3 generation from A549 cells was measured in response to an IL-4 challenge in many of the experiments in this study. Eotaxin-3 was measured using a specific Enzyme-linked Immunosorbent Assay (ELISA) for human Eotaxin-3 (DuoSet ®, Pharmaco, Auckland, New Zealand).

The antigen (eotaxin-3) was immobilized to a solid surface, the 96 well plate. Since the concentration of antigen is low, a two-antibody ‘sandwich’ ELISA was used where the specificity of antibodies is coupled with the sensitivity of an

enzyme assay. By using a purified antigen standard, the assay determined the absolute amounts of antigen in the unknown samples.

Non-specific binding of the capture antibody to the plate occurred, and unoccupied binding sites were blocked. Samples of cell supernate were then added and incubated, to allow specific binding of eotaxin-3 epitope sites to the capture antibody. Unbound supernate containing culture medium was then washed off, and detection antibody was added. This second antibody 'sandwiches' the antigen by binding to another epitope on the eotaxin-3 molecules, securing the chemokine at two sites when the biotin-labelled second antibody is bound to the antigen.

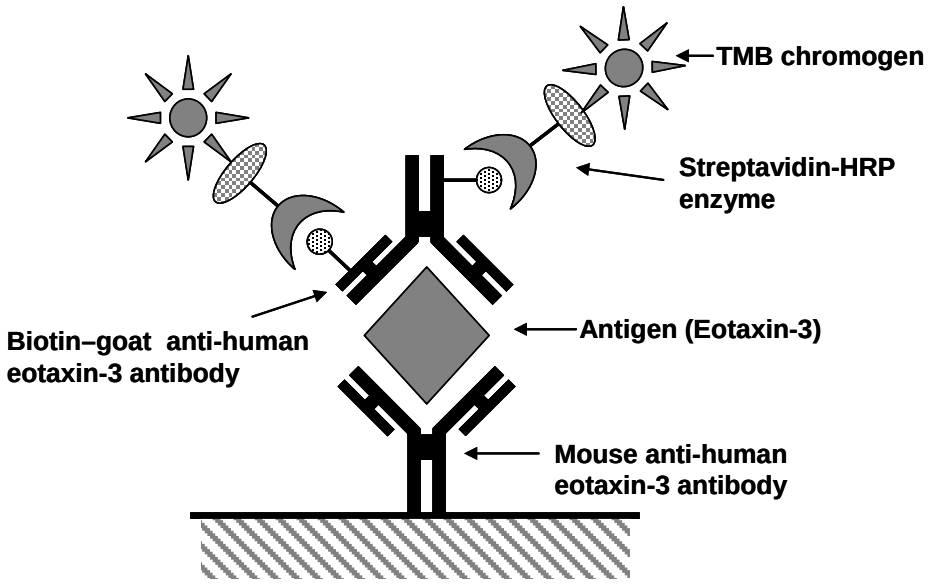


Figure 2.2. ELISA technique for quantification of eotaxin-3.

Quantification of the assay occured through measurement of the amount of labelled second antibody that was bound to the matrix, through the use of a colourimetric substrate (3,3',5,5'tetramethylbenzidine (TMB)). Streptavidin

protein conjugated to horseradish-peroxidase (Streptavidin-HRP) catalyzed the oxidation of substrates by hydrogen peroxide, resulting in a coloured TMB chromogen (Figure 2.2), which is measured by spectrophotometer at 450nm.

2.6.1 ELISA procedure

96-well tissue-culture treated plates (Falcon, North Ryde, Australia), which passively bind antibodies and proteins, were used for the assay. Reagent buffer was 1% bovine serum albumin (BSA) (Bio ICP, Auckland, New Zealand) diluted in PBS, and the blocking solution was a 10% BSA solution. The buffer for washing the plates (using a plate washer) was PBS adjusted to pH7.4.

Capture antibody (mouse anti-human Eotaxin-3) provided in the ELISA kit was diluted to a working concentration of 1µg/ml in PBS and pipetted into wells at 100µl/well to a final concentration of 100ng/well. The plate was sealed to avoid evaporation, and incubated overnight at room temperature. The plate was then aspirated and washed four times with PBS using an automated plate washer (ASYS Atlantis) (Alphatech Systems, Auckland, New Zealand), and then the reaction was blocked with the addition of 150µl/well of reagent buffer containing 1% BSA for 1 hour at room temperature. This was followed by a cycle of aspiration and washing.

A seven-point standard curve was prepared for each ELISA plate by diluting the recombinant human eotaxin-3 standard from 4000pg/ml in PBS, to serial dilutions in 1% BSA of 4000, 2000, 1000, 500, 250, 125 & 62.5 pg/ml.

100µl/well sample or standard was then added to the plate and incubated for 2 hours at room temperature, followed by 4 x aspiration/wash cycles.

Detection antibody (biotinylated goat anti-human Eotaxin-3) was diluted in 1% BSA reagent buffer and 100µl/well added, to a final concentration of 250ng/ml. The plate was then incubated at room temperature for 1.5 hours, followed by 4 aspiration/wash cycles.

Streptavidin-HRP enzyme (1:200 dilution) was added at 100µl/well as per manufacturer's instructions, and the plate was incubated in the dark for 20 minutes followed by 4 x aspiration/wash cycles. Finally, the peroxidise substrate 3,3',5,5'-tetra-methylbenzidine (TMB) (Sigma-Aldrich, St. Louis, U.S.A.), diluted in 0.1M sodium acetate buffer pH 5.4 (1.64g sodium acetate dissolved in 200ml distilled water and 120µl acetic acid) with an equal amount of hydrogen peroxide, was added at 100µl/well and incubated in the dark for 20 minutes. 50µl/well of 2M sulphuric acid was added as a reaction stop solution, and the resulting blue colour was read with a spectrophotometer (Organon Teknika, Boxtel, The Netherlands) at 450nm absorbance minus a background of 540nm.

2.7 Statistical analysis

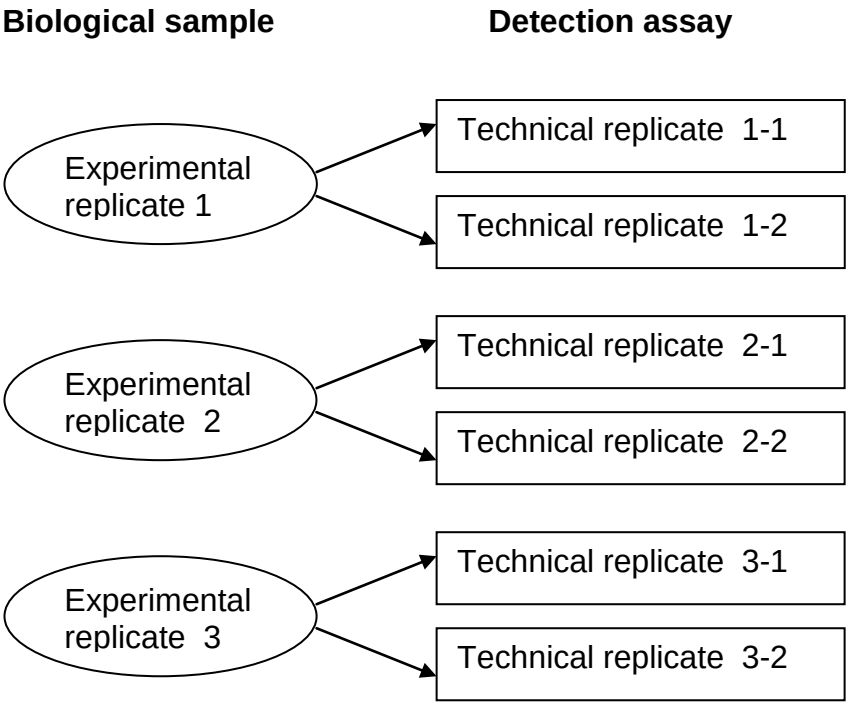


Figure 2.3. Format for experimental design.

The amount of eotaxin in a biological sample was measured as a technical replicate in a detection assay (Figure 2.3). For example, n=3 represents duplicate technical measurements of 3 biological samples, each of which was a set of 6×10^5 cells.

Standard error of the mean (SEM) is a measure of how accurate the sample mean is likely to be as an estimate of the biological sample, and was calculated for all results. $SEM = SD/\sqrt{n}$, where SD is sample standard deviation of the mean and n is number of samples.

2.7.1 Standard curves and linear regression

The coefficient of sample variation (CV) in the ELISA was determined for a representative standard curve. (CV=SD/mean) (See Results 3.1.1)

The response variable (eotaxin-3 amount in sample) per unit change in predictor variable (absorbance of chromogen complex) is plotted for each detection assay and the regression equation computed.

A large and significant coefficient of determination (R^2) portion of variance would be explained due to linear regression. The R^2 value can be interpreted as the proportion of percentage of the variance in absorbance that can be explained by variation in amount of eotaxin-3.

We explored possible relationships between ACN content of the BC cultivars and inflammation modulatory properties measured by eotaxin-3 levels generated in biological samples. Eotaxin-3 data for the BC cultivars (means ± SEM) were plotted against ACN levels for BC cultivars using linear regression (Microsoft Excel), and displaying trendlines and R^2 values. R^2 represented the strength of the association, and the slope of the regression line indicated a positive or negative trend.

2.7.2 Analysis of variance

The Student's t-Test (Microsoft Excel) was used to analyse data. This statistical procedure assumes the data follows a normal distribution, where 95% of the data fits within 2 standard deviations of the mean. The number of distribution tails was set at 2, and type at 2-paired (two different data sets), assuming equal sample variance. The analysis returns the probability (P) associated with the Student's t-Test, and is used to indicate significant differences from a parametric value other than zero. A confidence level of 95% was denoted as significant (*), with $P \leq 0.05$. If the P value was found to be less than 0.05, the null hypothesis was rejected and differences between arrays assumed to be significant. Other confidence levels used in this text were: ^ = $P \leq 0.01$, and $^{\#}$ = $P \leq 0.001$.

Chapter 3

Results

3.1 Optimisation experiments

3.1.1 Eotaxin-3 standard curve

Seven-point standard curves for each ELISA were created and plotted. The mean absorbance (450nm – 540nm) for duplicates of each standard were plotted against concentration of eotaxin-3.

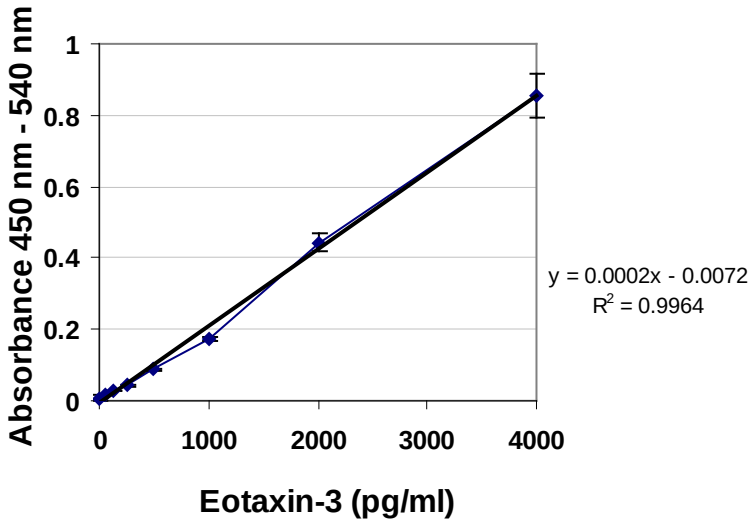


Figure 3.1. Representative eotaxin-3 standard curve (from IL-4 dose experiment). Data are means ± SEM (n=3 all samples). Regression line shown for data.

While the standard deviation (SD) between sample duplicates increased with concentration, the generally low SD values for this data indicated that duplicates were adequate for the detection ELISA measurement of individual biological samples (Table 3.1).

Table 3.1. Measures of accuracy of eotaxin-3 standard curve.

