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Dimeric procyanidins as modulators of airway inflammation in the context of allergic asthma

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

Doctor of Philosophy (PhD)

in

Human Physiology

at

Massey University

Manawatū, Palmerston North, New Zealand

Sara L Coleman, MS

2017

Declaration

It is hereby declared that this thesis has been composed by the undersigned Sara L. Coleman for the degree of Doctor of Philosophy (PhD) at Massey University. This work has not been presented in any previous application for a degree. All work was performed by the undersigned unless otherwise stated in the text. All sources of information have been specifically acknowledged in the text.

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A handwritten signature in black ink, appearing to read 'Sara L. Coleman', is written over a light blue horizontal line. The signature is stylized and cursive.

Sara L. Coleman

August 2017

Abstract

Dimeric procyanidins as modulators of airway inflammation in the context of allergic asthma

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Procyanidins are polyphenolic compounds that have come to be known as biologically active in the context of promoting human health. Epidemiological evidence suggests that populations that consume diets rich in procyanidins are less susceptible to inflammatory diseases. Allergic asthma is an inflammatory lung disease with an estimated 100 million affected individuals worldwide, with New Zealand having the world's second highest rate. Inflammation at the airway epithelium and infiltration of immune cells, specifically eosinophils, into the lung tissue are two central characteristics of allergic asthma. Thymic stromal lymphopoietin (TSLP) and eotaxin isoforms, eotaxin-1 (CCL11) and eotaxin-3 (CCL26), are three biomarkers of airway inflammation produced by the epithelium. Cell culture models were successfully optimized for CCL11 and CCL26 production in A549 cells. Investigation of procyanidins effect on epithelial TSLP production was not possible because TSLP production in A549 cells was undetectable. Data suggests that dimeric A-type linked procyanidin A2, but not B-type linked procyanidin B1 or B2, is capable of inhibiting IL-4-induced CCL11 production when incubated on A549 cells prior to an inflammatory insult. Co-incubation of A549 cells with procyanidin A2 and procyanidin B2 demonstrated no evidence of a synergistic relationship for inhibiting cytokine-

induced CCL11 production. Similarly, A549 cells exposed to procyanidin A2, and to a lesser extent procyanidin B2, had reduced production of cytokine-induced CCL26 production. An inhibition time course demonstrated procyanidin A2 had greatest inhibition efficacy on cytokine-induced CCL26 production when incubated for 2 h prior to an inflammatory insult. Comparison of procyanidin A2 inhibition to the known CCL26 inhibitor, IFN γ , demonstrated that procyanidin A2 and IFN γ did not share the same temporal inhibition patterns. Furthermore, experiments investigating concomitant incubation of procyanidin A2 and IFN γ demonstrated that procyanidin A2 could interfere with IFN γ -mediated CCL26 inhibition. Two possible mechanisms responsible for the procyanidin A-mediated inhibition of cytokine-induced CCL11 and CCL26 were investigated: the modulation of cytokine receptor expression, and modulation of plasma membrane fluidity. However, there was no evidence to support either of these modes of action. The data presented in this thesis collectively demonstrate the ability of procyanidin A2 to inhibit cytokine-induced eotaxin production from the lung epithelium *in vitro* and support further investigation of procyanidin A2 as a preventative approach for managing airway inflammation.

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Abbreviations

16HBE14o-	human airway epithelial cell-line
A549	human alveolar epithelial cell-line
AD	Alzheimer's disease
AP-1	activator protein-1
APC	allophycocyanin fluorophore
ARE	antioxidant response element
B2G2	procyanidin B2 3,3''-di-O-gallate
BALF	bronchoalveolar lavage fluid
BD	Becton Dickinson
BEAS-2B	human bronchial epithelial cell-line
BSA	bovine serum albumin
CA	catechin
Caco2	human colon epithelial cell-line
Calu-3	human lung epithelial cell-line
Calu-6	human lung epithelial cell-line
cAMP	cyclic adenosine monophosphate
CCL11	eotaxin-1
CCL24	eotaxin-2
CCL26	eotaxin-3
CCL3	macrophage inflammatory protein 1-alpha
CCR3	C-C chemokine receptor type 3
CD4+	cluster of differentiation 4 positive
CgC	common gamma chain
CO ₂	carbon dioxide
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CSB	cell staining buffer
-Cy TM 7	cyanine 7 fluorophore
DC	dendritic cell
DiO	3,3'-dioctadecyloxacarbocyanine perchlorate
DMEM	Dulbecco's modified eagle medium
DMEM/F-12	DMEM with Nutrient Mixture F-12
DMSO	dimethyl sulfoxide
DP	degree of polymerization
DPH	1,6-diphenyl-1,3,5-hexatriene
EC	epicatechin
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
em	emission

EPA	eicosapentaenoic acid
ERK1/2	extracellular-regulated kinase 1 and 2
ex	excitation
F1	first filial generation
FBS	foetal bovine serum
FcεRI	high-affinity IgE receptor
FITC	fluorescein
FLVR	<i>Faecalibacterium, Lachnospira, Veillonella, and Rothia</i>
FSC	forward scatter
GM-CSF	granulocyte-macrophage colony-stimulating factor
H ₂ O ₂	hydrogen peroxide
HeLa	human cervix epithelial cell-line
HepG2	human liver epithelial cell-line
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IFN γ	interferon gamma
IgE	immunoglobulin E
IgG1	immunoglobulin G1
IL	interleukin
IL-13R α 1	interleukin 13 receptor alpha one
IL-4R α	interleukin 4 receptor alpha
ILC	innate lymphoid cell
ILC2	ILC type 2
IM9	human peripheral blood B lymphoblast cell-line
JAK	janus kinase
JNK1/2	C-Jun N-terminal kinase 1 and 2
kDa	kilo Dalton
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption/ionization with time-of-flight mass spectrometer
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NAD ⁺	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NFAT	nuclear factor of activated T cells
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
Nrf2	nuclear factor E2-related factor 2
NS	not significant
NZ	New Zealand
OVA	ovalbumin
Ox40L	Ox40 ligand

PBS	phosphate buffered saline
PD-L1	programmed death ligand 1
PE	phycoerythrin fluorophore
PerCP	peridinin chlorophyll protein fluorophore
PFR	Plant & Food Research
PMT	photomultiplier tube
Procy	procyanidin
PSN	penicillin streptomycin neomycin antibiotic mixture
RPMI	Roswell Park Memorial Institute
SCFA	short chain fatty acid
SEM	standard error mean
SPTS	Science Publication Tracking System
SSC	side scatter
STAT	signal transducers and activation of transcription
T reg	T regulatory cell
TEER	transepithelial electrical resistance
Th1	T-helper 1
Th2	T-helper 2
THLE-2	human liver epithelial cell-line
TMB	3,3',5,5'-tetramethylbenzidine
TNF R1	tumour necrosis factor receptor one
TNF α	tumour necrosis factor alpha
TSLP	thymic stromal lymphopoietin
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
UV	ultraviolet
WST-1	water soluble tetrazolium-1

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Plant & Food
RESEARCH
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External Contributions

Portions of this thesis have been published elsewhere in the form of a book chapter, research articles, and a review article. Original drafts of these publications were solely written by Sara Coleman and then subsequently edited by co-authors of each publication. Manuscripts were submitted to Science Publication Tracking system (SPTS) at PFR, a compulsory internal process which includes scientific peer review and professional editing. All portions of this thesis not published elsewhere, are the product of Sara L Coleman alone.

A declaration of contribution detailing the specifics is included at the beginning of each chapter.

Outputs

Publications

Coleman, S.L.; Hurst, R.D.; Sawyer, G.M.; Kruger, M.C. Fruit Procyanidins: Modulating Inflammation to Promote Health. In: Sullivan I, editor. Proanthocyanidins: Food Sources, Antioxidant Properties, and Health Benefits. New York: Nova Science Publishers, Inc.; 2015. p. 73-97.

Coleman, S.L.; Hurst, R.D.; Sawyer, G.M.; Kruger, M.C.; The in vitro evaluation of isolated procyanidins as modulators of cytokine-induced eotaxin production in human alveolar epithelial cells. *J. Berry Res.* 2016; 5: 115-124; DOI: 10.3233/JBR-160121

Coleman, S.L.; Kruger, M.C.; Sawyer, G.M.; Hurst, R.D. Procyanidin A2 Modulates IL-4-Induced CCL26 Production in Human Alveolar Epithelial Cells. *Int. J. Mol. Sci.* 2016, 17, 1888; DOI: 10.3390/ijms17111888

Coleman, S.L.; Shaw, O.M. Progress in our understanding of allergic asthma pathology supports the potential of fruit proanthocyanidins as modulators of airway inflammation. 2017 (Under Review)

Abstracts for Publications can be seen in Appendix I

Presentations

Presentations:

- Dec 2014 Confirmation Presentation at Massey University, Palmerston North. New Zealand
- July 2015 Oral Presentation at NZASI Conference Auckland, New Zealand
- October 2015 Poster Presentation at Berry Health Benefits Symposium Conference, Madison, Wisconsin, USA
- June 2016 Oral Presentation at Joint Graduate School of Horticulture and Food Enterprise, Massey University, Palmerston North, New Zealand

Additional Scientific Education:

- July 2014 Attendance NZASI Conference Palmerston North, NZ
- October 2015 US:NZ Science Workshop- Building the Health Claims Dossier for Berry Fruits
- April 2016 Polychromatic Flow Cytometry Roadshow, Wellington, NZ

Chapter One

Introduction

1.1 Thesis origins

Previous work in the Food and Wellness laboratory investigated blackcurrant extracts in the context of allergic asthma. Experiments investigated the ability of multiple polyphenolic fractions to modulate the production of IL-4-induced eotaxin-3 (CCL26) in A549 lung epithelial cells. It was observed that a total phenolic fraction and a proanthocyanidin-enriched fraction of blackcurrant were able to reduce CCL26 secretion, whereas an anthocyanin-enriched fraction had no effect [1]. This doctoral project was borne from these findings and set out to evaluate structurally-defined proanthocyanidins, as opposed to fruit extracts, as potential modulators of airway inflammation *in vitro*.

1.2 Thesis outline

This doctoral thesis is principally an enquiry into procyanidin compounds found in fruit as modulators of inappropriate airway inflammation at the lung epithelium modelled *in vitro*. Chapter one provides the origins of the project and the research questions. Chapter two is a literature review of procyanidins and inflammation signal transduction. Chapter three provides a literature review of the pathology of allergic asthma and the potential of procyanidins as modulators of airway inflammation. Chapter four gives detailed information about the materials and methods that are used within the research chapters. Experiments regarding the effects of procyanidins on three biomarkers of airway inflammation produced by the lung epithelium, thymic stromal lymphopoietin (TSLP), eotaxin-1 (CCL11), and CCL26 are described in chapters five, six, and seven respectively. The project then shifts from modulating biomarker production to investigating possible cellular mechanisms of action for the observed procyanidin effects. In chapters eight and nine, procyanidin effect on cytokine receptor

expression and plasma membrane properties are explored. Finally in chapter ten, a summary of project findings is provided as well as descriptions of possible future work.

1.3 Initial research question

The initial research question for this project was:

What is the relationship between procyanidins and production of inflammation biomarkers *in vitro* in the context of allergic asthma?

1.4 Research hypotheses

The research question led to a null and alternative hypothesis:

Null:

There is no effect of dimeric procyanidins on the production of inflammation biomarkers in the context of allergic asthma *in vitro*.

Alternative:

Dimeric procyanidins can modulate the production of inflammation biomarkers in the context of allergic asthma *in vitro*.

Chapter Two

Literature Review (part 1) Fruit procyanidins: Modulating inflammation to promote health

2.1 Fruit procyanidins: Modulating inflammation to promote health

Contribution Declaration

This thesis chapter was published in whole as a book chapter and is reprinted with permission from Nova Science Publishers:

Coleman, S.L.; Hurst, R.D.; Sawyer, G.M.; Kruger, M.C. Fruit Procyanidins: Modulating Inflammation to Promote Health. In: Sullivan I, editor. Proanthocyanidins: Food Sources, Antioxidant Properties, and Health Benefits. New York: Nova Science Publishers, Inc.; 2015. p. 73-97.

The original manuscript of this chapter was written by Sara Coleman (90%), with content and grammatical editing from Roger Hurst, Greg Sawyer, and Marlena Kruger (10%). The revised manuscript was then internally reviewed by Dr. Simon Deroles (Discovery for Impact, PFR) and Dr. David Stevenson (Food and Wellness, PFR). The internally reviewed manuscript was entered into the SPTS at PFR which includes professional format and grammar editing. The final manuscript was published as cited above.

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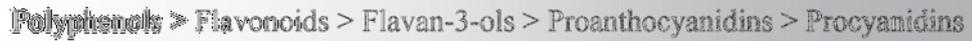
2.2 Abstract

Procyanidins are polyphenolic compounds found in relatively high concentrations in fruits that have come to be known as biologically active in the context of promoting human health. Epidemiological evidence suggests that people who consume diets high in fruits and vegetables are less susceptible to inflammation-related diseases and diseases of multi-factorial pathogenesis such as metabolic syndrome, atherosclerosis, asthma, and cancer. Procyanidins are one of three types of proanthocyanidins and differ from the others based on the hydroxylation pattern of their B ring. They differ amongst themselves according to the type of linkage that connects the flavan-3-ol monomeric units. Despite progress, there is still a lack of measurement technologies to delineate the structure of higher polymers accurately, although specific dimer designations have been identified. Research is working toward understanding the health-promoting properties of procyanidins, specifically moving beyond their antioxidant capabilities. Bioavailability of designated dimers in vivo is not well understood, though there are data to suggest that in vitro experiments could be relevant in the human system. Population studies have shown an inverse relationship between procyanidin consumption and ill health, especially inflammatory conditions. Here I present a focused review of the properties of individual procyanidins and suspected mechanisms by which they could potentially regulate inflammation. Modulation of cytokine secretion, mechanisms involving signal transduction pathways - NF- κ B, STAT, and MAPK, and procyanidin influence on the chemotaxis of inflammatory cells into the tissues, will all be discussed.

2.3 Introduction

For much of human history, secondary plant metabolites have provided inspiration for therapies to improve human health. As technologies progress and interest in nutrition has blossomed, research has moved towards understanding the biological activities of these plant-derived compounds and how their food sources can be better incorporated into the human diet to act as functional foods for maintaining or regaining optimal health. Fruits are of particular interest because it is now well established that a diet consisting of predominantly fruits and vegetables is advantageous for optimal health. Fruits are an abundant source of polyphenols, which are responsible for many of the health benefits gained from a high fruit and vegetable diet. Plant polyphenol is the generic title given to any plant compound that contains multiple phenolic rings in its structure. Polyphenols can be divided into many subcategories, most of which are outside the scope of this project.

Flavonoids, the most abundant subcategory of polyphenols (Figure 2-1), encompass a number of compounds, with flavan-3-ols being of particular interest because of their relatively high concentrations in fruits. When flavan-3-ol units, e.g. (+)-catechin and (-)-epicatechin, are combined in either a heterogeneous or a homogeneous manner, they form proanthocyanidins. Finally, procyanidins are proanthocyanidins that specifically have two hydroxyl groups attached to their B-ring (Figure 2-2), as opposed to one or three, in propelargonidins and prodelfinidins respectively. Procyanidins are also referred to as condensed tannins in the literature, depending on the source discipline. Interestingly, the mechanism by which plants polymerize flavan-3-ol units into proanthocyanidins is still to be fully elucidated [2]. This doctoral project focuses on



Polyphenols > Flavonoids > Flavan-3-ols > Proanthocyanidins > Procyanidins

Figure 2-1: Polyphenol nomenclature

The nomenclature used herein arranged linearly from the broadly encompassing term, polyphenols (on the left), to the most specific term, procyanidins (on the right).

procyanidins as a plant-based approach for modulating inflammatory events to improve health.

There are many challenges in designing experiments to assess the health-promoting properties of plant polyphenols, which have been reviewed [3]. However, there is much to be gained from exploring the potential of fruit procyanidins, as their possible role in mediating inflammation is now being recognized. The benefit of procyanidin intake was demonstrated in a large cohort study analysing data from eight European countries, which showed an inverse relationship between flavan-3-ol intake and type 2 diabetes [4]. Furthermore there has been work looking at the efficacy of procyanidin-rich extracts in inhibiting the progression of Alzheimer's disease (AD). Procyanidins from grape seed extract in combination with resveratrol and Concord grape juice extract were shown to be able to cross the blood-brain barrier and elicit an effect on a biomarker of AD progression. It was suggested by the authors of the study that while their grape seed/resveratrol/Concord grape juice extract did modulate a specific biomarker of AD progression, there is the possibility of more benefit being gained from the mixture's anti-inflammatory properties [5].

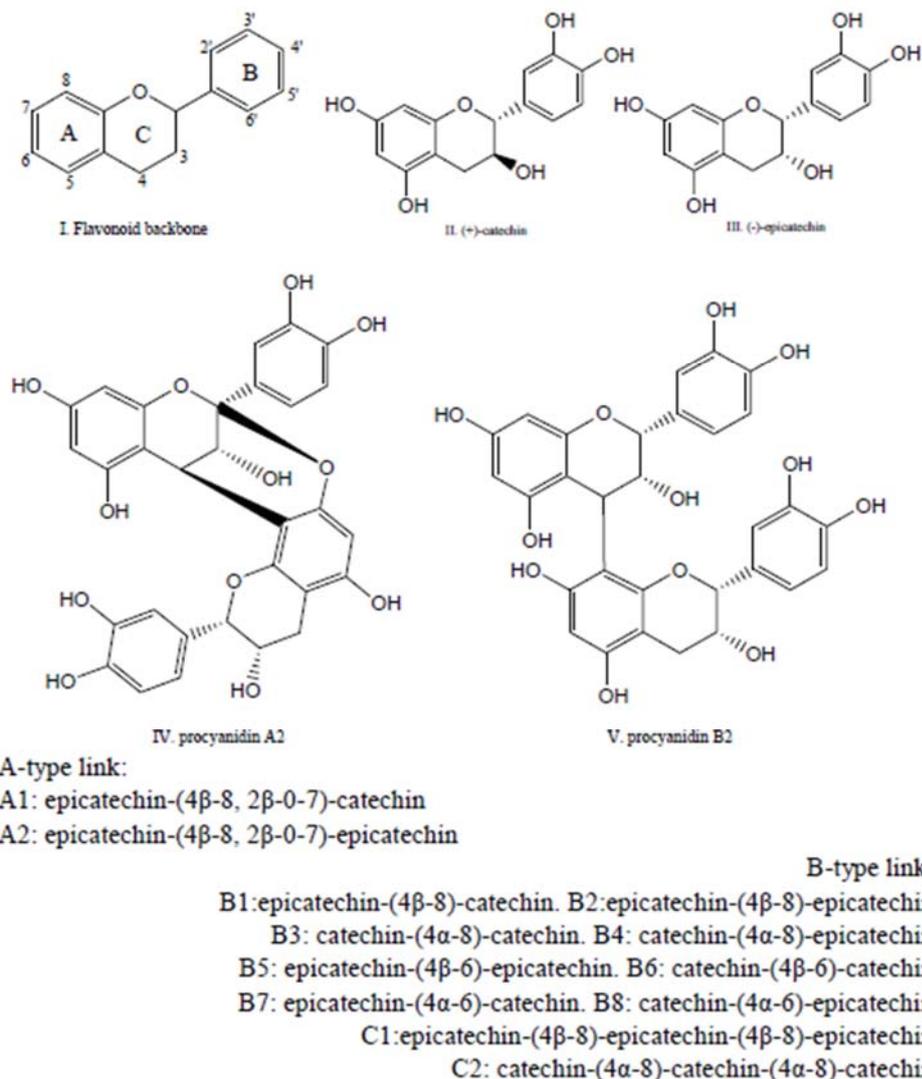


Figure 2-2: Chemical structure and designations of procyanidins

I) the backbone of flavonoid compounds; II) flavan-3-ol (+)-catechin; III) flavan-3-ol (-)-epicatechin; IV) procyanidin A2; V) procyanidin B2. An example of a 4 β -8, 2 β -O-7 A-type bond is illustrated in procyanidin A2. An example of a 4 β -8 B-type bond is illustrated in procyanidin B2.

Inflammation is the process by which stressed or injured cells and tissues are repaired, dead cells cleared, and tissue function restored. This would categorize inflammation as a beneficial process to the host; however, popular opinion perceives it as negative. An acute inflammatory response that allows for immediate mitigation of a threat and then a return to basal state is ideal. During the acute inflammation response,

tissue is subject to oedema and increased permeability of blood vessels, causing plasma proteins to aggregate. This process results in the cardinal signs of inflammation: red, hot, swollen, tender tissue that has a loss of function [6]. These factors reflect well the etymology of the word inflammation, which comes from the Latin *inflamare*, meaning to burn [6]. This acute response is essential to host survival. However, when inflammation persists beyond the acute response, the immune balance can skew, resulting in chronic inflammation. Chronic inflammation is an exacerbating factor in conditions such as AD, arthritis, psoriasis, eczema, inflammatory bowel disease (IBD), Crohn's disease, and particularly respiratory conditions such as chronic obstructive pulmonary disease (COPD) and asthma [7-9]. Chronic inflammation often lacks the outward signs of redness, heat, and pain associated with acute inflammation, but the microscopic picture in the tissue is highly abnormal. Dense masses of leukocytes, including neutrophils, eosinophils, basophils, monocytes, and lymphocytes infiltrate tissue during chronic inflammation [10].

This chapter will overview the nomenclature and chemistry of procyanidins, dietary sources of procyanidins with a focus on fruit, and then provide an in-depth review of the possible mechanisms by which specific procyanidins may balance inflammation and thereby potentially assist in managing inflammatory disease.

2.4 Procyanidin chemistry and descriptions used in health research

Procyanidins are oligomers and polymers built from monomeric flavan-3-ols (+)-catechin and (-)-epicatechin units. They vary according to the stereochemistry and hydroxylation pattern of the flavan-3-ol backbone and extension units and the bond position between units [2]. Structures of two common procyanidins can be seen in Figure 2-2. Specifically to be termed a procyanidin, as opposed to other

proanthocyanidins such as propelargonidins or prodelphinidins, a compound will have two (3', 4') B-ring hydroxyl groups [2]. The monomeric units can be combined by either A-type or B-type linkages (Figure 2-2).

As research on procyanidins develops, it is imperative that scientists use the most accurate and agreed-upon descriptors, to be able to distinguish clearly which compounds are eliciting effects in a particular model of health or disease. Ideally, monomers would be listed by name, (+)-catechin or (-)-epicatechin; dimers would be listed by designations; trimers would be listed as either C1, or C2, or as a trimer with accompanying major linkage type; and polymers listed by approximate degree of polymerization (DP) and major linkage type. When technologies to delineate compound designations are unavailable, the minimum standard should be to distinguish linkage type and DP.