Standard (pg/ml)	Mean	SD	CV %	SEM
0	0.008	0.0099	124	0.007
63	0.0165	0.0007	4.3	0.0005
125	0.0265	0.0007	2.7	0.0005
250	0.0425	0.0021	5.0	0.0015
500	0.089	0.0042	4.8	0.003
1000	0.171	0.0085	5.0	0.006
2000	0.4425	0.0361	8.2	0.0255
4000	0.854	0.0863	10.1	0.061

The coefficient of subject variation (CV) for each serial dilution in the eotaxin-3 calibration curve represents the difference between sample values. The CV was shown to be large at 0 (124%), consistently low between 63 and 1000pg/ml, then increased above 5% variation from 2000pg/ml (Table 3.1).

These data confirm the manufacturer's recommendation to set the lower limit for the detection assay at 62pg/ml. This is the lowest concentration that can be detected with reasonable certainty in terms of the variation associated with absorbance measurement in the detection assay.

From the R^2 value of the regression line (Figure 3.1), the proportion of analytical error was 0.36%. Since the value for this graph (R^2=0.9964) was close to 1.00, the assumption can be made that the standard curve provided a reliable estimate of the amount of eotaxin-3 contained in the biological samples.

3.1.2 Activity of tetramethylbenzidine (TMB)

In the early stages of this study we found some variability in the colourimetric detection assay substrate. Hence we performed an analysis of the TMB product.

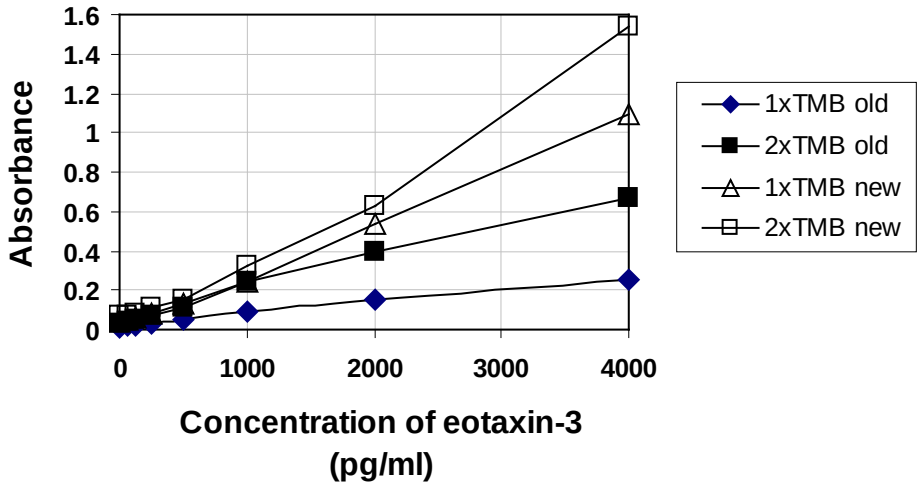


Figure 3.2. Evaluation of activity of detection assay chromophore TMB. Data are means (n=2 all samples).

These data indicate that a new stock of TMB would be appropriate (Figure 3.2). However, as twice-strength TMB approaches 2000pg/ml eotaxin-3, the relationship to absorbance was no longer linear. Therefore original strength TMB from new stock was used for further experiments.

3.1.3 A549 cell number optimisation

In order to optimise cellular conditions for determination of the effect of BC samples on IL-4-induced eotaxin-3 generation by A549 cells, we set out to

optimise the cell numbers required for good detection of eotaxin-3. To determine optimum cell numbers to use in the biological assay, A549 cells (6×10^5, 3×10^5, 1.5×10^5, 7.5×10^4, 0) were grown to confluency and then co-incubated for 24 hours with IL-4. Biological replicates (n=6) were measured in triplicate in the ELISA detection assay to determine the amount of Eotaxin-3. Samples were undiluted for the ELISA detection.

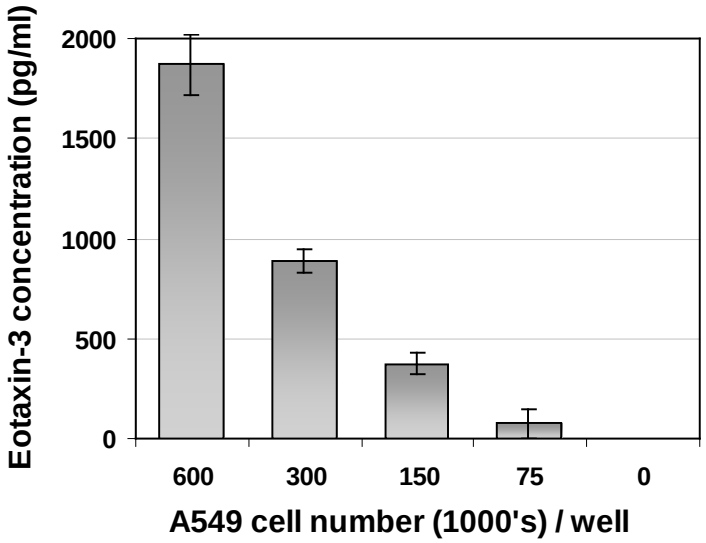


Figure 3.3. Response to IL-4 by cell number. 24 hour incubation time course. Data are means ± SEM, (n=6 all samples).

As shown, 6×10^5 A549 cells gave a quality eotaxin-3 response (1,869 pg/ml ± 150) to IL-4 challenge (Figure 3.3), as it was within the linear region of the ELISA standard curve and easily detectable.

3.1.4 Cell supernatant for ELISA assay

In order to validate the ELISA detection assay for measurement of samples, supernatants from two concentrations of A549 cells (6×10^5 and 3×10^5) were

diluted in reagent buffer (neat, 1:2 & 1:4) to establish how the ELISA should be calibrated for the biological samples.

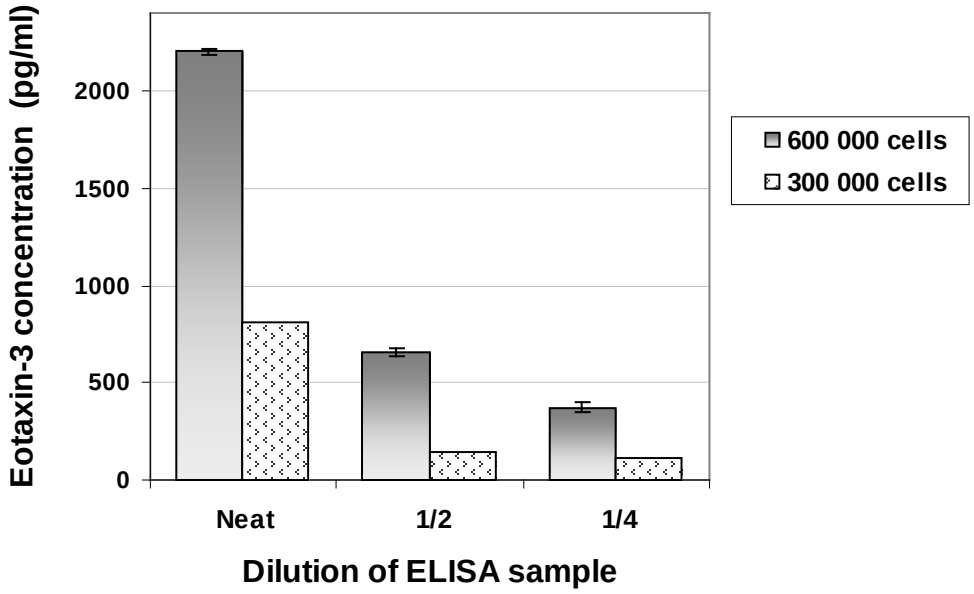


Figure 3.4. Cell culture supernatants (6 x 10^5 cells and 3 x 10^5 cells) and their dilution factors (neat, 1:2, 1:4) compared by ELISA. Data are means ± SEM, for 600,000 cells (n=3), except for 300,000 cells, (n=1).

Undiluted samples of 6 x 10^5 cells were chosen for further ELISA experiments, since this supernatant sample gave good eotaxin-3 detection values (Figure 3.4). All samples were measured in triplicate, and error bars indicated reliability of measurement for 6 x 10^5 cells.

3.2 IL-4 cytokine challenge to cells

3.2.1 Dose response

A549 cells (6 x 10^5 cells/ml) were stimulated with varying doses of IL-4 (50, 25, 10, & 2.5ng/ml) in order to determine optimum dose for A549 cell activation.

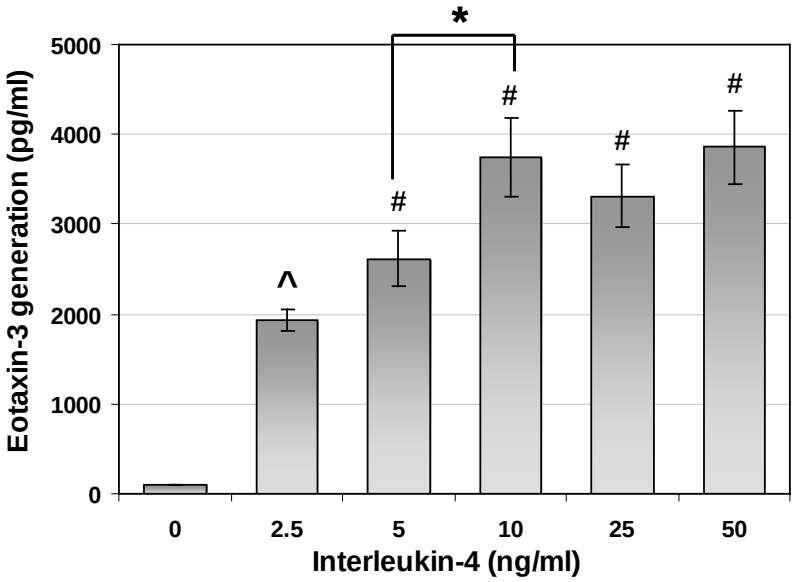


Figure 3.5. Determination of optimum IL-4 concentration to stimulate eotaxin-3 generation by A549 cells. IL-4 doses (50, 25, 10, 5, 2.5 ng/ml) were incubated with cells for 24 hours (n=14 all concentrations). Results are means ± SEM. ^ = P≤ 0.01 and # = P≤ 0.001 vs. control, * = P≤ 0.05 for 5ng/ml vs. 10ng/ml.

Eotaxin-3 generation dose-dependently increased with IL-4 dose, to a maximum response at 10ng/ml (P≤ 0.001 vs. control). All doses tested were significantly different from 0, and there was a significant difference between the cell response to 10ng/ml vs. 5ng/ml IL-4 (P=0.04) (Figure 3.5). Therefore, it could be assumed that at 5ng/ml, receptors for IL-4 on A549 cells were not saturated, and that 10ng/ml was the lowest dose with maximum receptor binding site occupancy.

10ng/ml IL-4 was therefore considered the optimum IL-4 dose for stimulated generation of eotaxin-3 at 24 hours incubation with A549 cells, for all further experiments.

3.2.2 Incubation time course for IL-4

Previous IL-4 incubations with the cells were carried out for 24 hours, but the response over time needed to be explored. In order not to affect the exposure of cells by changing ratios of media and IL-4, separate samples for each time point were prepared in the following time course experiments.