Using chemistry techniques to define procyanidins involved in a study accurately was demonstrated by Feliciano and colleagues [11]. Their experiment characterized proanthocyanidins from both cranberry and apple using matrix-assisted laser desorption/ionization with a time-of-flight mass spectrometer (MALDI TOF MS). They created proanthocyanidin treatments that were described as having one or more A-type interflavan bonds originating from cranberry, or containing only B-type interflavan bonds originating from apple [11]. The importance of such detailed description can be seen from other studies that showed the ability of isolated procyanidins B1 and B2 to form stereospecific binding directly to the p50 subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), a predominant transcription factor that signals inflammatory events (see section 2.6.1) [12]. Procyanidins inhibiting NF- κ B's ability to bind DNA would hinder pro-inflammatory signals from being executed. Procyanidins A1

and A2, however, were not capable of binding the p50 subunit in the same manner. In work investigating the anti-microbial properties of A-type and B-type procyanidins, A-type procyanidins at physiologically relevant doses inhibited an *Escherichia coli* bacterial strain from entering Caco2 human gut epithelial cells *in vitro*, whereas B-type proanthocyanidins were capable of such inhibition only at much higher concentrations [11]. However, both A-type and B-type procyanidins had similar hypoglycaemic activities when glucose tolerance of human liver HepG2 cells *in vitro* was measured [13]. These studies collectively show that procyanidins are extremely diverse in their abilities to influence cellular signalling and to alter cellular physiology and function. Providing the most accurate procyanidin description in research studies will lead to more robust data reporting, which will assist the success of translating *in vitro* and animal findings to the human situation and end-user utilization.

2.5 From fruit bowl to blood stream

Procyanidin consumption varies widely depending on both cultural food traditions and the individual [14]. Epidemiological analyses from the United States (US) and Europe have provided rough estimates of typical dietary proanthocyanidin intake. Available data are organized by generic proanthocyanidin content and not specifically by procyanidins; however, they do provide a starting point from which to determine relevant doses for scientific experimentation. Controversy surrounds the bioavailability of procyanidin compounds; they are shown to be stable during gastric transit, and monomers up to tetramers are believed to be absorbed via paracellular, passive diffusion in the small intestine, with larger polymers continuing into the large intestine [15]. However, it is currently unknown if bioavailability is a limiting step or if increased consumption of procyanidins could enhance absorption. Work has been done to estimate the proanthocyanidin content in the human diet. The average total

proanthocyanidin intake for adults in the US has been reported as 95 mg/day [16]. The major food sources of proanthocyanidins in the US are tea, legumes, and wine. Tea is the primary source of monomers and dimers, whereas legumes are the major source of polymers [16]. For this study, data were partially collected by a 24-hour food recall; the authors listed participants that did not claim to eat a specific food as non-consumers [16] and 78% of the 8,809 people involved in the study were non-consumers of tea. Using tea as the main source of procyanidins is not a prudent choice when looking to improve human health in the US, as drinking tea is not a cultural norm as it is in many other countries, e.g. the United Kingdom (UK) or New Zealand (NZ). However, the same study also showed that only 34% of the participants were non-consumers of fruits or fruit juices, which would make fruit sources of procyanidins much more likely dietary sources, despite fruits having lower total concentrations of proanthocyanidins. Furthermore, studies looking at Finnish and Spanish diets, showed fruits (specifically apples and berries) as the major contributors to total proanthocyanidin intake [17,18]. A large case-cohort study analysing data from eight European countries showed results similar in nature to the US study, in that tea was the primary source of monomers, (+)-catechin and (-)-epicatechin and their gallated derivatives; whereas fruits accounted for 38% of proanthocyanidin dimers of unspecified linkage [4]. It is common for population studies to use the United States Department of Agriculture (USDA) database for the proanthocyanidin content of select foods to estimate the proanthocyanidin content in the diet [4,16,18]. Unfortunately, the database is not able to give specific information on the dietary content of procyanidins by designation. If we are to imagine fruits as therapeutic tools to assist in the management of health and wellbeing, it will be necessary to identify procyanidin chemistry, specifically linkage type. Furthermore, when looking for the procyanidin content of fruits, it is pertinent to be mindful of the stage of fruit

ripeness, as this has been demonstrated to relate to procyanidin concentration. Unripe white cranberries, for example, contain 184.1 mg/100 g fresh weight of total procyanidins, whereas red ripe cranberries contain 115.5 mg/100 g fresh weight of total procyanidins [19]. Proanthocyanidin content is four times higher in unripe green blackberries, and declines as they ripen to mature black fruit [20].

2.5.1 Procyanidins in fruits

Identifying the concentrations of specific procyanidins in foods can be a challenge. Total proanthocyanidin content is typically listed in studies but with little information, until recently, available on designations or linkage type. *Rubus* and *Fragaria* genus Berries, apples, grapes, peaches, pears, plums, apricots, kiwifruit, mangoes and bananas are among the fruits that have been found to contain procyanidins [21]. Berries and apples have among the highest concentrations, with bananas having the least [22]. Fruits are now being investigated for specific procyanidins. Table 2-1 shows the concentrations of known A-type linkage dimers and B-type linkage dimers as well as concentrations of higher polymers that are found in fruits. The data presented are assumed to apply to the whole fruit unless specified, and for some fruits (e.g. plums), procyanidin content has been evaluated in the peel and the flesh. Five cultivars of apples, ‘Annurca’, ‘Red Delicious’, Pink Lady®, ‘Fuji’, and ‘Golden Delicious’, were studied, and all but ‘Red Delicious’ showed higher total procyanidin content in the flesh than in the peel [19]. This is in contrast to the common notion that the majority of polyphenolic compounds are in the skins of fruits [23].

Indeed, data suggest that procyanidin content is one of the most variable characteristics between individual fruit of the same species and is influenced by the weather, sun exposure, rootstock, and cultivar [23]. For example, a study evaluating

104 varieties of apples showed a procyanidin B2 content ranging from 0.64 to 32.43 mg/100 g fresh weight [24]. For agricultural breeding to promote health, it would be advantageous to breed for plant cultivars that contain higher concentrations of procyanidins; however, even this comes with challenges for plant scientists, which have been reviewed elsewhere [25].

Most research on A-type procyanidins has occurred in cranberries, but they have also been identified in plums, and avocados [21]. Among sources of A-type procyanidins, total procyanidin content was highest in lingonberries and then cranberries, but this was mostly in the form of higher polymers. When looking at absorbable components, plums had 31.5 mg/100 g fresh weight of dimers, as opposed to 25.9 mg/100 g fresh weight of dimers in cranberries [21]. Raspberries and strawberries are known fruits to contain propelargonidins, and blackcurrants and grapes are known fruits to contain prodelfinidins, which are the other two types of proanthocyanidins besides procyanidins. Grape seeds are one of the only sources that have been demonstrated to contain all B-type procyanidins B1-B8; however, concentrations of individual compounds have not been quantified [26]. Total proanthocyanidin contents of fruits and other foods not included here can be found from studies and reviews elsewhere [4,16-22,24,27,28].

Table 2-1: Concentrations of catechin (CA), epicatechin (EC), epicatechin (EC), and procyanidins in fruits

	Concentration (mg/100 g fresh weight) unless stated otherwise														Polymers
	CA	EC	B1	B2	B3	B4	B5	B6	B7	B8	C1	C2	A2		
Apple	1.38 ^a - 1.66 ^b	7.70 ^{ab}	2.71 ^b - 3.40 ^b	8.58 ^b - 11.53 ^a	0.00	0.00	0.89 ^a	0.00	0.63 ^a	0.00	5.80 ^a	0.00	0.00	0.00	74.96 ^c
Apricot	2.51	4.62	1.09	3.05	0.19	0.41	0.41	0.00	0.19	0.00	1.61	0.00	0.05	7.90	
Avocado	0.06	1.34	0.31	2.13	0.00	0.22	0.20	0.00	0.09	0.00	1.18	0.00	0.00	3.60	
Banana	0.00	0.08	0.00	0.09	0.00	0.00	0.02	0.00	0.00	0.00	0.06	0.00	0.00	2.30	
Blackberry	0.91	20.68	0.19	5.03	1.35	8.85	0.89	0.29	0.00	0.00	1.30	1.40	0.00	13.00	
Blueberry	0.77-2.10 ^d	0.60	3.50	0.94 ^d -1.15	0.00	0.00	0.09	0.00	0.33	0.00	0.99	0.00	0.00	163.00	
Boysenberry															
Seed	5.43	7.41	-	-	3.44	13.40	-	-	-	-	-	7.41	-	11.90	
Juice (mg/100 ml)	0.88	1.06	-	-	0.35	0.62	-	-	-	-	-	0.09	-	-	
Cherry	6.79	7.11	3.96	3.96	0.15	0.41	0.49	0.00	0.60	0.00	3.22	0.00	0.00	8.40	
Cranberry															
American	0.43 ^e - 0.49	3.33 ^e -3.83	0.29 - 0.37 ^e	1.56-3.23 ^e	0.04	0.41	0.36 - 0.61 ^e	0.00	0.09	0.00	0.37 ^e - 4.00	0.00	4.61 ^e - 6.90	5.97 ^e A-type trimer 0.61 ^e B-type trimer	
European	1.38 ^f	0.48 ^f	0.78 ^f	0.41 ^f	-	-	0.07 ^f	-	-	-	0.04 ^f	-	0.17 ^f	0.31 ^f A-type trimer 0.12 ^f B-type trimer	
Currant															
Black	1.39 - 6.28 ^g	0.72	0.14	0.19	0.13	0.42	0.03	0.05	0.04	0.00	0.06	0.00	0.00	-	
Red	0.77 ^h - 1.37	0.29	0.27	0.06	0.24	0.09	0.01	0.03	0.03	0.00	0.03	0.10	0.00	-	
Fig	0.67	0.13	0.23	0.03	0.13	0.06	0.00	0.02	0.05	0.00	0.10	0.04	0.00	-	
Grape	1.30 ⁱ	0.27 ⁱ	1.32 ⁱ	0.11 ⁱ	0.13 ⁱ	0.00	0.00	0.00	0.13 ⁱ	0.00	0.00	0.00	0.00	66.70	
Seed	-	-	-	-	-	-	-	-	-	-	-	-	-	2164.00	

Table references: [19,21,24,29-37]

Table 2-1 (Cont.): Concentrations of catechin (CA), epicatechin (EC), and procyanidins in fruits

	Concentration (mg/100 g fresh weight) unless stated otherwise														Polymers
	CA	EC	B1	B2	B3	B4	B5	B6	B7	B8	C1	C2	A2		
Lingonberry	10.10 ⁱ	6.58 ^l	10.44 ^j	5.40 ^j	-	-	0.93 ^l	-	-	-	0.59 ^l	-	5.04 ^j	8.85 ⁱ A-type trimer 2.02 ⁱ B-type trimer	
Litchi	0.00	1.22	0.00	1.77	0.00	0.00	0.26	0.00	0.00	0.00	0.95	0.00	0.74	-	
Mango	1.44	0.01	0.20	0.00	0.00	0.00	0.00	0.06	0.06	0.00	0.00	0.00	0.00	7.20	
Nectarine	0.38-9.30 ^k	0.03-5.30 ^k	0.39-12.41 ^k	0.01	0.06	0.00	0.00	0.00	0.05	0.00	0.00	0.01	0.00	16.90	
Papaya	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	
Peach	2.12-13.57 ^l	0.10-6.20 ^l	2.18-18.80 ^l	0.07	0.23	0.00	0.02	0.00	0.21	0.00	0.00	0.05	0.00	50.60	
Pear	0.58	6.14	0.50	2.75	0.04	0.08	0.42	0.00	0.16	0.00	1.89	0.00	1.14	24.20	
Plum															
Whole Fruit	2.81 ^m	4.32 ^m	3.33 ^m	4.74 ^m	0.75 ^m	6.61 ^m	0.47 ^m	0.00	1.91 ^m	0.00	2.05 ^m	0.32 ^m	3.14 ^m	149.10	
Flesh	10.86 ⁿ	4.06 ⁿ	8.65 ⁿ -14.22 ⁿ	7.15 ⁿ -7.70 ⁿ	-	8.95 ⁿ	-	-	1.38 ⁿ	-	-	-	-	-	
Skin	-	-	22.87 ⁿ	16.09 ⁿ	-	32.53 ⁿ	-	-	-	-	-	-	-	-	
Quince															
Fruit	0.03	2.06	0.27	3.00	0.04	0.35	0.27	0.00	0.09	0.00	2.04	0.00	0.00	-	
Juice (mg/100 ml)	3.00 ^p	45.00 ^p	17.80 ^p	78.90 ^p	-	-	-	-	-	-	94.80 ^p	-	-	1120.00 ^p	
Raspberry	0.75	8.37	0.00	0.30 ^q	0.63	12.13	0.06	0.13	0.00	0.20	0.00	0.35	0.00	8.60	
Rose Hips (dry weight)	2.25 ^r	0.15 ^r	-	2.43 ^r	-	-	-	-	-	-	-	-	-	-	
Strawberry	4.27 ^r	0.14 ^r	1.62 ^r	0.00	3.15 ^r	0.00	0.00	0.36 ^r	0.33 ^r	0.00	0.72 ^r	3.31 ^r	0.00	127.80	

Values listed as "0.00" were measured and found to be zero. A dash (-) is used for values that were not measured in available literature. a) five cultivars of apples, b) 104 cultivars of apples, c) five cultivars of apples with and without peel, d) three cultivars of blueberries, e) six cultivars of *Vaccinium macrocarpon*, f) two cultivars of *V. oxycoccus*, g) two cultivars of blackcurrant, h) one cultivar of redcurrant, i) three samples of grapes, j) two cultivars of *V. vitis-idaea* (lingonberry), k) 10 cultivars of nectarines measured at both mature and ripe stages from peel and flesh, l) 10 cultivars of peaches measured at both mature and ripe stages from peel and flesh, m) five samples of plums, n) five cultivars of plums, o) five cultivars of plums measured at both mature and ripe stages, p) 12 cultivars of quinces, q) 'Muskoka' cultivated raspberry, r) five cultivars of rose hips, expressed as mg/100 g dry weight, s) two cultivars of rose hips, expressed as mg/100 g dry weight, t) three samples of strawberry.

2.5.2 Bioavailability and metabolites as bioactives

Bioavailability is defined as the proportion of compounds that are absorbed and enter the blood stream so they can be distributed and metabolized within body organs. The term bioaccessible is used to refer to a compound being released from a food matrix during digestion [38]. The bioaccessibility of procyanidins depends on their degree of polymerization (DP), which can range from 1 up to 25+. Higher polymers are doubted ever to leave the food matrix because of entanglement with the plant cell wall polymers, and affinity for covalent attachment with other proteins. Flavan-3-ol monomers, (+)-catechin and (-)-epicatechin along with dimers up to tetramers are believed to be absorbed into the systemic circulation from the small intestine in humans, although in low concentrations [15]. Rodent experiments have demonstrated that more than 95% of consumed procyanidins remain in the intestinal lumen [39]. Procyanidins that do make it into systemic circulation are thought to undergo metabolic conjugation in the liver, producing methyl-, glucuronoyl-, and sulphoconjugates [40]. Very little work has been done *in vitro* with procyanidin metabolites, as they are not commercially available, and isolating the compounds for research can present problems such as stability. One metabolite, methyl-epicatechin glucuronide, has been shown to have protective effects over AD, working through the cyclic adenosine monophosphate (cAMP) response element binding protein [5]. If procyanidin metabolites and not the parent compounds are responsible for cellular activity, little is known of the nature of the metabolites and their modes of action.

Beyond the uncertainty of procyanidin metabolites, catechin, epicatechin, dimers and trimers have been shown to be available in plasma in their free forms at concentrations ranging up to 8.55 μM [40]. A couple of studies have shown that catechin, epicatechin and procyanidin dimers, A1, A2, and B2 are absorbed from the

small intestine without being altered [41,42]. The bioavailability of isolated procyanidins B2 and B3 in distilled water was studied in rats, and at 1 μ M plasma recovery was 89.5% and 90.8% respectively [43]. Plasma concentrations peaked at 30 min and 45 min respectively [43]. Accumulation of procyanidins was demonstrated after long-term intake, with the greatest concentrations of metabolites being found in the liver, kidney, lung, and brain [5,44]. Furthermore, Serra and colleagues showed that both procyanidin metabolites and free-form procyanidins are bioavailable in rat plasma and that tissue accumulation varies depending on the organ [44]. These studies provide support for *in vitro* experiments showing biological activity with procyanidins, which may then be more translatable to the human situation.

The majority of procyanidins consumed are not absorbed in the small intestine and continue through the gastrointestinal tract to the large intestine. It is in the large intestine that the microbiota significantly alters parent procyanidin compounds, degrading them into smaller phenolic acids. Products generated by the microbiome have been reviewed in detail elsewhere [15]. Microbiome products can then be absorbed as these smaller phenolic acids, or excreted. It is possible that some of the health benefits seen as a result of a high procyanidin diet may result from the bioactivity of phenolic acids created by the microbiome. Human intervention studies clearly show beneficial effects from procyanidin intake, although it has been suggested that it may not be through mechanisms that require absorption into systemic circulation [15,45]. Polymeric procyanidins have recently been shown to change the physical properties of phospholipid bilayers and interact with lipid rafts in the gastrointestinal tract [14]. Hexameric procyanidins were shown to hinder a permeability insult by inhibiting extracellular-regulated kinase 1 and 2 (ERK1/2) (see section 2.6.4) activation in Caco-2 human intestinal epithelial cells [46]. Only a few studies have been carried out

investigating procyanidin impact on lipid rafts *in vitro* and they all used specific commercially generated hexameric procyanidins; however, absorption may not be a prerequisite for cellular impact by the higher polymers found in large amounts in fruits [15]. Non absorption-mediated mechanisms such as these would side-step some of the potential issues with the systemic bioavailability of procyanidins.

2.6 Signal transduction: the dance of inflammation and procyanidins

In plants, procyanidin compounds are suggested to play a role in mitigating stresses, such as drought, excess UV exposure, and/or pathogen invasion [14]. It is now clear that these compounds are also capable of modulating the human stress responses as well. As mentioned earlier, inflammation is the process by which the human body responds to injury and it involves a suite of complicated and interconnected cell-signalling cascades. Cellular signalling is an elaborate, complex network of communication mechanisms that enables cells to recognize and respond appropriately to events in their environment. When acute inflammation escalates to chronic inflammation, a variety of disease states can present with aberrant cell signalling processes, such as constitutively activated signal transducer and activators of transcription 3 (STAT3) and NF- κ B in neoplastic cells (see section 2.6.3) [47]. Dietary procyanidin intervention could be a non-pharmaceutical approach to prevent cellular malfunction and maintain a well-controlled cell signalling process.

Initial research focused on health effects of procyanidins acting as antioxidants [48,49]. A hawthorn extract consisting of 18% procyanidin B2, and 17% (-)-epicatechin was shown to promote binding of transcription factor nuclear factor E2-related factor 2 (Nrf2) with the antioxidant response element (ARE) in THLE-2 cells, a human liver cell line [50]. The ARE is a cis-acting element in the promoter of genes encoding major cell

detoxification enzymes [51]. Nrf2 is one of the signals that link oxidative stress with inflammation and cancer [52].

Recent efforts have revealed the ability of procyanidins to modulate inflammatory signal pathways. Effects on the key signal transduction players NF- κ B, activator protein-1 (AP-1), STAT, and mitogen-activated protein kinase (MAPK) will be discussed below.

2.6.1 NF- κ B signalling

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is a family of transcription factors that promote the expression of over 150 important genes [53,54]. Five members of the NF- κ B family, p50, p52, RelA, RelB, and c-Rel, are predominant players in the progression of the human inflammatory response [53]. The active forms are heterodimers and of these, p50/RelA and p52/RelA are the most common. Recently Martinez-Micaelo and colleagues provided a review that covers much of what is currently known regarding procyanidins and their interactions with NF- κ B, summarized as their ability to promote accumulation of p50/RelA in the cytoplasm restricting access to the nucleus, which would decrease the transcript of pro-inflammatory mediators [55]. I offer the distinction between effects of A-type and B-type dimers in reference to NF- κ B here. Experiments in Jurkat T cells showed that only procyanidins B1 and B2, not A1 and A2, were able to inhibit the nuclear binding activity of NF- κ B in response to an inflammatory insult [12]. The minimum energy conformers of procyanidin A1, A2, B1, and B2 were investigated for their ability to bind to the p50 component of the NF- κ B heterodimer, thus restricting translocation to the nucleus. It was illustrated that the structure of the B-type linkages made it possible for hydrogen bonding with three arginine residues in the p50 subunit, while the A-type

linkages did not allow for these hydrogen bonds. Procyanidins B1 and B2 are both compounds that connect via a 4 β - 8 link. It could be important to model the B-type procyanidins that connect via a 4 β - 6 link (e.g. procyanidins B5, B6, B7, B8) to predict if their minimum energy conformations would also be capable of inhibiting NF- κ B nuclear binding activity.

2.6.2 Effects on pro-inflammatory signalling events

Signalling pathways are often not distinct. They interact with and influence each other, a spider's web of communication within the cell that can be very complex to unravel. Many cytokine genes are regulated cooperatively by the transcription factors AP-1 and nuclear factor of activated T-cells (NFAT), with interleukins IL-2, IL-3, IL-4, IL-5, IL-13, interferon γ (IFN γ), tumor necrosis factor α (TNF α), granulocyte-macrophage colony-stimulating factor (GMC-SF), and chemokine IL-8 all having been shown to have NFAT-dependent gene regulation [56]. AP-1 is a transcription factor that is involved with the control of cellular differentiation, proliferation, and apoptosis. A study using a procyanidin B2 derivative found in grape seed extract, procyanidin B2 3,3''-di-O-gallate (B2G2), showed an inhibition of AP-1 expression after pre-treating prostate cancer cell lines, albeit in high doses [57]. This B2 derivative also inhibited NF- κ B. In a further report, the anti-inflammatory properties of isolated procyanidin A1 or apple procyanidin extracts on serum markers of the allergic response were investigated in a mouse model. While the authors did not identify their apple extract chemistry, it can be assumed that B-type linked compounds were in the majority. Mice were sensitized with intraperitoneal injections of ovalbumin, and treated orally with either a control, isolated procyanidin A1, or apple extracts for 21 days. Procyanidin A1 at 3 mg/kg/day and 10 mg/kg/day as well as apple procyanidins at 30 mg/kg/day inhibited IgE production; however, only 10 mg/kg/day A1 was able to inhibit IgG1

production [58]. Furthermore, the study looked at IFN γ and IL-4 serum concentrations. The cytokine IFN γ is secreted from T-helper 1 (Th1) cells and natural killer cells. IFN γ is needed for the maturation of undifferentiated CD4⁺ cell into new Th1 cells and antagonizes the development of Th2 cells. The cytokine IL-4 is associated with the development of Th2 cells, and conversely inhibits the development of Th1 cells. The balance between Th1 and Th2 cell responses is considered a major factor in the progression of pathological allergic conditions, such as allergic asthma [7-9]. Procyanidin A1 at 10 mg/kg/day and apple procyanidins at 30 mg/kg/day were both able to inhibit the production of IL-4 compared with the control. Interestingly, only procyanidin A1 at 3 mg/kg/day or 10 mg/kg/day, and not the apple extracts, increased systemic IFN γ [58]. No molecular mechanisms were proposed by the authors, but it could be speculated that NFAT or AP-1 could be involved and that A-type and B-type procyanidins could interact with signal transduction elements in differing ways.