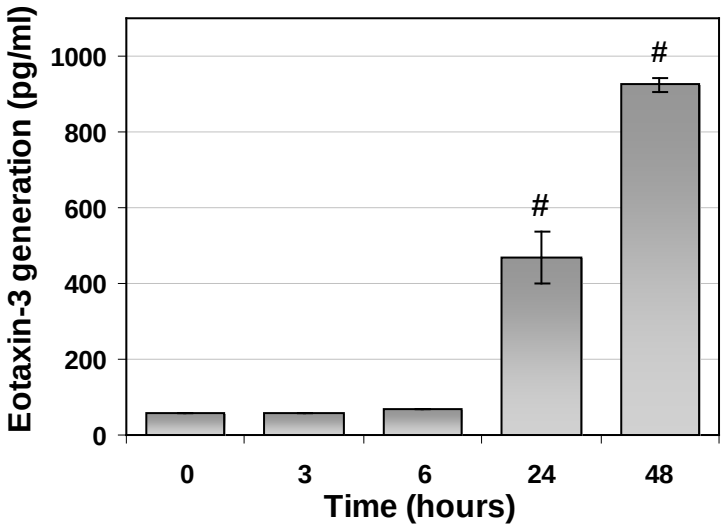


Figure 3.6. Time course for IL-4 incubation with A549 cells (n=6 all time points). Results are means ± SEM. # = P≤ 0.001 vs. control.

Eotaxin generation remained constant at baseline levels for the first 6 hours of incubation with IL-4 (10ng/ml), then increased significantly in a time-dependent manner from 24 to 48 hours incubation (P≤ 0.001), (Figure 3.6, & Appendix 1). Less than 24 hours incubation with IL-4 did not stimulate the cells sufficiently to be able to generate eotaxin-3 in response. The release of eotaxin-3 from the A549 cells increased over time from 24 to 48 hours, but it was deemed sufficiently responsive as an assay to choose 24 hours incubation with the cells for further experiments.

3.3 Cytotoxicity

Since eotaxin-3 was released from granules inside the A549 cells, and IL-4 stimulation was via ligand-receptor interactions, it did not necessarily follow that the granules released their contents in response to IL-4. Therefore, we needed to establish that the IL-4 was of a sufficient purity so that lipopolysaccharide (LPS) cell wall fragments from the bacteria it was produced by, did not contribute to cell rupture and granule release. In addition, the solvent dimethyl sulfoxide (DMSO) used to reconstitute the BC extracts may have toxic effects, and the BC extracts themselves may be toxic to cells. Polyphenolic extracts showed considerable toxicity to hepatic cells at 50µg/ml concentration (Kanazawa et al., 2006), and we needed to find BC extract concentrations that were non-toxic to the human lung cells in this study.

Since the product information for the *E. coli*-produced Recombinant Human IL-4 used in this assay stated that endotoxin (eg. LPS) level was less than 0.1 ng/µg, it was assumed that the IL-4 was not highly toxic to the cells. Hydrogen peroxide (H_2O_2) was used as a positive control for the cytotoxicity (WST-1) assay, but concentration of H_2O_2 and response by the cells needed to be established so that a comparison to maximum cell death over the WST-1 incubation time with A549 cells could be made.

The manufacturer's recommendation for the WST-1 assay, measuring percent cell viability, was for an incubation time of four hours with the cells.

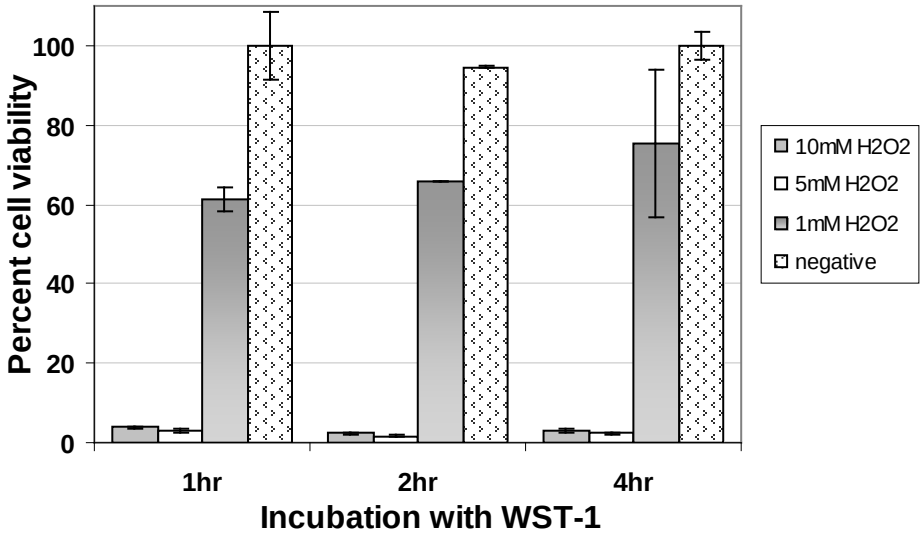


Figure 3.7. Establishing the positive control for determination of cytotoxicity in A549 cells (n=3 all time points). Results are means ± SEM.

Over a four hour incubation period with WST-1, it was shown that the 5mM concentration of H_2O_2 was effective as a cytotoxic agent for A549 cells (2.4% ± 0.3% cell viability) (Figure 3.7). This concentration of H_2O_2 was therefore chosen as the cytotoxic positive control agent for A549 cells in the WST-1 assay.

3.3.1 Cell toxicity of selected blackcurrant cultivars

The polyphenolic BC extracts used in the subsequent assays may have been toxic to cells at high doses. In addition, the DMSO solvent may exert effects independent of the BC extracts. We therefore tested two blackcurrant extracts (0129-02 & 0129-06) at varying concentrations (50, 25, 10 & 1µg/ml), and DMSO in excess (5%), for cytotoxicity to A549 cells.

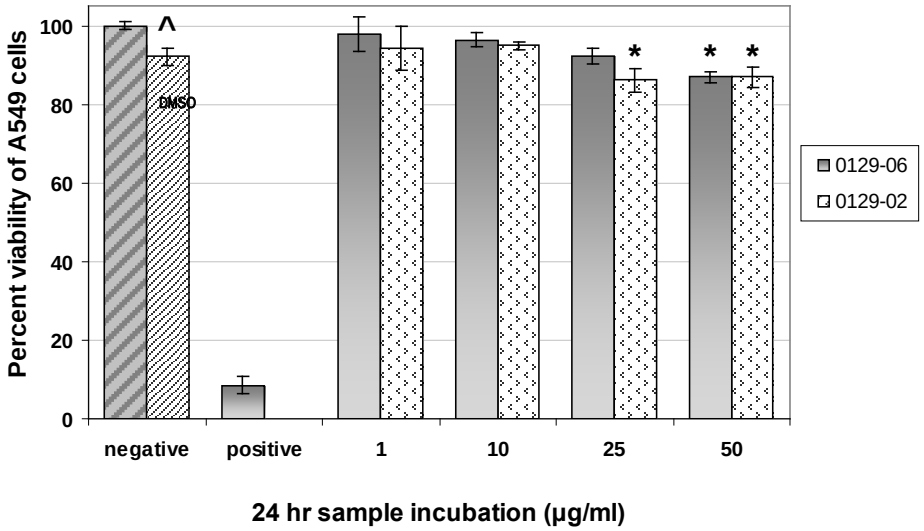


Figure 3.8. A549 cells exposed to successive doses of polyphenolic blackcurrant (BC) extracts for 24 hours and assessed for cell viability. DMSO sample was in excess (5%) (bar 2). Positive control was H_2O_2 (5mM), (bar 3), (n=8 all samples except for BC 0129-02, 25 µg/ml where n=4). The WST-1 assay measured the product formed when a tetrazolium salt was reduced to a formazan dye by metabolically active cells. Data are means ± SEM. * = P≤ 0.05, ^ = P≤ 0.01 vs. negative control.

There was a statistically significant difference between the negative control and the DMSO sample (P≤0.01), indicating that this solvent was slightly toxic to cells (7.8 ± 2.1%) (Figure 3.8). While the differences between the negative control and both of the 50µg/ml BC samples, and BC 0129-02 at 25µg/ml were significant (P≤0.05), they were not statistically different from the DMSO control. This meant that no further cytotoxicity was due to the polyphenolic BC extracts at any of the concentrations tested.

Therefore, DMSO was exerting a slight (≤10%) cytotoxic effect on the cells, with no further significant increase in cell death due to BC extracts. However, since a trend in cell death was observed for the BC extracts from lower to higher

concentration, 10µg/ml was chosen as the non-toxic dose for further experiments.

3.4 Co-incubation with IL-4

The aim of this study was to find a polyphenolic BC extract dose that was within a physiologically relevant range, was non-toxic to cells and was able to effectively attenuate the A549 cellular response to inflammatory challenge.

3.4.1 Blackcurrant extract dose response

A549 cells (6 x 10^5 cells/ml) were grown to confluency and exposed to a range of polyphenolic BC extract doses (1, 10 & 50µg/ml), (DMSO 0.1, 1 & 5% respectively) in a series of co-incubation experiments with IL-4 (10ng/ml) for 24 hours. The supernatant was collected and quantified for eotaxin-3 by specific ELISA.

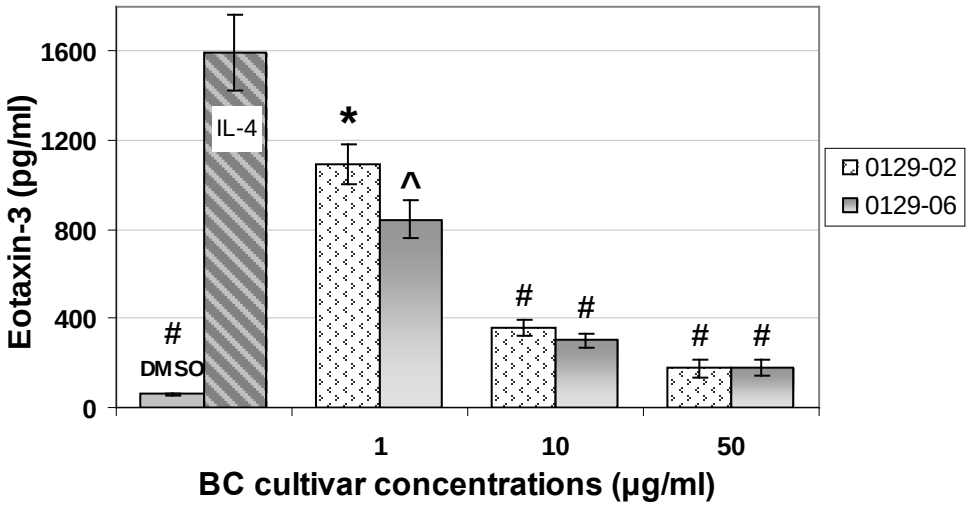


Figure 3.9. Comparison of BC extract doses (1, 10 & 50 µg/ml) after co-incubation with IL-4 and A549 cells for 24 hours. Data are means ± SEM (n=9 all samples). * = P≤0.05, ^ = P≤0.01, # = P≤0.001 vs. stimulated control (IL-4). DMSO control = 5% in culture medium.

It can be seen from these results (Figure 3.9 & Appendix 3) that a clear and dramatic response by cells co-incubated with polyphenolic BC extracts is possible. A significant difference between eotaxin-3 production by IL-4-stimulated cells and BC extract 0129-02 at 1µg/ml concentration was demonstrated (P=0.02). All other BC extracts further reduced the amount of inflammatory cytokine produced by cells in a dose-dependent and statistically significant manner. While the variance between 0129-02 and 0129-06 cultivars at 1µg/ml was not significant, intriguing differences in cell response to BC cultivars were achieved at low concentrations.

3.4.2 Dimethyl sulfoxide (DMSO) effects

Further experiments to determine the extent of influence of DMSO on the observed BC extract effects on cells was required. We therefore compared A549 cell response to a range of DMSO concentrations (0.01, 0.1, 1 & 5%) co-incubated with IL-4 (10ng/ml).

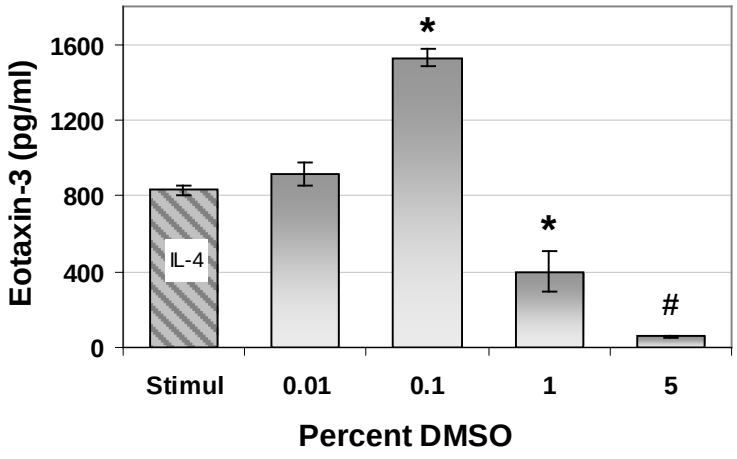


Figure 3.10. Co-incubation of DMSO (0.01, 0.1, 1, & 5%) and IL-4 with A549 cells for 24 hours. Data are means ± SEM (n=3 all samples). * = P≤ 0.05, # = P≤ 0.001 vs. stimulated control (IL-4).

From these results (Figure 3.10), it was clear that DMSO had a significant effect on cellular eotaxin-3 production that was independent of the BC extracts at all concentrations except 0.01% (P≤ 0.05 at 0.1% and 1% DMSO, P≤ 0.001 at 5% DMSO).

The higher concentrations of DMSO could have interfered with IL-4 production and therefore influenced eotaxin-3 production, with eotaxin-3 production alone, or killed the cells so they were unable to produce eotaxin-3. DMSO at 5% also interfered with binding of the eotaxin-3 standard to substrates in the ELISA detection assay, independent of exposure to cells (data not shown). Therefore the DMSO concentrations in the BC extracts were limited to 0.01% for all further experiments.

It was important to discover whether the DMSO (0.01%) affected IL-4 stimulation of cells independent of the BC extracts. To address this issue, we compared IL-4 stimulated DMSO controls with a selected BC extract (BC 0129-03) dissolved in PBS with 0.01% DMSO.

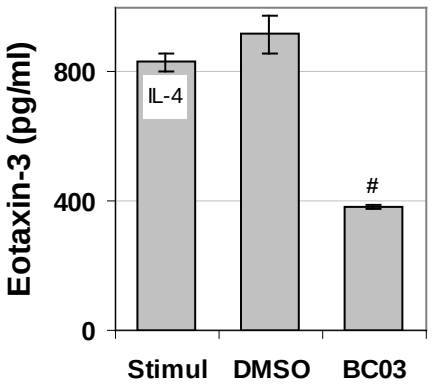


Figure 3.11. Comparison of DMSO control (0.01%) with a BC extract (BC 0129-03) containing equivalent amount of DMSO. Data are means ± SEM (n=3 all samples), # = P≤ 0.001 vs. stimulated control (IL-4).

These results showed that the minimum amount of DMSO in the BC extracts (0.01%) did not affect the IL-4 stimulated eotaxin-3 generation by cells (Figure 3.11). It was also demonstrated here that a BC cultivar (BC 0129-03) containing 0.01% DMSO, significantly reduced the amount of eotaxin-3 produced by A549 cells compared to IL-4 (P≤ 0.001). Therefore, an inflammatory modulation effect was demonstrated in this cell system.

3.4.3 All blackcurrant cultivars co-incubated with IL-4

A comparison of ten polyphenolic BC extracts was made with respect to their ability to reduce eotaxin-3 generation by IL-4 stimulated cells.

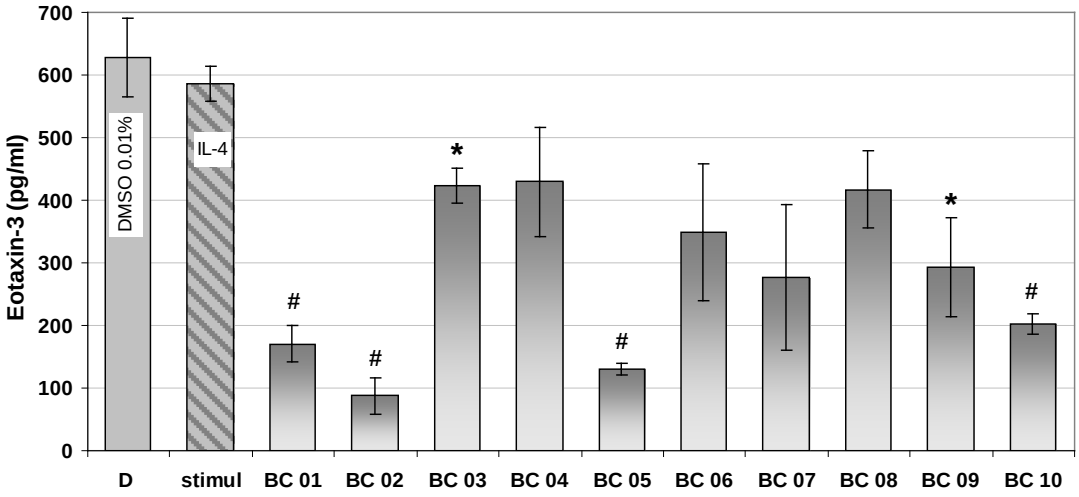


Figure 3.12. All blackcurrant extracts (BC 0129-01 to 10, [10µg/ml]) were co-incubated with IL-4 (10ng/ml) and A549 lung epithelial cells (6 x 10^5 cells) for 24 hours (n=3 all cultivars), (DMSO =0.01%). Results are means ± SEM. ***** = P≤ 0.05, $^{\#}$ = P ≤ 0.001 vs. stimulated control (IL-4).

The strongest relationship between reduction of IL-4 stimulation (10ng/ml) and BC cultivar (10µg/ml) in this analysis was achieved by BC 0129-02 (P=0.0002), but BC 0129-05 (P=9 x 10^{-5}), BC 0129-01(P= 0.0005) and BC 0129-10

(P=0.0003) were also impressive in reducing the amount of inflammatory chemokine (eotaxin-3) generated by lung epithelial cells in culture (Figure 3.12). All BC cultivars reduced the amount of eotaxin-3 in response to IL-4, and six BC cultivars (BC 01, 02, 03, 05, 09 & 10) significantly reduced eotaxin-3 levels in stimulated cells (P≤ 0.05).

Table 3.2. Most effective inflammation modulating BC cultivars from this study.

Blackcurrant cultivar	Percent reduction from control
0129-01	71 ± 4.9
0129-02	85 ± 5
0129-05	78 ± 1.5
0129-10	66 ± 4.9

The four most effective BC cultivars identified in this study reduced eotaxin-3 levels from IL-4 stimulated controls by more than 65%, and are presented in Table 3.2.

Four BC cultivar results were not significantly different from stimulated controls (BC 04, 06, 07 & 08), largely due to error bars. This variation could possibly have been improved with more experimental repeats.

3.5 Pre-incubation

Previous studies by other scientists in the laboratory indicated that pre-incubation of cultivars with the cells, followed by IL-4 stimulation, would produce

an effective eotaxin-3 response. This concept was explored in a set of experiments with selected BC cultivars in order to make a comparison with co-incubation data.

3.5.1 Pre-incubation of selected BC cultivars

Three BC cultivars (BC 0129-01, -02 & -03) were pre-incubated with A549 cells, washed off, and the cells were then stimulated with IL-4.

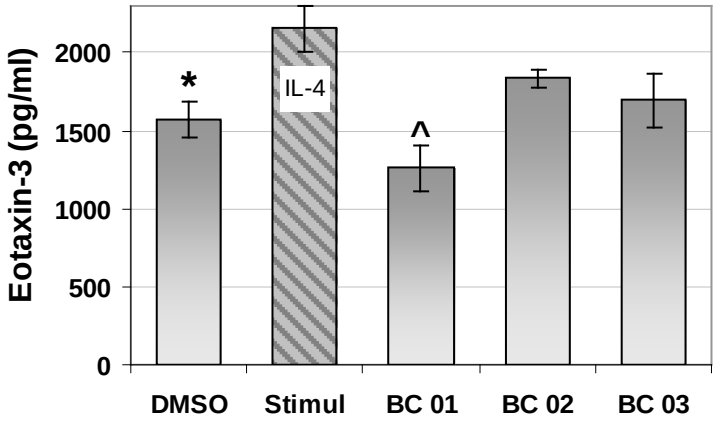


Figure 3.13. Pre-incubation of blackcurrant cultivars (10µg/ml) with A549 cells for 24 hours, then stimulated with IL-4 for 24 hours. Results are means ± SEM (n=4 all samples), (DMSO=0.01%). * = P≤ 0.05, ^ = P≤ 0.01 vs. stimulated control (IL-4).

The three BC cultivars tested (BC 0129-01, -02 & -03) reduced cellular eotaxin-3 generation by pre-incubation before IL-4 stimulation (Figure 3.13), and BC-01 reduced eotaxin-3 levels by statistically significant levels compared to the IL-4 control (P=0.008). However, the DMSO control was significantly different from the IL-4 control (P= 0.02), and none of the BC extracts were different from the DMSO control. Therefore, this technique was not explored further with respect to identifying inflammation modulation effects of the BC cultivars.

3.6 Comparison of co- and pre-incubation treatments

A comparison between cellular incubation treatments was made, normalising results to 100% of IL-4 stimulation by A549 cells to identify any similarities or differences between BC cultivar responses.

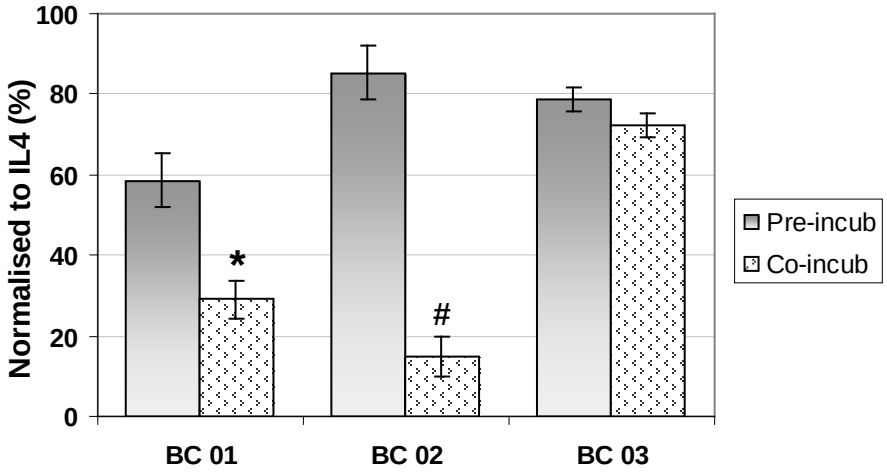


Figure 3.14. Comparison of BC extract treatments with respect to IL-4 stimulation. Cellular responses (eotaxin-3 generation) for selected BC extracts (BC 0129-01,-02 & -03 [10µg/ml]) were normalised to 100% IL-4 stimulation for each treatment (co-incubation and pre-incubation). Results are means ± SEM (n=4 pre-inc., n=3 co-inc.), (DMSO=0.01%). * = P≤ 0.05 and # = P≤ 0.001 for pre-incubation vs. co-incubation.

This comparison of cellular treatments for BC extracts with respect to percent reduction from IL-4 stimulated controls revealed different sensitivities for individual BC cultivars (Figure 3.14). While there was no difference between co- and pre-incubation for BC-03, significant differences between treatments were shown for BC-01 (P≤ 0.05), and BC-02 (P≤ 0.001).

3.7 Influence of anthocyanins (ACNs) on inflammation modulation

Compositional data of the BC extracts were provided by Plant and Food Research in order to explore possible relationships between either individual ACNs or total ACN glycosides and inflammation modulation properties that were established in this study (Table 3.3).

Table 3.3. Anthocyanin (ACN) levels in BC cultivars. Quantities (mg) per gram of polyphenolic BC extract.