AP-1 has a reported role in differentiation of naive T cells into Th1 cells and Th2 cells [56]. Furthermore, AP-1 is suspected as a key player in the signal transduction interplay during respiratory epithelium carcinogenesis [59]. The role AP-1 has in the mechanism by which A-type procyanidins influence IFN γ has not yet been tested directly.

Procyanidins were also studied in the context of IL-6, a pro-inflammatory cytokine important in the acute inflammation response. Research with THP-1 cells, a human monocytic cell line, and primary human monocytes showed the ability of procyanidin B1 and C1 to inhibit lipopolysaccharide (LPS)-induced IL-6 production after pre-treatment with procyanidin isolates for 48 hours at 17.3 μ M and 11.5 μ M respectively [60]. B2 showed an inhibitory effect only in primary human monocytes,

not in the THP-1 cell line. Collectively these studies validate the conclusion that procyanidins' anti-inflammatory effects are widespread and will probably be specific to the individual procyanidin designation.

2.6.3 Influence on Signal Transducers and Activators of Transcription

Immune cell infiltration into tissues is a hallmark of chronic inflammation. Chemokines are small cytokine molecules that are secreted by various cells and tissues and promote chemotaxis of immune cells into tissue. Eotaxin-3 is one such chemokine, which promotes chemotaxis of immune eosinophils into lung tissue and is a clinical marker of asthma. There are three isoforms of eotaxin, with eotaxin-3 being the one known to remain 24 hours after an allergen challenge and hence is associated with chronic airway inflammation [1]. Work in our laboratory has shown that fruit proanthocyanidins in dimethyl sulphoxide (DMSO) at 2.5 µg/ml and 7.5 µg/ml are able to inhibit CCL26 secretion from the human lung epithelium *in vitro* after an inflammatory insult of IL-4 [1]. The antagonistic nature of IFN γ toward the Th2 cell perpetuated process was investigated, with proanthocyanidin exposure working with IFN γ to suppress CCL26 secretion. The mechanism of action of proanthocyanidin effects in alveolar epithelial cells was shown to be mediated through stimulation of STAT6 [1]. There are seven members of the STAT family in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [61]. STATs receive an extracellular message by binding to a cellular receptor and after phosphorylation by janus kinase (JAK), dimerize and translocate to the nucleus, where they act either to activate or to repress transcription [62]. Further research using an extract from litchi fruit, a tropical fruit native to southeast Asia, demonstrated an ability of A-type procyanidins to modulate cellular events through STAT3 in RAW 264.7 macrophages [63]. The authors showed an inhibition of the pro-inflammatory mediators nitric oxide

(NO), prostaglandin E2, TNF α , IL-1 β , IL-6, and cyclooxygenase-2 (COX-2) production when cells were pre-treated [63]. Investigation into the cellular signalling pathway demonstrated the involvement of NF- κ B, and STAT3, but not STAT1 [63]. The lowered incidence of phosphorylated STAT3 was through inhibited activation of JAK2, which is the molecular species that interacts with the cellular receptor [63]. Another study, while not investigating isolated procyanidin compounds, used a non-fruit source of procyanidins with 9.7 mg/g procyanidin B1, and 84.3 mg/g procyanidin B2 (concentrations that could be achieved through a 100-mL serving of quince juice, Table 2-1) to investigate procyanidin effects on pro-inflammatory mediators in the context of IBD modelled in mice. The oral procyanidin treatment reduced leukocyte infiltration into the colon, abrogated NO production, and inhibited COX-2 secretion [64]. The molecular mechanisms responsible were shown to be a reduction in phosphorylated STAT1 and STAT3, but not in NF- κ B [64]. Peritoneal macrophages were collected from the mouse model of IBD and treated *ex vivo* with the same B-type procyanidin extracts for 1 h. Experiments showed a significant decrease in the production of IL-6, IL-1 β , and TNF α , but not of IFN γ [64]. It has been suggested that a balance between Th1 and Th2 responses is the optimal scenario for managing inappropriate inflammation and its associated disease [65]. Procyanidins may play a role in re-balancing skewed Th2 responses through utilizing the antagonistic powers of Th1 mechanisms. With respect to cancer development, procyanidin-mediated systemic inhibition of both STAT3 and NF- κ B could result in increased damage to normal cells and tissue [64]. Simultaneous inhibition of all major inflammatory mechanisms is undesirable. Procyanidins could act as mediators of health through selective-partial inhibition of inflammatory pathways – an effect that can vary from tissue to tissue - to balance and bring back appropriate inflammatory responses.

2.6.4 Mitogen-Activated Protein Kinase pathways

The MAP kinases are a conserved family of enzymes that convert extracellular signals into intracellular responses through serial phosphorylation cascades, with immune function and stress responses being only two of the many cellular processes they control [66]. Three distinct pathways exist: C-Jun N-terminal kinase 1 and 2 (JNK 1/2), the p38 pathway, and extracellular-regulated kinase 1 and 2 (ERK 1/2). The A-type procyanidin litchi fruit extract that inhibited NF- κ B, and STAT3 in RAW 264.7 macrophages, was also able to inhibit pro-inflammatory mediator production by inhibiting the phosphorylation of ERK1/2, but not of p38 or JNK [63]. Moreover, procyanidins effect on ERK1/2 phosphorylation in THP-1 human monocytes has been investigated [67]. In these studies, THP-1 cells were pre-treated with grape seed procyanidin extracts or isolated compounds for 4 h and then insulted with 1 μ g/ml LPS. Both grape seed procyanidin extracts and isolated procyanidin B1 were able to inhibit LPS-induced phosphorylation of ERK 1/2. Procyanidins B2 and C1 were also able to inhibit LPS-induced phosphorylation of ERK 1/2 but not to the same degree as the grape seed procyanidin extracts or procyanidin B1. Procyanidin B2- and C1-treated cells expressed phosphorylated ERK 1/2 at c. 10% higher than the control and 60% lower than LPS-only treated cells. While these results are promising, it should be noted that these experiments used 10 μ g/mL of isolated procyanidin compounds, which would correspond to only a supplemental dose. Furthermore, a whole grape seed extract is a known source of both A-type and B-type linked procyanidins [68]. It would have been interesting to discern the results from an isolated A-type linked procyanidin such as A2. The relevance of procyanidins being capable of controlling the phosphorylation on ERK 1/2 cannot be overstated. Activated ERK 1/2 is capable of phosphorylating over 80

known substrates in the cell cytoplasm and the nucleus, affecting a variety of cellular processes such as cell survival, differentiation, metabolism, and transcription [69,70].

2.7 Conclusions

Many chronic diseases that burden our society today are perpetuated by chronic inflammation. Fruit procyanidins have great potential in the appropriate modulation of inflammation to maintain and return health. There is evidence for procyanidins attenuating three major inflammatory pathways, NF- κ B, MAPK, and STAT, with possible collaboration from AP-1. Research into the modulation of inflammation by procyanidins will probably be tissue specific (with defined mechanisms of action being proposed). Asthma, which is an inflammatory disease, can be used as a model system to investigate isolated procyanidins health promoting properties. A cell culture model of allergic asthma could provide evidence for molecular mechanisms by which isolated procyanidins could modulate inflammation. Understanding the mechanisms by which each procyanidin influences cellular signalling in more detail would allow for the focused use of single and multiple procyanidins in fresh and processed foods as a natural means to prevent inflammatory illness, limit reliance on pharmaceutical interventions, and assist with improving human health.

Chapter Three

Literature Review (part 2)

Progress in our understanding of allergic asthma pathology supports the potential of fruit proanthocyanidins as modulators of airway inflammation

3.1 Progress in our understanding of allergic asthma pathology supports the potential of fruit proanthocyanidins as modulators of airway inflammation

Contribution Declaration

This thesis chapter is a prepared manuscript which is under review for publication.

The original manuscript of this chapter was written by Sara Coleman (95%), with content and grammatical editing from Odette Shaw (5%). The revised manuscript was then internally reviewed by Dr. Paul Blatchford (Food and Nutrition, PFR) and Dr. Roger Hurst (Food and Wellness, PFR) as is standard practice at PFR. The internally reviewed manuscript was entered into SPTS at PFR, which includes professional format and grammar editing.

3.2 Abstract

Allergic asthma is a chronic inflammatory lung disease characterized by sensitization of the airways and the development of immunoglobulin E antibodies to benign antigens. The established pathophysiology of asthma includes unresolved inflammation at the lung epithelium, bronchial smooth muscle hyperreactivity, and chronic lung tissue remodelling that collectively restrict airflow in genetically susceptible individuals. Immune cells, including eosinophils and the recently characterized type 2 innate lymphoid cells, infiltrate lung tissue as part of the inflammatory response in allergic asthma. It is well established that a diet consisting predominantly of fruits and vegetables reduces the risk of inflammatory diseases, with recent evidence for the involvement of the microbiota in modulating allergic airway inflammation. Secondary plant metabolites, such as fruit proanthocyanidins, derived from the diet or through supplementation, are a promising intervention for the reduction and prevention of allergic asthma airway inflammation. In this review, the most recent advances in our understanding of the pathophysiology of allergic asthma are discussed. Furthermore, recent progress for utilizing proanthocyanidins to modulate the airway inflammation of allergic asthma is examined, with emphasis on proanthocyanidins mitigating eosinophil cell recruitment to the lungs.

3.3 Introduction

Our understanding of asthma as an airway disease has progressed through history from a collection of symptoms dominated by restricted airflow with little knowledge of causation [71,72], to the belief it was a psychosomatic disorder [73], and then to the understanding that asthma is an inflammation-based lung disease [74,75]. It was the identification of the innate lymphoid cells (ILCs) [76,77] and increased understanding of the roles of T-helper 2 (Th2) cells, as well as IgE adaptive immunology [78], along with advances in our understanding of the human microbiome that allowed a shift from classifying asthma as two phenotypes, allergic and non-allergic, to multiple endotypes clustered by phenotype [8], clinical presentation, and molecular mechanisms associated with the pathophysiology [8,79]. Thus, we now recognize that asthma is no longer a single disease [80,81].

Proanthocyanidins are a sub-classification of plant secondary metabolites within flavonoids, the largest group of polyphenol compounds. Proanthocyanidins are oligomeric in structure, built from flavan-3-ol monomeric units, catechin and epicatechin, and their gallated derivatives. These monomeric units can be linked together through A-type or B-type linkage, and the number of linked monomers is referred to as the degree of polymerization (DP), with 2-10 DP being typical. The sub-classification of proanthocyanidins can be reduced further, such as when referring to the structurally defined dimeric compounds procyanidin A2 or procyanidin B2 (Figure 3-1). Fruit consumption is the most common, though not the most concentrated, source of proanthocyanidins [82], with chocolate, tea, and wine all having high concentrations. Proanthocyanidins are heavily supported as being biologically active in regard to human health [27,83-86]. Their bioactivity is well established and is reviewed elsewhere [87-91].