BC cultivar	Dp-glu	Dp-rut	Cy-glu	Cy-rut	Tot rut	Tot glu
			(mg/g)			
O129-01	11	108	10	167	298.3	35.5
O129-02	3	74	3	195	315.9	20.5
O129-03	19	144	15	135	296.5	49.1
O129-04	34	114	19	121	249.8	61.6
O129-05	23	146	11	145	310.9	47.3
O129-06	36	145	15	135	302.1	61.4
O129-07	24	112	12	120	253.2	48.6
O129-08	24	122	12	140	278.7	46.1
O129-09	14	118	6	89	230.6	28.1
O129-10	8	68	11	173	274.5	39.7

3.7.1 Four major ACNs in BC extracts

We explored the relationships between the four major ACNs (mg/g) and the inflammation modulation responses generated for the BC cultivars (eotaxin-3, pg/ml) in the figures presented.

Since reduced values of eotaxin-3 (low *y* co-ordinate) for the regression lines represented an inflammation modulation effect, the *x* co-ordinate (amount of

ACN) was reversed so that a positive slope correlated with positive inflammation modulation.

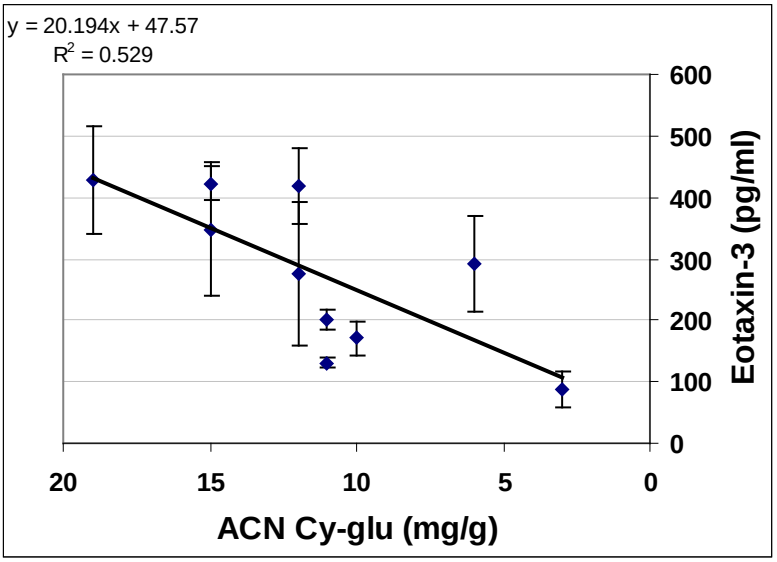


Figure 3.15. Relationship between cyanidin glucoside component of BC extracts vs. immune modulatory effect (eotaxin-3). Values for BC extracts are means ± SEM (n=3 all BC cultivars).

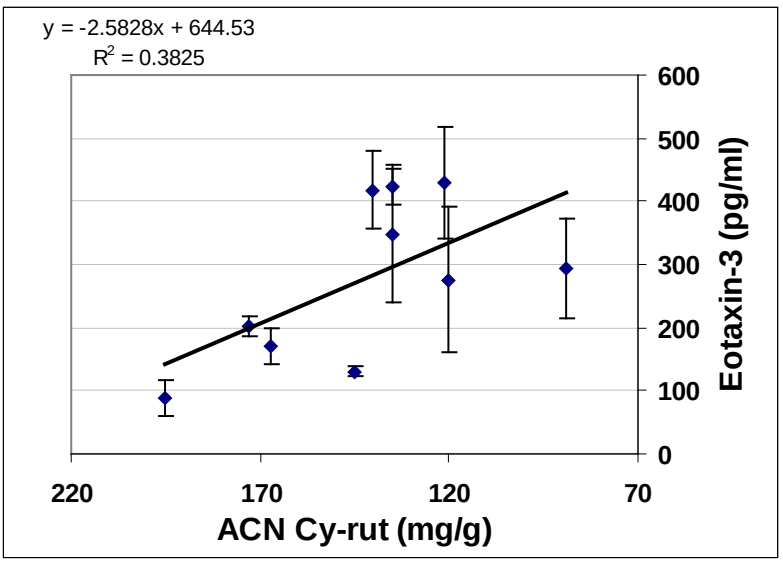


Figure 3.16. Relationship between cyanidin rutinoside component of BC extracts vs. immune modulatory effect (eotaxin-3). Values for BC extracts are means ± SEM (n=3 all BC cultivars).

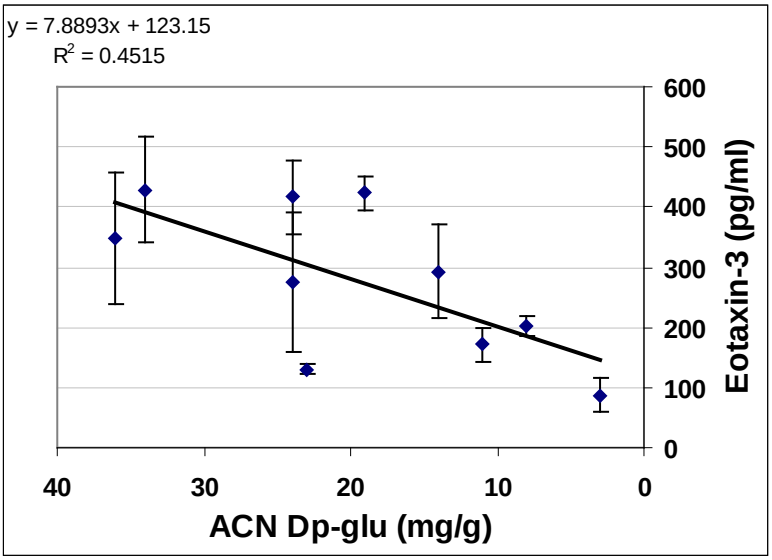


Figure 3.17. Relationship between delphinidin glucoside component of BC extracts vs. immune modulatory effect (eotaxin-3). Values for BC extracts are means ± SEM (n=3 all BC cultivars).

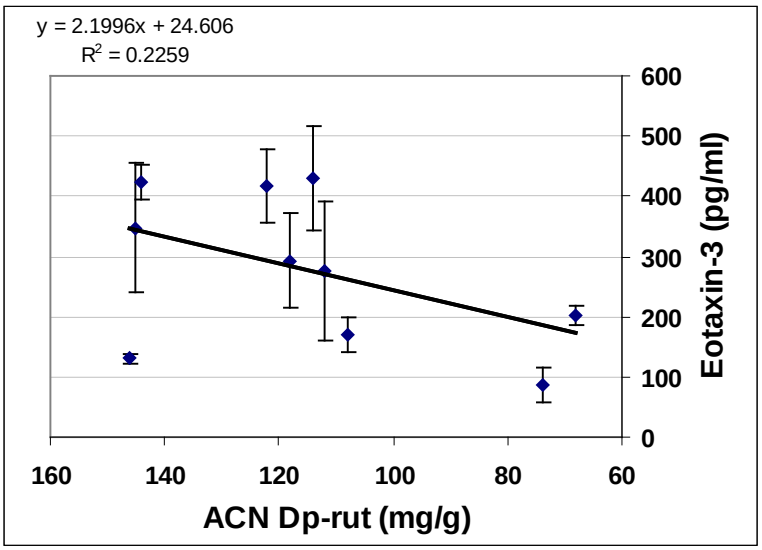


Figure 3.18. Relationship between delphinidin rutinoside component of BC extracts vs. immune modulatory effect (eotaxin-3). Values for BC extracts are means ± SEM (n=3 all BC cultivars).

A weak positive correlation was demonstrated between cyanidin rutinoside (Cy-rut) and eotaxin-3 (R^2=0.38), indicating a positive association between this ACN and inflammation modulation (Figure 3.16). However, the slopes of the

regression lines for the other major ACNs contained in the BC extracts were negative (Figures 3.15, 3.17 & 3.18). This indicated a possible antagonistic effect for these ACNs (cyanidin glucoside (Cy-glu), delphinidin glucoside (Dp-glu) & delphinidin rutinoside (Dp-rut)) to the positive modulation shown for Cy-rut. Therefore, within one BC cultivar, the ratio of ACN components was shown to influence the inflammation modulation effect.

3.7.2 Total ACN glycosides in BC extracts

In order to assess possible influences of glycosylation of ACNs in the BC extracts on inflammation modulation, a comparison was made between total glucosides vs. eotaxin-3, and total rutinosides vs. eotaxin-3.

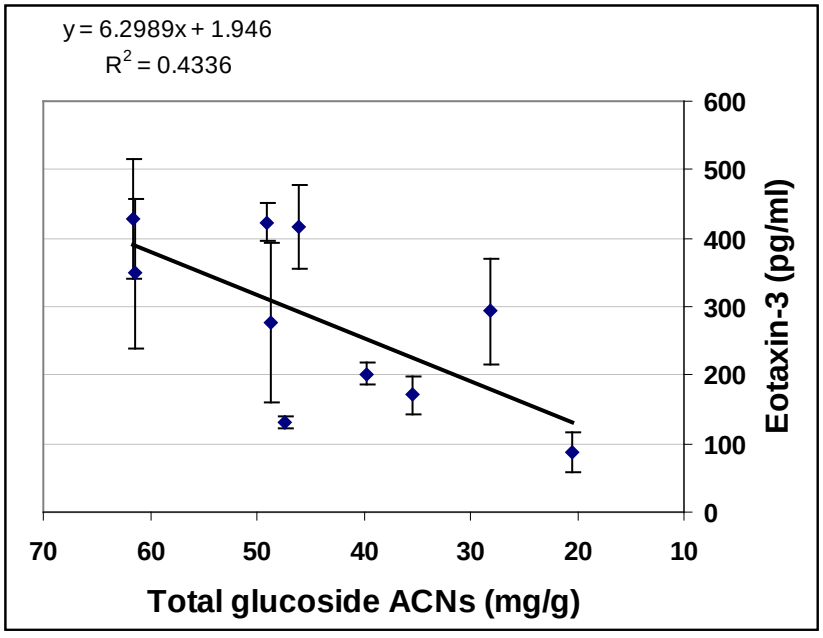


Figure 3.20. Relationship between total glucosides in BC extracts vs. immune modulatory effect (eotaxin-3). Values for BC extracts are means ± SEM (n=3 all BC cultivars).

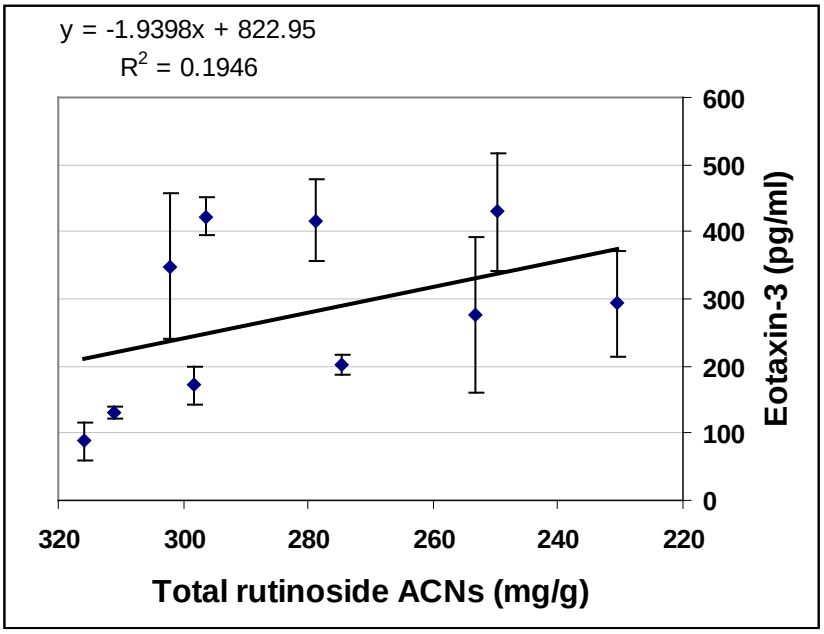


Figure 3.21. Relationship between total rutinosides in BC extracts vs. immune modulatory effect (eotaxin-3). Values for BC extracts are means ± SEM (n=3 all BC cultivars).

While a positive trend was shown between rutinoside content of BC extracts and immune modulation (Figure 3.21), no relationship was established, which was indicated by a low correlation coefficient (R^2= 0.195). On the other hand, the weak negative correlation between total glucosides vs. eotaxin-3 (R^2 = 0.43) (Figure 3.20), represented a negative impact within the BC cultivars on inflammation modulation due to glucoside moieties of ACNs.

In considering the rutinosides as a group, a weak positive inflammation modulation effect for the Cy-rut component of the BC extracts was made (Figure 3.16), but the trend was weaker for total rutinosides (Figure 3.21). This meant that one particular rutinoside ACN (Cy-rut) could be largely responsible for the positive trend in the relationship between inflammation modulation and ACN content of polyphenolic BC extracts (10µg/ml).

Chapter 4

Discussion and conclusions

4.1 Interpretation of the results

This study of “an analysis of polyphenolic BC extracts for the potential to modulate allergic airway inflammation” was designed to evaluate the effectiveness of BC cultivars in reducing a marker of inflammation in human lung epithelial cells after simulated allergen challenge. If these berryfruit extracts are shown to reduce the inflammatory marker eotaxin-3, then a non-toxic cellular response is identified. This information may be useful for formulation of a nutritional product of benefit to asthmatics.

In this study, polyphenolic extracts of ten BC cultivars reduced eotaxin-3 in response to IL-4 challenge to lung cells, and therefore demonstrated inflammation modulatory properties associated with asthma. Six BC cultivars significantly reduced levels of the marker of inflammation, revealing differences in efficacy between the BC cultivars. One particular anthocyanin (ACN) was weakly but positively associated with inflammation modulation.

4.1.1 Effects of polyphenolic blackcurrant extracts

All ten polyphenolic BC extracts (0129-01 to -10, [10µg/ml]), co-incubated with IL-4 (10ng/ml) and A549 lung epithelial cells, reduced eotaxin-3 levels compared to stimulated controls. Six of the extracts (0129-01,-02, -03, -05, -09 & -10) reduced the inflammatory chemokine levels to statistically significant

levels (P< 0.05), and four BC cultivars were identified as being particularly effective at reducing IL-4 stimulation (P≤ 0.001), (Table 3.2).

4.1.2 Eotaxin-3 release by stimulated lung epithelial cells

The objective of this study was to measure the effectiveness of BC polyphenolic extracts in the context of TH_2 cell recruitment and activation associated with sustained inflammation.

In this study, it was shown that cells stimulated with IL-4 at 10ng/ml produced eotaxin-3 at a significantly higher level than at 5ng/ml (P=0.04), but no change was observed after increasing the IL-4 concentration further. Therefore, it could be assumed that at 5ng/ml, receptors for IL-4 on A549 cells were not saturated, and that 10ng/ml was the lowest dose with maximum receptor binding site occupancy.

Other researchers demonstrated that although IL-4 and IL-13 shared receptors, IL-4 was more potent at inducing eotaxin-3 release (van Wetering et al., 2007), and at STAT-6 activation with significantly lower doses than IL-13 (LaPorte et al., 2008). Therefore cell response to IL-4 stimulation was chosen as representative of a TH_2 physiological response.

IL-4 incubation for 6 hours did not raise eotaxin-3 production by the cells to a measurable extent above baseline, whereas at 24 hours a significant increase was seen (P≤ 0.001), which continued in a time-dependent manner to 48 hours.

The cellular production of eotaxin-3 in response to inflammatory cytokine stimulation largely involves gene transcription signals via phosphorylation of the STAT-6 kinase subunit associated with the IL-4 receptor. Since the STAT-6 binding site of IL-4 was shown to be necessary for full eotaxin-3 promoter activity in intestinal epithelial cells (Blanchard et al., 2005) and bronchial epithelial cells (Mullings et al., 2001), it was assumed that insufficient time had evolved before 24 hours stimulation of cells for signal transduction effects to have occurred.

There was a background level of pre-formed eotaxin-3 released by the cells from cytoplasmic granules at the lower detection limit of the ELISA assay up until 6 hours of IL-4 incubation. Stimulus-induced exocytosis of secretory proteins from nascent vesicles, as required by cytokines, is a tightly regulated process. In many cell types, a large reserve pool exists, but only a small portion of the granules are mature and rapidly releasable, and the process of granule maturation alone does not confer plasma membrane fusion competence (Burgoyne et al., 2003).

In order for synthesis of eotaxin-3 to have occurred, IL-4 receptors on the epithelial cells needed to be engaged with their cytokine ligand to signal transcription and translation of the eotaxin-3 peptide, followed by granule maturation and release. This possibly explains the time lag before a significant eotaxin-3 cellular response to cytokine challenge was observed.

Other researchers used 24 hour incubation of IL-4 at 10ng/ml in A549 cells to measure eotaxin-3 response (Abonyo et al., 2006), which confirmed the validity of the IL-4 incubation dose and time-course used in these studies.

4.1.3 Cytotoxicity, and effects of DMSO

One objective of this study was to provide evidence towards including BC extracts in a healthful berry product. Therefore it was of importance that the nutritional dose of polyphenolics was not toxic to cells and tissues. While significant losses are incurred from ingested polyphenolics, some are absorbed intact.

It was demonstrated here that the polyphenolic BC extracts at 10µg/ml were not toxic to cells, but concentrations above that began to cause cell death for some cultivars. The solvent DMSO was shown to be slightly toxic to the cells (8 ± 2%, P=0.004), however this was in excess of final concentrations of DMSO used in BC extracts. The BC extracts (1,10, 25 & 50µg/ml) did not show cytotoxic increases above the level of DMSO, and therefore further increase in cell death was not attributable to the polyphenolic BC extracts. However, since a trend in cell death was observed for the BC extracts from lower to higher concentration, 10µg/ml was chosen as the non-toxic dose for further experiments.

In agreement with these results, a concentration of 10µg/ml of purified polyphenolics (PPs) was found to be a non-toxic, physiologically relevant dose to human hepatic cells *in vitro* (Kanazawa et al., 2006), and a high PP fruit and

vegetable juice was found to be non-toxic to hepatic cells at concentrations from 3 to 30µg/ml (García-Alonso et al., 2006a).

DMSO is an oxidant, and the thiol regulatory domains of the kinase and phosphatase enzymes of cytokine receptor molecules are particularly responsive to modification by oxidants (Suzuki et al., 1997). IL-4 activation can therefore be stimulated by oxidants, and receptor interactions can produce RONS.

In this study it was demonstrated that DMSO interfered with IL-4 binding at concentrations above 0.01% (Figure 3.10). DMSO could have stimulated the addition or removal of phosphate moieties by enzyme interaction within the IL-4 receptor complex. Alternatively, changes in the level of RONS may have influenced eotaxin-3 production by the cells. DMSO may also have caused suppression of eotaxin-3 secretion by directly binding with IL-4 or masking its receptors.

In pre-incubation experiments, a significant difference between the IL-4-stimulated cells and the DMSO (0.01%) control was shown (P=0.02), (Figure 3.13). With pre-incubation, the cells were exposed to DMSO for 24 hours, washed, and then incubated with IL-4. This means the DMSO was taken up by the cells independently of IL-4 to influence eotaxin-3 generation. This could indicate oxidation within cell membranes, or priming of enzyme complexes involved in the eotaxin signal transduction cascade within the IL-4 receptor.

Intriguing differences in cellular response between BC cultivars, with respect to reduction from IL-4 stimulated controls, for two incubation techniques was demonstrated (Figure 3.14). While there was no difference between co- and pre-incubation for BC-03, significant variation between treatments were shown for BC-01 ($P \leq 0.05$), and BC-02 ($P \leq 0.001$). Since it was demonstrated that DMSO at 0.01% affected the cells by pre-incubation but not co-incubation, DMSO could have influenced the cellular response to BC cultivars. With an altered cellular oxidative environment, the distinctive polyphenolic compositions of individual BC cultivars may have responded uniquely to the oxidative insult.

4.1.4 Antioxidant and modulatory properties of polyphenolics

While not all inflammation is detrimental to the body, the focus in this study was on inappropriately sustained inflammation associated with cellular damage in asthma. The preliminary results with boysenberry and blackcurrant fruit extracts in the A549 cell system showed a reduction in eotaxin-3 production by IL-4 stimulated lung epithelial cells. Down-regulation of eotaxin-3 transcription, and reduction in IL-4 receptor STAT-6 phosphorylation was previously demonstrated (Plant and Food Research, unpublished data). However, since these berryfruit juices were not polyphenolic extracts, they would also have contained other bioactive nutrients and phytochemicals.

In this study the effects of BC polyphenolic extracts on a marker of TH_2 cell-mediated inflammation were tested. The extracts of ten BC cultivars all demonstrated a reduction in production of eosinophil-selective chemokine in human lung epithelial cells, but to varying degrees. This implies that

differences in polyphenolic composition, and/or synergistic effects in the BC cultivars influenced inflammatory modulation.

In human hepatocyte cultures, antioxidant properties of high-polyphenolic juice were demonstrated when the cells prevented lipid peroxidation caused by H_2O_2 (García-Alonso et al., 2006a), but this could have been due to the high vitamin C content along with other bioactive substances present in the juice. Lower doses of PPs were not found to have significant antioxidant activity, but the phenolics were shown to be rapidly taken up by the cells.

Dietary intake of a juice designed to deliver the equivalent of 10 servings of fruit and vegetables per dose improved the antioxidant status of healthy individuals after a short-term trial (García-Alonso et al., 2006b). But the anthocyanins (ACNs) in the phenolic-rich juice were poorly absorbed, and were shown to make a negligible contribution to total antioxidant capacity of subjects. ACN fractions were shown to be minor contributors to antioxidant capacity in a study of phenolics in raspberries (Mullen et al., 2002).

The consumption of berries high in ACNs could affect inflammatory pathways by several different mechanisms unrelated to antioxidant activity. A diet of freeze-dried berries inhibited oesophageal and colon cancer progression in rodents (Stoner et al., 2007). The ACN fraction containing cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, was shown to be effective, indicating that cyanidin glycosides accounted for some of the disease prevention activity of the berry extracts.

In this study, the polyphenolic BC extracts were complex mixtures of PPs containing anthocyanins (≈30%), with major ACNs being the cyanidin and delphinidin glycosides. Extraction methods of PPs from the berryfruit involved specific binding to resin columns followed by washing and reconstitution to control reaction parameters, including pH and other bioactives such as vitamin C. Therefore we can have confidence that the inflammation modulatory properties demonstrated in these studies were attributable to PPs.

4.1.5 Cellular environment and RONS

While RONS generation is a necessary part of the immune defence in inflammatory states, prolonged generation of these free radicals is destructive to cells. For example, inducible NO synthase (iNOS) gene expression and production is up-regulated in asthmatics, which leads to airway hyperresponsiveness and epithelial tissue damage (Nevin & Broadley, 2002). RONS metabolites produced by cells in close proximity to epithelial cells can influence chemotactic factors. For example, the $OONO^-$ radical has been shown to modulate eosinophil migration by inhibiting eotaxin ligand/receptor binding (Sato, Simpson, Grisham, Koyama & Robbins, 2000).

One confounding factor in studies of RONS in biological systems is protein nitration and modulation of phosphorylation reactions in signal transduction cascades, measured as 3-nitrotyrosine. In allergic inflammation in mice, formation of 3-nitrotyrosine was shown to be dependent on peroxidase enzyme activity within eosinophils (Duguet et al., 2001), and independent of iNOS

upregulation. While most of the protein nitration was located in the airways of mice after challenge, none was located in the epithelium.