Within this review, I will focus on the recent progress in our understanding of the pathology of one specific endotype of asthma, eosinophilic allergic asthma [92]. Specifically, I will examine the role of ILC type 2 (ILC2) cells in controlling eosinophil fate in allergic asthma, as well as explore the role the microbiota has on the progression of allergic asthma and as an avenue for therapeutic intervention. The use of dietary support to influence disease development or progression is not a novel concept; but delineating the appropriate use of functional foods has proved to be challenging; however, the progress gained in our understanding of allergic asthma pathology has strengthened the potential of fruit-derived proanthocyanidins as dietary support for lung health.

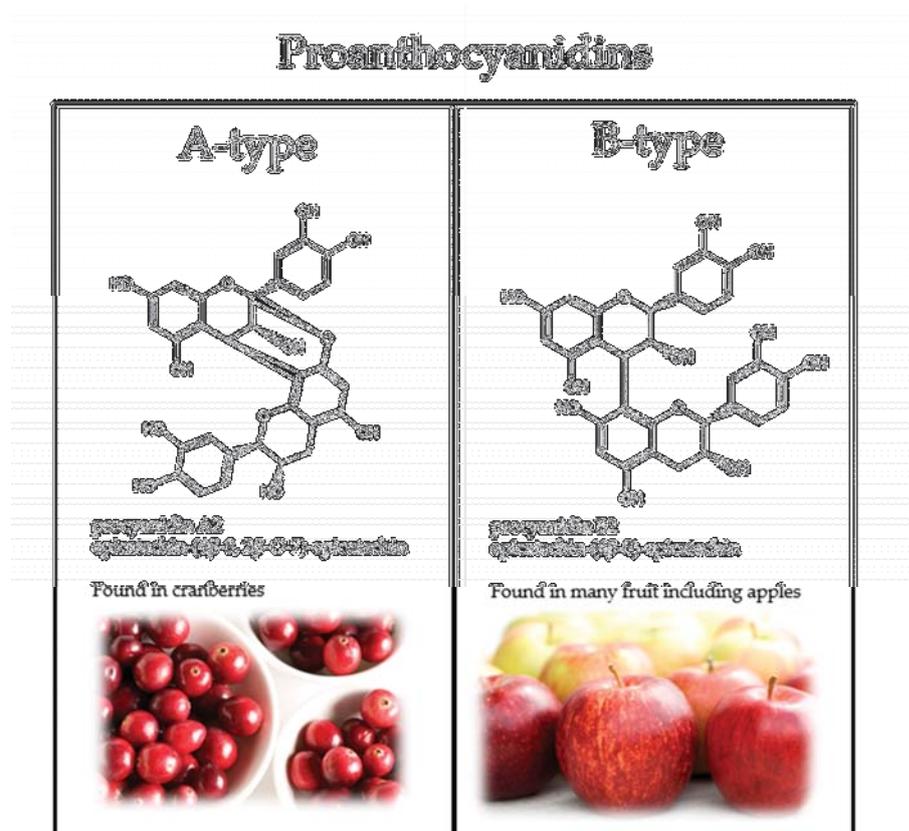


Figure 3-1: Procyanidin linkages and fruit

The chemical structure of procyanidin A2 and procyanidin B2. These two polyphenols are examples of proanthocyanidins A-type and B-type linkages.

3.4 Pathology of allergic asthma

The established pathology of asthma includes repetitive exacerbations of inflammation at the lung epithelium [93], bronchial smooth muscle hyperreactivity [75], mucous production [94], and lung tissue remodelling resulting in reversible airway obstruction. These symptoms are traditionally associated with asthma being an eosinophilic airway disorder perpetuated by overactive T_H2 cells, and their cytokines IL-4, IL-5 and IL-13 in response to inhaled allergens. Now, there are distinctions between different endotypes that are perpetuated by other immune cells, such as neutrophils [95], and that have non-allergen triggers, such as cold air [8,96]. Here, I will concentrate on the pathology of eosinophilic allergic asthma.

3.4.1 The lung epithelium and eosinophilia

The airway epithelium is a central regulator of allergic asthma pathology [97]. Allergen exposure initiates the release of the cytokines interleukin 33 (IL-33), IL-25, and thymic stromal lymphopoietin (TSLP) by epithelial cells [98]. IL-33 induces multiple immune cell types, including Th2 cells, basophils, mast cells, and ILC2 cells which secrete IL-4, IL-5 and IL-13 [99,100]. IL-25 induces eosinophilic inflammation, including: airway hyperresponsiveness, airway remodelling, goblet cell hyperplasia, sub epithelial collagen deposition, and angiogenesis in a Th2 cytokine-dependent manner [101,102]. The presence of TSLP at the epithelium stimulates OX40 ligand (Ox40L) expression on lung tissue-resident dendritic cells facilitating naïve T cells' maturation toward a Th2 phenotype in the lymph nodes [103,104]. Since the 1980s, Th2 cells have been regarded as the primary source of type 2 pro-inflammatory cytokines which perpetuate allergic asthma, although other T helper-cell subtypes have been more recently implicated in inappropriate airway inflammation [105,106].

Lung epithelial exposure to allergens also stimulates the release of eotaxin, a family of chemotactic signalling proteins that are responsible for mediating eosinophil cell infiltration into inflamed tissue. Eotaxins are a C-C-chemokine protein family with three members: CCL11 (eotaxin-1), CCL24 (eotaxin-2) and CCL26 (eotaxin-3). Eotaxins recruit eosinophil cells into the epithelium, where the eosinophils secrete cationic peptides and reactive oxygen species, causing epithelial injury; eotaxins also mediate the release of IL-4 stores from within the eosinophil granules [107]. The expression of each isoform is cell specific. CCL11 is secreted by eosinophils, macrophage, lymphocytes, fibroblasts, smooth muscle endothelial cells, and epithelial cells [108]. CCL24 and CCL26 are functional homologues that are primarily secreted by epithelial and endothelial cells [109]. CCL11 transcription is controlled by both the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and signal transducer and activator of transcription 6 (STAT6), and is the initial eotaxin isoform produced, whereas CCL26 is predominantly controlled by STAT6 and is the late-stage eotaxin remaining present up to 24 h after allergen exposure [110,111]. All three eotaxins interact with the CCR3 receptor on eosinophils to attract them to the site of inflammation [108].

Eosinophils are formed in the bone marrow, migrate into the blood, and are responsible for numerous inflammatory processes, including during parasitic helminth, bacterial and viral infections, tissue injury, tumour immunology, and allergic disease [112]. Compared with other immune cells, they are a small but stable population [113]. Cytokine IL-5 mediates eosinophil development and survival, and it is ILC2s (see section below) that are suspected as the source of the IL-5 which sustains eosinophil presence [80,114].

In a clinical setting, eosinophilia is a demonstrated reliable biomarker for predicting asthma endotype, which is useful when prescribing treatment [115]. Blood eosinophilia can be consistently quantified through serum IL-5 and IL-13 concentration [116]. During allergic asthma exacerbations, the excess eosinophil presence in the lung tissue causes damage to lung epithelium through the release of cationic peptides such as eosinophil-derived neurotoxin (EDN), and peroxidases [113]. The role of eosinophils across asthma endotypes and other mucosal pathologies has recently been reviewed [113,117].

In conjunction with eosinophil infiltration of lung tissue, other immune cells also have a role in allergic asthma initiation and progression. Macrophages are critical for the development of inflammation and fibrosis [118], as Borthwick et al. demonstrated that IL-13-driven tissue inflammation and fibrosis can resolve quickly if not for macrophages recruiting T-cells, which actively maintain the tissue-localized immune responses [118]. Additionally, basophil populations are implicated as a bridge between the commensal-bacterial populations (see section below on microbiome) and allergic inflammation, as antibiotic use increased serum IgE concentrations, whereas depletion of basophil populations reduced the exaggerated responses [119]. Dendritic cells are integral in crosstalk between innate and adaptive immunity and involved in the induction of asthma through antigen sampling across the airway epithelium [104]. The involvement of IgE antibody to benign antigens, and the roles of mast cells and B-cells in allergic asthma are not the focus of this review and have been discussed elsewhere [120-122].

3.4.2 Type 2 innate lymphoid cells

Eosinophil cell survival in peripheral tissues requires IL-5 and concentrations of IL-5 are maintained by ILC2 cells [114,123,124]. ILCs are a recently characterized family of effector lymphoid cells that protect against pathogens, and restore tissue integrity [125,126]. Three subsets have been characterized based on function, development, and cytokine production: group 1 ILCs producing type-1 cytokines (IFN γ , TNF), group 2 secreting type-2 cytokines (IL-4, IL-5, IL-9, and IL-13), and group 3 ILCs producing IL-22, IL-17, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [125]. During homeostasis, ILC2s and ILC3s are the predominant subsets that promote immune regulation and control tissue homeostasis and repair. However, dysregulated ILCs shift towards pro-inflammatory pathology that has been associated with asthma [127]. Bal et al. proposed that IL-12 and IL-4 govern the functional identity of ILCs and that imbalance between these cytokines towards IL-4 results in type 2 inflammation [125]. ILCs act as an innate correlate to T cells [128]. It has been suggested that while T_H2 cells are the main source of type-2 cytokines for atopic (meaning an IgE-mediated reaction) eosinophilic asthma, ILC2 are the main source in non-atopic eosinophilic asthma, as ILC2 cells are non-antigen specific [78]. In a BALB/c mouse model of allergic airways inflammation, T-cell –derived IL-4/IL-13 was essential for IgE production, recruitment of eosinophils and basophils to the lung, goblet cell hyperplasia, and the development of airway hyperreactivity; whereas, ILC2s were recruited to the lung in the same efficiency in wild-type and IL-4/IL-13 T-cell-specific knockout mice [129]. In patients with severe eosinophil non-atopic asthma, ILC2s are thought to be the predominant source of type 2 cytokines [130], although Gordon et al. have shown that basophils and mast cells, not ILCs, may be the cellular source of type 2 cytokines in chronic asthma [99]. The shared roles of ILCs and T cells has been

reviewed [128]. Peripheral blood ILC2s were recently demonstrated to be an accurate biomarker of eosinophilic airway inflammation [131].

3.5 Asthma and dietary intervention

The prominent role of the microbiome in human health has become clear over the last decade. There is a demonstrated association between reduced diversity in the intestinal microbiota and allergic disease [132] and evidence that antibiotic use increases susceptibility to allergic airway inflammation [133]. It has also been shown that asthmatics are more likely to have an inflammation-promoting diet (a diet high in saturated fats and cholesterol and low in fibre, fruits, and vegetables), which may contribute to perpetuation of asthma pathophysiological features [134-136]. Furthermore, high adherence to a Mediterranean diet (a diet high in fibre, fruits, vegetables and fish) is associated with increased concentrations of faecal short-chain fatty acids, and *Prevotella* and *Lachnospira* genera in the microbiome [137].

3.5.1 The gut and lung microbiota

The intestinal microbiota has been suggested as a potential target in the prevention of the development of childhood asthma [138]. Gut community composition analysis in 3-month-old infants showed that atopic wheezing subjects, who had high risk of developing childhood asthma, had a lower abundance of four specific genera *Faecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia* (FLVR) with an accompanying reduction in fecal acetate production compared with age-matched controls, although overall taxa diversity was not different between the groups [138]. Interestingly, when adult germ-free mice were inoculated with the human faeces from one of the atopic wheezing subjects supplemented with the four above-mentioned FLVR genera, the F1 offspring retained the modified microbiota, and after undergoing an ovalbumin (OVA)

immunization regime were protected from airway inflammation [138]. The FLVR-supplemented animals had reduced lymphocytes in the bronchoalveolar lavage fluid (BALF) and reduced histopathology compared with mice inoculated with faeces from an atopic wheezing subject without supplemented FLVR. The authors suggested that because the vast majority of the intestinal bacteria detected at 3 months of age are gram-positive, the reduction in a gram-negative species (*Veillonella*) may not allow for adequate LPS biosynthesis, which has been demonstrated to induce proliferation of IL-12-producing dendritic cells and promote the T_H1 arm of the immune response, providing opposition to Th2 mechanisms. Thus the microbiome's influence on asthma susceptibility could be through priming of the immune system for either tolerance or hyper-reactivity. Indeed, the microbiota is engaged in a complex, bilateral interaction with the host and has a demonstrated effect on the innate immune system [139].