Because of the heterogeneous nature of lung tissue and chemotactic influences, RONS generation by cells surrounding epithelial type II cells in the inflamed lung micro-environment may influence the effectiveness of PP berryfruit extracts *in vivo*.

Precursors of ACNs found in nutritional supplements of grape extract were analysed in a human cancer cell line. Several metabolites including methylated products and conjugates were produced by the cells after incubation with the PPs (Lizarraga et al., 2007). In the same cell line, variations in flavonoid antioxidant activities were observed. Some PPs affected both cells and nuclei, while others influenced the cells only (Kanazawa et al., 2006). Structural changes that favoured activity against the cancer-associated telomerase enzyme *in vitro* were demonstrated for low concentrations of the green tea phenolic EGCG (Naasani et al., 2003).

Radical-scavenging activities of PPs have been shown to differ between the lipid phase and an aqueous phase (Cheynier, 2005), and the nature of the sugar moieties on ACNs affect their bioabsorption (McGhie et al., 2003). PP structure has a significant influence on antioxidant capacity, protective capacity, and particularly on the mechanism of action in cellular environments.

Variations in PP metabolism by different species, and structural differences in cellular metabolites *in vitro* have been discussed. Events involved in the influence of PPs on inflammatory markers has been increasingly clarified. By demonstrating that human lung epithelial cells are able to alter their response to allergen challenge under the influence of blackcurrant PPs, this study has added to the body of knowledge by linking the bioactive properties of berryfruit with a marker of modulation of allergic airway inflammation.

4.1.6 Fruit , polyphenolics and asthma

In population studies, fruit intake was inversely related to markers of inflammation (Holt et al., 2009), and kiwifruit consumption was linked to the reduction of respiratory symptoms and asthma (Forastiere et al., 2000). A large population-based survey of dietary antioxidant intake (n=9,709), found associations between apple and red wine consumption and asthma, but no links to asthma with vitamin C and vitamin E (Shaheen et al., 2001).

The antioxidant properties of vitamin E, selenium and fruit have not explained associations with IgE and atopic sensitization (Devereux et al., 2005). But non-antioxidant properties exerted on TH cells have been shown to relate to asthma and atopic disease. Human TH cells supplemented with physiologic quantities of vitamin E down-regulated IL-4 mRNA expression and subsequently reduced IL-4 secretion (Li-Weber, Giaisi, Treiber & Krammer, 2002).

Chronic allergen exposure that leads to sustained inflammation and hypersensitivity involves the TH_2 type immune response, and maturation of TH

cells into the TH2 phenotype is directed by IL-4. The recruitment of immune effector cells in the TH2 reaction cascade is directed by chemokines such as eotaxin-3.

Alveolar type II lung epithelial cells and A549 cells express the IL-4Rα high affinity binding subunit of the IL-4 receptor, and not other forms found on haematopoietic T cells. The IL-4Rα type II receptor complex was shown to be the more active isoform in regulating cells that mediate airway hypersensitivity (LaPorte et al., 2008).

In this study, BC extracts and IL-4 were incubated with A549 lung epithelial cells in a time-course experiment. We demonstrated that eotaxin-3 levels did not increase above background expression levels before 24 hours incubation in A549 cells. This was observed for the IL-4 stimulated control, and the berryfruit co-incubation experiments (data not shown). Therefore, the PP blackcurrant extracts did not induce eotaxin-3 production independent of IL-4 receptor mediated pathways. A549 cells possessed both IL-4 receptors and eotaxin-3 receptors, and were able to manufacture and release eotaxin-3 after TH2 cytokine stimulation.

While eotaxin-3 was not the only eotaxin or chemokine released by alveolar type II cells into the extracellular milieu, it was the specific peptide targeted in these studies because of its competitive regulation of other eotaxins, and its specificity for attracting eosinophils to the site of inflammation.

The manufacturer's instructions for the human eotaxin-3 ELISA development kit used in these studies stated that no cross-reactivity or interference with other human chemokines, or mouse eotaxins was exhibited at 50ng/ml. Therefore we can have confidence that these results are specific for eotaxin-3.

Of the eotaxins, only eotaxin-3 gene expression was shown to be up-regulated in asthmatics 24 hours after allergen challenge. (Berkman et al., 2001). The eotaxin receptor (CCR3) is expressed at high levels on eosinophils. Serum IgE levels and peripheral eosinophil counts are raised in asthmatics.

In a mouse model of chronic asthma, kiwifruit extracts inhibited bronchial hyperresponsiveness and eosinophil influx (Lee et al., 2006). After mice were exposed to chronic allergen exposure, CCR3 was expressed on T lymphocytes. The kiwifruit extract down-regulated CCR3 expression, and reduced the number of IgE B cells in bronchoalveolar lavage fluid (BALF).

In another mouse model of asthma, high levels of purified ACNs reduced mRNA levels of various cytokines, and reduced numbers of eosinophils recruited to lung tissue in airway hyperresponsiveness (Park et al., 2007). Since this decrease was not demonstrated for other inflammatory cells, beneficial effects of ACNs were linked to eosinophil counts. However, high levels of ACNs have been shown to be toxic to cells.

While providing evidence for associations between PPs and aspects of asthma, mouse studies may not be particularly relevant to humans. Species differences

in enzymes involved in metabolic pathways at the genetic level (polymorphisms) cause variation in enzyme activities and specificities. There are also polymorphisms in the transcriptional machinery that dictate levels of enzyme production. For example, the eotaxin-3 molecule shares only ~68% amino acid homology between human and mouse forms (Teran, 2000).

A human study was designed to assess the therapeutic value of a natural source of PPs in terms of relieving symptoms of asthma. High-ACN extracts of passionfruit peel were administered in a four week, placebo-controlled trial to asthmatics (n=43) (Watson et al., 2008). The PP extracts significantly reduced prevalence of wheeze, cough and shortness of breath compared to placebo. PPs administered (150mg/day) were deemed safe in animal studies, and showed no adverse effects. While therapeutic benefits of a PP supplement from a fruit product to asthma symptoms were demonstrated, the ACNs were not derived from the fleshy portion of the fruit usually eaten.

4.1.7 Anthocyanins

Phenolics were shown to be rapidly taken up by cells (Garcia-Alonso et al., 2006a), and 80-90% of the ACN compounds from blackcurrants that existed as rutinoside were excreted in the urine as the intact molecule in pigs (Wu et al., 2005). In a human study, plasma concentrations of ingested BC anthocyanins were reflected in the urinary excretion profiles (Matsumoto et al., 2001), indicating that ACN glycosides can be absorbed rapidly (within 2 hours of ingestion) and subsequently excreted in the urine as intact forms. Hence, ACNs could be exposed to cells and tissues as the intact molecule in humans.

The relative proportions of ACN metabolites may depend on concentrations in the berry, quantities consumed, and the glycoside moiety. While the cyanidin ACN fraction of freeze-dried berries was shown to be chemoprotective in rodents (Stoner et al., 2007), the total composition of ACNs in berryfruit have been demonstrated to affect their metabolism, as measured by percentages of cyanidin glycosides excreted in the urine (Wu et al., 2005).

In a study of ACN-fed rats, various metabolites were detected in plasma (native ACNs, methylated and/or glucuronidated derivatives, and aglycones) (Talavéra et al., 2005). A high proportion of methylated ACNs in rat liver and low amounts of these ACNs in plasma suggested that some ACNs are metabolised in the liver and excreted directly into the bile.

Rutinosides are diglycosides, and ACNs with a diglycoside attached were shown to be excreted in the urine primarily as the intact molecule. Cyanidin 3-*O*-rutinoside was not metabolised to ACN derivatives, as was the case for cyanidin 3-*O*-glucoside (Wu et al., 2005). Therefore, it can reasonably be assumed that a proportion of intake of BC rutinosides will reach target tissues.

In the BC extracts used in this study ACNs constituted ≈30% of total phenolics, and the rutinosides accounted for the majority (~90%) of those ACNs. We have identified one particular rutinoside ACN (Cy-rut) in the polyphenolic BC extracts as having a weak positive correlation with inflammation modulation.

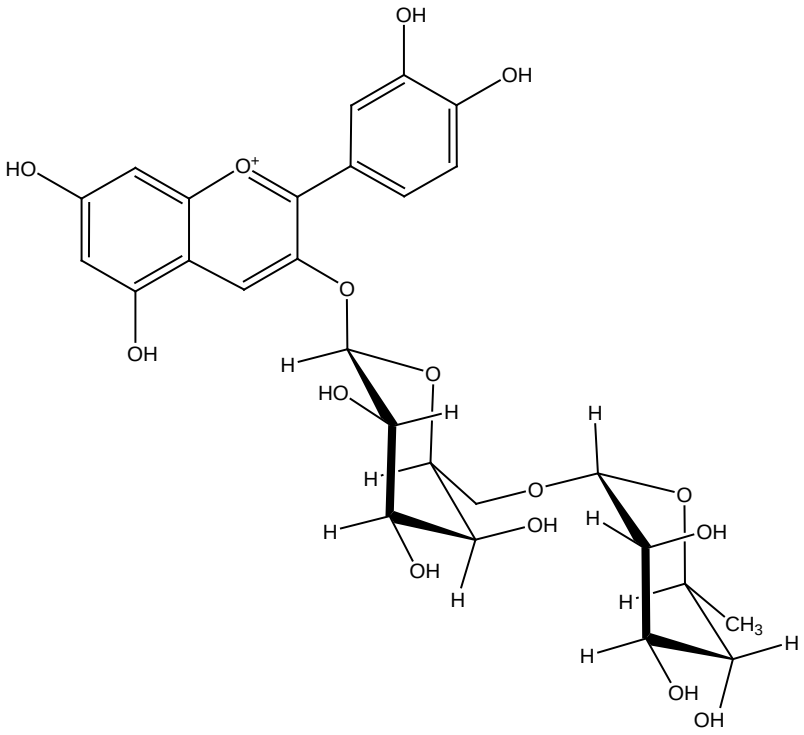


Figure 4.1. Structure of cyanidin 3-*O*-rutinoside (Cy-rut).

4.2 Conclusions

All ten blackcurrant cultivars tested in this study reduced a key cell marker of inflammation after lung cells were challenged with IL-4 for 24 hours. This indicated the potential modulation of allergic airway inflammation by berrryfruit extracts, and that blackcurrants could be of benefit in the control of inappropriate inflammation of asthma.

Blackcurrant cultivar differences in cell response to cytokine challenge were demonstrated in this study, and this could be of interest to berryfruit growers in New Zealand. We have identified a nutritional dose of polyphenolic blackcurrant extracts with potential health benefits to asthmatics.

This study has demonstrated the beneficial inflammation modulatory effects of polyphenolic BC extracts, which could be related to cyanidin 3-*O*-rutinoside content. However, since only ten BC cultivar samples were analysed, more work is needed to define the correlation further.

4.3 Limitations and suggestions for future improvements

The objective of this study was to analyse polyphenolic berryfruit extracts for their potential to modulate allergic airway inflammation. An analysis of previous literature suggested a physiologically relevant dose of PPs that lung cells might be exposed to, and that eotaxin-3 was a marker for eosinophil involvement in asthma.

Although we succeeded in demonstrating modulation of eotaxin-3 production by human lung epithelial cells, *in vitro* cell techniques have limitations that need to be understood.

4.3.1 Tissue culture limitations

Some *in vivo* features of the cells can be altered or lost under artificial growing conditions. Cell lines could have become adapted to the culture medium, and successive passaging of cells could have caused genetic selection from the heterogeneous population supplied in the initial A549 cell culture. The lung epithelial cells formed an adherent monolayer on the surface of polystyrene flasks, as opposed to an *in vivo* air/plasma interface, and being in close proximity to other cell types.

Although epithelial cell differentiation has been shown to be important for the secretion of inflammatory mediators by A549 cells (van Wetering et al., 2007), A549 cells have been used for over 20 years in the study of alveolar type II lung epithelial cells (Abonyo et al., 2006). Therefore an existing body of evidence and experimental procedures has been built to validate the use of these cells as a tool for studying inflammatory chemokines such as eotaxin-3.