The immune system at mucosal sites across the body including: the gut, lung, oral, and cervical epithelium are understood as one collective functioning system [140]. One example of the cross-talk between mucosal sites is the development of T regulatory (T reg) cells in the gut following oral probiotics which then migrate to the lung and protect against allergic airway inflammation [141]. The lower airways of the lung were once thought sterile, but have been revealed as a host of commensal bacteria and an important component in the overall human body microbiota. It is typical of infant's lungs to be initially colonized with *Staphylococcus* or *Corynebacterium* before stable colonization with *Alloiococcus* or *Moraxella* [142].

The risk for developing allergic asthma is determined early in life, with the underlying cause of asthma being associated with a disruption of microbial communities which can then lead to airway inflammation [143]. Viral infections and shifts in bacteria

abundance in the lower airways increase susceptibility to respiratory illness, with early life changes to host-microbiota interactions leading to long-term predisposition to airway inflammation [144]. The interactions between host and airway microbiome train the maturing infant immune system for tolerance through a cycling process involving Th2 cells and T reg cells and requires programmed death ligand 1(PD-L1) [145]; however, viral infections, antibiotic use, and day-care attendance can interfere with this development and result in airway mucosal dendritic cells with increased expression of the high-affinity IgE receptor (FceRI) leading to a greater allergic response. Similarly, epidemiological evidence has suggested reduced risk of experiencing allergic asthma from early childhood exposure to farm environments, barn-microbes, and unpasteurized milk [146]. Thus, establishing a tolerance-promoting airway environment is crucial for reducing risk of allergic asthma.

3.5.2 Dietary intervention to modify microbiota

Evidence supporting dietary intervention to modify the gut microbiota to improve health is growing [137]. A study demonstrated that high-fibre diets yield a distinct acetate-producing gut microbiota profile in mice [147]. Additionally, maternal consumption of a high-fibre diet protected the F1 generation from developing allergic airway disease as a result of increased exposure to acetate *in utero* [147]. Fruit are rich sources of soluble fibre in the diet; they are also a common source of proanthocyanidins in the diet [17,18,82]. Their consumption satisfies two of the three ‘P’s’ for gut health: probiotics, prebiotics, and polyphenols [148]. The interaction between polyphenols and the gut microbiota related to human health has been reviewed [149-156].

It is possible that consumption of fruit-derived proanthocyanidins could beneficially modulate the immune system, reducing susceptibility to inflammatory

diseases, including allergic asthma. This concept has been demonstrated with Concord grape polyphenol extracts that improve health outcomes in relation to metabolic syndrome. The improved health outcomes were demonstrated to be mediated via the gut microbiota [157]. Additionally, fruit are regarded as beneficial for targeted sites, such as cardiovascular health [158]. A similar benefit could exist for lung health.

Biological effects of proanthocyanidin consumption are clear despite administered radio-labelled proanthocyanidin studies showing minimal absorption with 88-94% being recovered from the gastrointestinal tract or the faeces [157]. Thus, the gut microbiota has been suggested as the missing link between the poorly absorbed proanthocyanidins and improved health outcomes as a result of their consumption [157]. The consensus is that only a small portion (< 10 %) of parent polyphenolic compounds are found in circulation, and that smaller microbiota-produced metabolites are responsible for much of the health-promoting activity of proanthocyanidins. An *in vitro* fermentation model, which was used to investigate metabolism of procyanidins by human faecal microbiota, indicated that catechin, epicatechin, procyanidin B2, procyanidin A2, and partially purified apple procyanidins as well as partially purified cranberry procyanidins can all be metabolized by human faecal bacteria [159]. Comparatively, A-type linked and high-degree polymerization compounds were more resistant to microbial catabolism than B-type linked and low-degree polymerization compounds [159]. Both procyanidin A2 and partially purified cranberry extracts show at least 50% original compounds after 24 h of fermentation [159]. Further supporting this, lingonberry supplementation, a fruit high in A-type link proanthocyanidins, has been shown to prevent low-grade inflammation in high-fat diet fed mice through increasing the relative abundance of *Akkermansia* and *Faecalibacterium*, two genera associated with anti-inflammatory activity and a healthy gut [160]. Similar anti-

inflammatory effects through increased *Akkermansia* gut colonization of mice were demonstrated with cranberry extracts [161]. Deciphering the interplay between the gut microbiome and dietary components will allow for advancements in personalized nutrition [162]. Modulation of lung microbiota by dietary intervention and/or polyphenols is still in its infancy and requires further attention.

3.5.3 Proanthocyanidin inhibition of eosinophilia biomarkers

In plants, procyanidin compounds are suggested to play a role in mitigating stresses, such as drought, excess UV exposure, and/or pathogen invasion, and are demonstrated as biologically active in the context of human health [14,55,82]. Many plant-derived food polyphenols have been investigated for their ability to attenuate allergic airway inflammation [163], with recent investigation of proanthocyanidins' role in inhibiting eosinophilia, a hallmark feature of allergic asthma. Eosinophil cell infiltration of the lungs can be predicted in humans most reliably with serum measurements of IL-13 and IL-5 as well as CCL26 [116]. Various blackcurrant cultivar extracts, and specifically proanthocyanidin-enriched blackcurrant extracts, have been shown to inhibit the *in vitro* production of CCL26 in type-2 cytokine-stimulated human alveolar epithelial cells [1,164]. Similarly procyanidin A2 inhibits both CCL11 and CCL26 in type-2 cytokine-stimulated human alveolar epithelial cells [165,166].

Proanthocyanidins have also been investigated in both murine models of OVA and house dust mite-induced allergic asthma [167]. A commercially purchased grape seed proanthocyanidin extract was shown to ameliorate a comprehensive set of biomarkers for inflammation, bronchial hyper-responsiveness, and lung tissue fibrosis in both an acute and a chronic murine model of allergic asthma [168]. Grape seed proanthocyanidins extracts typically consist of B-linked proanthocyanidins, although no

specific chemistry was given in this study. The Th2 cytokines IL-13 and IL-5 were reduced while the Th1 cytokine IFN γ was increased, suggesting that grape seed extract supported the rebalancing of the Th1/Th2 antagonistic relationship [168]. Similar results with another grape seed extract were shown by Zhou et al. [169]. Grape seed extract-treated BALB/c mice sensitized with OVA for eight weeks to model chronic inflammation had reduced eosinophil count and decreased IL-4 and IL-13 in BALF compared with controls. Work from the Food and Wellness group has complementary results, with blackcurrant inhibiting eosinophilia through reduced production of CCL11 in an OVA-induced allergic airway inflammation mouse model [170].

Proanthocyanidins also influence allergic asthma biomarkers other than eosinophilia. Boysenberry extracts in a chronic model of allergic airways inflammation reduced lung tissue fibrosis by supporting the development of pro-fibrotic alternatively activated macrophage [171]. The data from animal studies for Boysenberry have yet to be replicated in human intervention studies. The data from *in vitro* and murine *in vivo* models are promising; however, few human trials have been conducted to investigate a proanthocyanidin supplement or food preparation. Although there is not yet a clear protocol for a proanthocyanidin-based therapeutic intervention, there is epidemiological evidence in humans that clearly demonstrates that proanthocyanidin exposure is advantageous for health and results in a decreased risk of inflammatory disease [172-177].

A future approach for investigating proanthocyanidin inhibition of airway inflammation could be the direct application of structurally defined polyphenolic compounds to the lung via inhalation. A recent study used A-type procyanidins in a preparation administered intranasally to a mouse model of allergic rhinitis, which has

T_H2-dominated pathology similar to asthma [178]. Investigating proanthocyanidins administered through inhalation would complement oral ingestion by possibly allowing biologically active parent compound structures access to pulmonary target tissue while also gaining benefit from the complex mixtures of metabolites generated by the microbiota.

3.6 Concluding remarks

Understanding the therapeutic benefit of fruit proanthocyanidins still remains a challenge in respect to dietary consumption, because of the low bioavailability of parent compounds, the complexity of resultant metabolites of the microbiota, and their ability to gain access to or influence tissues outside the gastrointestinal tract. It is imperative for research in this area to progress for investigators to clearly identify biologically active polyphenolic moieties within a fruit extract. *In vitro* work must consider the physiological relevance of investigated concentrations, and *in vivo* work should monitor the microbiota profiles. Additionally, human trials should use appropriate biomarkers for measuring allergic asthma outcomes. As our understanding of chronic inflammatory diseases, including allergic asthma, continues to progress, we can continue to characterize the use of foods and structurally defined proanthocyanidin compounds for therapeutic interventions to support tissue-specific and overall health.

Chapter Four

Materials and Methods

4.1 Introduction to materials and methods

The materials and methods listed in this chapter include information that has not been detailed in the data-generating research chapters (chapters 5-9). Each of the research chapters contains its own materials and methods section which provides the necessary information for the body of research contained within the respective chapter.

4.2 Materials

Epithelial cell lines were purchased from the American Type Culture Collection (A549: ATCC® CCL-185™; BEAS-2B: ATCC® CRL-9609™; Calu-3: ATCC® HTB-55™ (c/o Cryosite, New South Wales, Australia)). Cell culture media, phosphate buffered saline (PBS), penicillin-streptomycin-neomycin antibiotic mixture, 100× L-glutamine, and 2.5% trypsin were purchased from Life Technologies (Auckland, New Zealand). Fetal bovine serum (FBS) was purchased from Moregate Biotech (Hamilton, New Zealand). Low-endotoxin bovine serum albumin (BSA) was purchased from MP Biomedicals (Auckland, New Zealand). The TSLP, CCL11, and CCL26 DuoSet ELISA kit, human recombinant IL-4 and IFN γ were purchased from R&D Systems (Pharmaco, Auckland, New Zealand). Ecoteric T20 (Tween 20), 0.5% trypsin, and 30% hydrogen peroxide were purchased from ThermoFisher Scientific (Auckland, New Zealand). Sodium acetate tri-hydrate was from BDH Laboratory Supplies (Poole, UK). Procyanidin A2 (HPLC \geq 99%, epicatechin-(4 β -8, 2 β -0-7)-epicatechin), procyanidin B1 (HPLC \geq 80%, epicatechin-(4 β -8)-catechin, and procyanidin B2 (HPLC \geq 90%, epicatechin-(4 β -8)-epicatechin) were purchased from Extrasynthese (Genay, France). WST-1 reagent was purchased from Roche (Auckland, NZ). β -nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), sodium pyruvate, dimethyl sulfoxide (DMSO), 3,3',5,5'-tetramethylbenzidine (TMB) and all other chemicals not specifically listed were purchased from Sigma-Aldrich (St. Louis, MO, USA). The materials listed

here are not an exhaustive list. Additional materials used in this project are detailed in chapters 5–9.