Proteins in the calf serum component of the culture medium could have slightly affected peptide binding studies. This could have affected the levels of IL-4 that cells were exposed to, or eotaxin-3 levels generated by cells. Nevertheless, A549 cells were shown to be responsive in this study, and sensitive differences in eotaxin-3 generation with specific BC cultivars were able to be measured.

4.3.2 Blackcurrant cultivars

While all ten BC cultivars reduced eotaxin-3 production by A549 cells after IL-4 stimulation, not all results were significant. Further experimental repeats may have reduced variation and strengthened results.

Since the actions of PPs are structure and cell specific (Huang et al, 2006), we analysed the four major ACNs in the BC extracts for possible relationships to inflammation modulation effect demonstrated for the BC cultivars. Since a weak correlation to this effect was found for one particular ACN (Cy-rut), (Figure 3.16) for these ten BC cultivars, more samples tested may strengthen the positive trend identified for Cy-rut.

Since the ACN Cy-rut contained in the polyphenolic BC extracts may be largely responsible for the beneficial effects demonstrated in this study, a comparison of PP blackcurrant extracts from different growing seasons may reveal the proportion of cultivar difference attributable to environmental conditions.

By identifying the phylogenetic relationship between BC cultivars that exhibited the greatest inflammation modulatory effects in this study, we could indicate the proportion of cultivar differences attributable to genetic variation. Intriguing variation between co- and pre-incubation techniques for three BC cultivars were identified here (Figure 3.14). Mechanistic studies may reveal the underlying causes of these differences or the proportion attributable to antioxidant activity.

In this study, it was demonstrated that polyphenolic structure influenced inflammation modulation of BC extracts (Figures 3.15 to 3.18), and that ACNs within the extracts may have antagonistic effects. Previously, in the A549 cell system, synergistic inflammation modulation effects of berryfruit juices were demonstrated (Plant and Food Research, unpublished data). Various concentration ratios of ACNs from berryfruit mixtures, particularly the di- and tri-glycosides such as Cy-rut, may deliver powerful nutritional products for asthma.

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Appendices

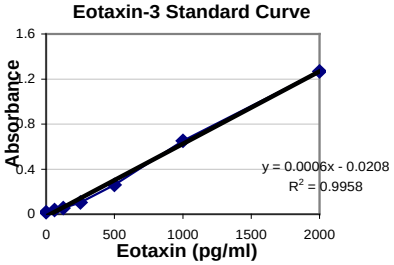
Appendix 1. Working spreadsheet for IL-4 incubation time course.

Date: 24th September 2008

Calibration Curve Eotaxin-3

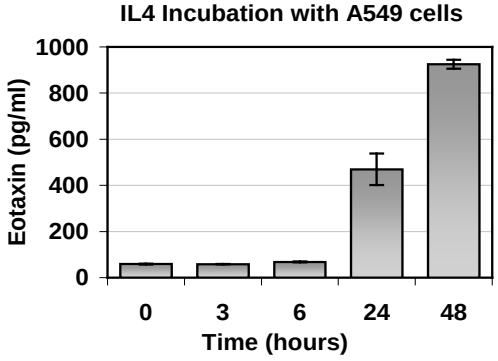
(pg/ml)	Absorbance		Av	SEM
0	0.015	0.018	0.0165	0.0015
62.5	0.036	0.038	0.037	0.001
125	0.053	0.054	0.0535	0.0005
250	0.108	0.099	0.1035	0.0045
500	0.261	0.26	0.2605	0.0005
1000	0.611	0.691	0.651	0.04
2000	1.26	1.275	1.2675	0.0075
4000	1.763	1.722	1.7425	0.0205

slope 0.000644
intercept -0.020814



A549 cells at 600 000 cells/ml
IL4 at 10 ng/ml

Incubation time with IL4			Average	SEM	Eotaxin-3	Mean	SEM
0	0.015	0.016	0.0155	0.0005	56.40035		
	0.016	0.021	0.0185	0.0025	61.05975		
	0.015	0.015	0.015	0	55.62378		
	0.014	0.016	0.015	0.001	55.62378		
	0.018	0.023	0.0205	0.0025	64.16602		
	0.019	0.016	0.0175	0.0015	59.50662	58.73005	1.41781
3hrs	0.017	0.016	0.0165	0.0005	57.95348		
	0.019	0.016	0.0175	0.0015	59.50662		
	0.017	0.017	0.017	0	58.73005		
	0.016	0.016	0.016	0	57.17692		
	0.02	0.015	0.0175	0.0025	59.50662		
	0.016	0.016	0.016	0	57.17692	58.34177	0.436999
6hrs	0.029	0.026	0.0275	0.0015	75.03795		
	0.021	0.02	0.0205	0.0005	64.16602		
	0.025	0.026	0.0255	0.0005	71.93169		
	0.022	0.02	0.021	0.001	64.94258		
	0.02	0.026	0.023	0.003	68.04885		
	0.02	0.021	0.0205	0.0005	64.16602	68.04885	1.859441
24 hrs	0.281	0.326	0.3035	0.0225	503.7028		
	0.422	0.46	0.441	0.019	717.2587		
	0.241	0.256	0.2485	0.0075	418.2805		
	0.227	0.221	0.224	0.003	380.2287		
	0.195	0.186	0.1905	0.0045	328.1987	469.5339	68.23234
48 hrs	0.583	0.563	0.573	0.01	922.2723		
	0.557	0.552	0.5545	0.0025	893.5393		
	0.596	0.598	0.597	0.001	959.5475	925.1197	19.10803



Appendix 2. Cytotoxicity assay raw data

Cytotoxicity assay for Blackcurrant PP extracts

Date: 20th January 2009
Signature: Janet Taylor

Cells at 500 000cells/ml x 100uL=50 000cells/well
WST-1 at 10uL added to wells and incubated 4 hours

A450

(ug/ml)	Cultivars: 0129-06				0129-02			
50	3.713	3.759	3.810	3.599	3.810	3.868	3.759	3.810
25	3.713	3.713	3.412	3.759	3.483	3.509	3.434	3.412
25	3.634	3.483	3.759	3.391	3.391	3.434	3.333	3.251
10	3.537	3.567	3.759	3.810	3.483	3.458	3.391	3.391
1	3.935	3.810	3.599	3.759	3.483	3.483	3.483	3.391
neg	3.868	3.810	3.713	3.599				
DMSO	3.391	3.351	3.351	3.483		Av blanks 450nm		
H2O2	1.093	1.066	1.099	1.309		0.71		
blank	0.727	0.719	0.716	0.685				

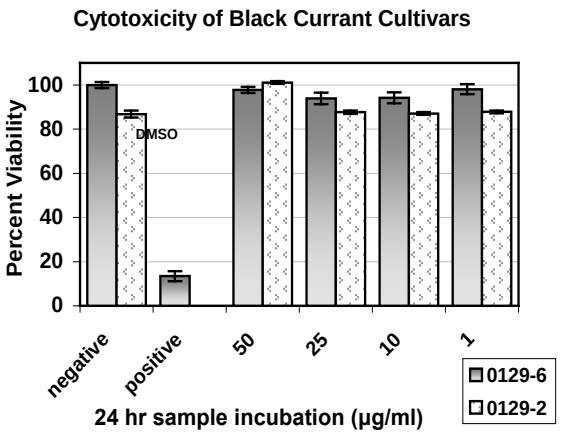
A620-blanks

(ug/ml)	Cultivars: 0129-06				0129-02			
50	0.023	0.004	-0.001	-0.012	0.007	-0.007	-0.020	-0.012
25	0.062	0.046	0.046	0.048	0.036	0.061	0.051	0.059
25	0.070	0.072	0.074	0.047	0.048	0.035	0.033	0.044
10	0.078	0.067	0.052	0.049	0.050	0.053	0.031	0.034
1	0.045	0.048	0.048	0.053	0.053	0.050	0.053	0.036

(A450-A620)/Average negative control *100 = Individual well % cell viability

(ug/ml)	Cultivars: 0129-06				0129-02			
50	96.906	99.001	100.813	94.32276	100.5596	102.9216	99.79829	101.1875
25	95.614	96.158	86.339	97.56643	88.96435	89.00339	86.90493	85.92564
25	92.777	87.806	96.721	85.64585	85.58729	87.42873	84.21434	81.16912
10	89.37428	90.69518	97.43955	99.20942	88.51863	87.63044	86.15338	86.03951
1	103.3933	99.22243	92.36094	97.394	88.42428	88.50562	88.41127	85.97444
neg	103.1949	101.4673	98.14554	97.19229				
DMSO	86.05578	85.09276	84.5104	91.51504		Av negative control		
pos	11.44477	10.55333	11.47405	20.26483		3.07		

		Av	SEM
	negative	100	1.404826
(H_2O_2)	positive	13.43424	2.286864
	DMSO	86.7935	1.605777
BC cultivar			
0129-6	50	97.76082	1.396649
	25	93.91933	2.560091
	10	94.17961	2.435135
	1	98.09267	2.286086
0129-2	50	101.1167	0.665282
	25	87.69958	0.768
	10	87.08549	0.599566
	1	87.8289	0.618505

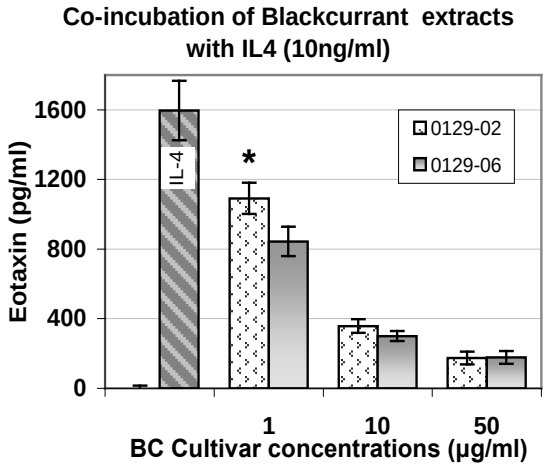


Appendix 3. Blackcurrant cultivar raw data

Date: 16th February 2009
Co-incubation of BC cultivars with IL4 (10ng/ml)

24 hour incubation
A549 cells at 600 000cells/ml

	Eotaxin-3	Av	SEM
Unstim	35.36957		
	39.54053		
	40.93085		
	-63.5353		
	-60.52862		
	-69.54868		
	30.22288		
	23.39256		
	21.68498	-0.274579	16.22646
Stimul	1734.342		
IL4	2490.677		
(10ng/ml)	929.3462		
	1755.51		
	2170.433		
	1131.623		
	1488.496		
	1100.876		
	1568.753	1596.673	170.4781



0129-02	Eotaxin-3	Av	SEM
1ug/ml	986.3494		
	1261.633		
	990.5204		
	816.7302		
	1372.859		
	765.2883		
	1117.951		
	1578.998		
	931.8252	1091.351	89.37467
10ug/ml	330.1177		
	382.9499		
	288.4081		
	220.2823		
	257.821		
	280.0661		
	465.6558		
	595.4319		
	400.7678	357.9445	39.28376
50ug/ml	157.7179		
	207.7694		
	268.9436		
	291.1887		
	281.4565		
	248.0887		
	43.88353		
	43.88353		
	30.22288	174.795	36.46074
0129-06			
1ug/ml	910.6311		
	337.8573		
	719.7065		
	688.1363		
	836.9673		
	1124.106		
	998.4208		
	1187.962		
	793.5112	844.1443	85.27536
10ug/ml	208.5698		
	250.6634		
	274.7169		
	292.757		
	262.6902		
	207.0665		
	467.3634		
	387.1071		
	349.5404	300.0528	28.76299
50ug/ml	161.9662		
	291.2537		
	222.0999		
	294.2604		
	213.0799		
	289.7504		
	38.76078		
	50.71385		
	42.17594	178.229	36.57195