4.3 Airway epithelial cell culture

Human airway epithelial cell culture models were utilized to gain insight into the relationship between dimeric procyanidins and physiological responses of the pulmonary epithelium during airway inflammation. There are a number of airway epithelial cell-lines commonly used in biomedical research, such as A549, BEAS-2B, and Calu-3. The *in vitro* experiments for this project used predominantly the A549 human alveolar epithelial cell-line. The lung can be divided into the conducting airway (bronchiolar system) and the alveolar system. The alveolar sacs sit at the end of the bronchi and are responsible for gas exchange. The bulk of O₂ enters into the lungs through alveolar cells and diffuses across the epithelium into the blood to be picked up by haemoglobin and distributed to the rest of the body. The alveolar surface of the lungs is comprised of two epithelial cell types, the terminally differentiated type I pneumocytes cell and its progenitor, type II pneumocyte cells [179]. Type I cells are responsible for gas exchange and cover approximately 96% of the surface area of the pulmonary epithelium but are unable to divide. Type II cells are more numerous than type I cells, but constitute less surface area. The A549 cell-line is derived from an adenocarcinoma in a 58 year-old Caucasian male and has been shown to have properties similar to type II pneumocytes [180]. Primary type II cells will after 5-6 days in culture reduce proteolytic activity and increase their transepithelial electrical resistance (TEER); in short, they start to resemble type I cells. Cell cultures of A549 cells do not have this shift following long-term culture; they maintain proteolytic activities and low TEER levels compared to type I cells [181]. A549 also have been shown to have surfactant containing lamellar bodies, which is also a characteristic of type II

pneumocytes [180]. It was because of their distinct functions and substantial characterization in the literature that A549 cells were chosen for development into a model for airway inflammation here.

Similarly, two other lung epithelial cell-lines were utilized during this project. The BEAS-2B human bronchial epithelial cell-line is virus transformed from normal lung tissue. The BEAS-2B bronchial cell-line was investigated as a complement to the alveolar A549 cells. Additionally, the human bronchial submucosal cell-line, Calu-3, was derived from an adenocarcinoma in a 25 year-old Caucasian male and is suggested to have greater secretory capacity compared with A549 cells [182]. Calu-3 is commonly utilized in drug absorption studies because of the expression levels of proteins that make up the major intercellular junctions (i.e. tight junctions); they differentiate, and express essential drug transporter proteins [183]. In the event of research involving application of procyanidins by aerosol and not as a consumed food, it would be advantageous to utilize this cell-line. The most translatable model system would be primary cultures of mixed populations of human airway epithelium. However, even this model has limitations because of the lack of tissue availability, limited amount of cells generated by primary cell cultures, and donor variation [184]. Additionally, the type I pneumocytes cell line, TT1, was not used in this project but could be a complementary model to that of A549 [185]. Furthermore, a distal lung epithelial cell-line with type II properties but which are electrically tight could be useful for complimentary experiments [186].

4.4 Methods

The methods described here are detailed accounts of methods utilized in the research chapters. Additionally, I provide background information necessary for

understanding how data were analysed and describe assay development which established controls.

4.4.1 Cell culture aseptic technique

Strict aseptic technique was followed for the culturing of all cell-lines. Aseptic technique is a series of technical steps that are employed to keep the cell cultures free of contamination by microorganisms such as bacteria, fungi, or mycoplasma and the user safe from the cancerous cell-line. These steps include a designated work area, personal hygiene, and specific clean handling practices of reagents and tools.

All culture work was performed inside either a Heraeus HS18 class II biological cabinet or a Bio-Cabinet Bioair top safe 1.8 class II biological cabinet. Both of these provided protection for the external personnel as well as the sample within the cabinet. The airflow of a class II cabinet is designed such that sterile air falls over the samples within the cabinet and exiting air is HEPA-filtered. Prior to starting cell culture work, the cabinet was turned on and air cycled for 15-30 mins. The work surface was sprayed with 70% ethanol and only essential tools (i.e. sterile pipettes and tips) were kept within the cabinet space. Any tool or reagent to be used was sprayed with 70% ethanol before entering the cabinet space.

Personal hygiene practices were also used to maximize protection of both personnel and sample. Wearing a lab coat and gloves, and tying back hair reduced the risk of contact with potentially hazardous cancer cell-lines. The cabinet space was set up such that all reagents could be reached easily and to minimize reaching over any sample.

All pipette tips, reagents, flasks, and plates used during culture were purchased as sterile. The culture media arrived sterile from the manufacture and were only opened

within the cabinet space to maintain sterility. Lids to reagents, flasks, and plates had minimal contact with work surfaces and were replaced to cover their containers as soon as possible to maintain sterility of cells and liquids inside. All pipette tips were single use and contact with reagents bottles or culture plates was kept to a minimum.

The cell stocks used in this study were tested for mycoplasma in July 2013 and demonstrated (by polymerase chain reaction and resolving samples by agarose gel electrophoresis) to be negative. The culture environment was visually monitored for the presence of bacterial and fungal contamination. Signs of contamination include cloudy and discoloured cell culture media, and unpleasant smells. Contamination of cell cultures never occurred during this doctoral project due to the diligent use of aseptic technique and the well maintained equipment.

4.4.2 Routine cell culture

Cell culture stocks are maintained for long-term storage by cryopreservation in liquid nitrogen. Cells were stored in complete growth medium supplemented with 5% DMSO (v/v). To revive cell stocks for active culture, a 1 mL vial of cryopreserved cells was removed from liquid nitrogen and quickly thawed inside a biological cabinet. For A549 cells, 10 mL of culture media was added to a 75cm² (0.2 µm vented plug seal cap), polystyrene cell culture flask (T-75 flask) and then 1 mL of thawed cryopreserved cells was pipetted into the flask. The flask was then incubated for 3-12 h for cells to adhere and then culture medium was refreshed. Depending on the specifics of the cell-line, it may be necessary to start initial culture in a smaller flask (T-25 flask) as some cells grow better when in closer proximity to other cells at this early post revival stage.

Mammalian cell cultures were maintained in incubators which keep atmospheric conditions at 37°C with 5% CO₂. The constant 37°C represents basal human body

temperature and allows for maximal proliferation of most mammalian cells. The atmosphere was supplemented with 5% CO₂ because it aided in maintaining the pH balance of the cell culture media. Regulating pH was critical for optimal proliferation of cell cultures. The gaseous CO₂ balances with the CO₃²⁻/HCO₃⁻ content of the culture medium to maintain an optimal 7.4 pH. Chemical buffering agents such as HEPES can also be used in conjunction with controlled gaseous atmosphere. Additionally, the culture media used for this project contained phenol red which was a visual indicator for pH. The red culture media can change to yellow at low pH levels or to purple at high pH levels.

Following revival from cryopreservation, it is generally recommended that adherent cell cultures have media changes every other day except in the event they have recently been passaged or seeded for an experiment, then they would receive fresh media the next day. I passaged cells as needed (once every week or two) as A549 cells display contact inhibition, which means once the entire culture surface of a flask is covered with cells, their proliferation slows and they do not grow on top of each other. Depending on the specific characteristics of a cell-line, they may be susceptible to overgrowth if not passaged regularly.

Passaging or detachment of cells for seeding an experiment commonly involves the use of the serine protease, trypsin. Trypsin cleaves peptides on the C-terminal side of lysine and arginine residues. Trypsin cleaves proteins which bind the cells to the flask's plastic surface. To start detachment, cell culture media is removed and cells are washed with Ca²⁺Mg²⁺-free PBS. These metal ions decrease trypsin's enzymatic activity; the use of ethylenediaminetetraacetic acid (EDTA) with trypsin is also common - EDTA is a chelating agent and has an affinity for the metal ions. Trypsin

(0.5%) was diluted (50%) in $\text{Ca}^{2+}\text{Mg}^{2+}$ -free PBS and incubated on cells at 37°C for approximately 5-10 mins or until the cell layer was visibly detached from the bottom of flask. Complete media was then added into the cell flask and gently pipetted up and down to disperse clumps of cells and create a single cell suspension. The resultant cell suspension was then spun down for 5 min at 1200 RPM to create a cell pellet. The supernatant was poured off to remove the remaining trypsin. The cell pellet was then resuspended in fresh media. For stock cell cultures that weren't involved in an experiment, 1 mL of resuspended cells was added back to a new flask with fresh complete media, allowed to adhere again for 3-12 h, and then media refreshed. Passaging was always done using trypsin; however detachment during experimentation can involve other methods if appropriate which are detailed in chapter 8.

For an experiment, cells were seeded into 6 or 12-well plates as needed. They were incubated for 24 h at 37°C with 5% CO_2 in complete media and then culture media was switched to serum free media for 24 h. FBS is a complex mixture of different growth factors, proteins, vitamins, trace elements, and hormones which are used for the proliferation of cell cultures; however, serum components can interfere with accurate measurement of subtle changes in cytokine production. Thus, it is common practice for experimental purposes to remove serum from the culture media [187].

4.4.3 Cell counting

Epithelial cells were counted when seeded for an experiment to maintain consistency between experiments. Cells per volume were enumerated using a hemocytometer and manual counting through an Olympus CKX41 inverted microscope. Four-1 square mm corner areas were counted for cells and then averaged to give a 10^4

cell count per 1 mL. Cells were diluted to desired seeding concentration with complete media.

4.4.4 Quantitative analysis of biomarker production by ELISA

Enzyme-linked immunosorbent assays (ELISA) were used to quantify epithelial cell production of biomarkers of interest from the extracellular media. The commercially purchased sandwich ELISA kits used consisted of seven steps to produce a chromogenic reaction with washing between each step (Figure 4-1). First, a capture antibody for target antigen bound to the plastic assay plate. Transparent, polystyrene, flat-bottom, high-binding Greiner 96 well ELISA microplates from Greiner bio-one (Medi'Ray, Auckland, New Zealand) were used for this project and were demonstrated as producing the most consistently reliable results (see section 5.5.1 for more details) for the biomarkers investigated. Next, blocking with 5% BSA was required to prevent subsequent non-specific binding to the plastic. Third, the complex mixture sample containing the target antigen (extracellular media) was added to each well and the target antigen bound with the capture antibody. Fourth, a biotinylated detection antibody bound to the target antigen to provide a binding site for step five a streptavidin labelled

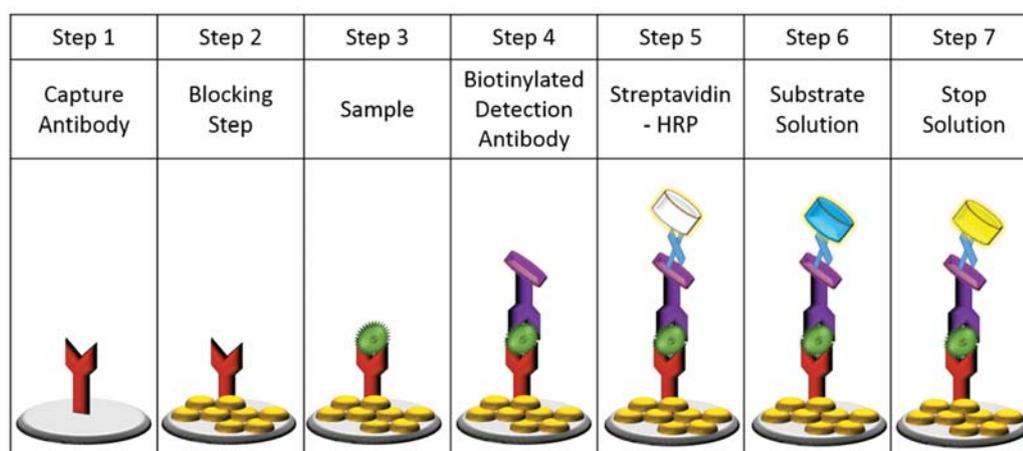


Figure 4-1: Schematic of sandwich ELISA reaction

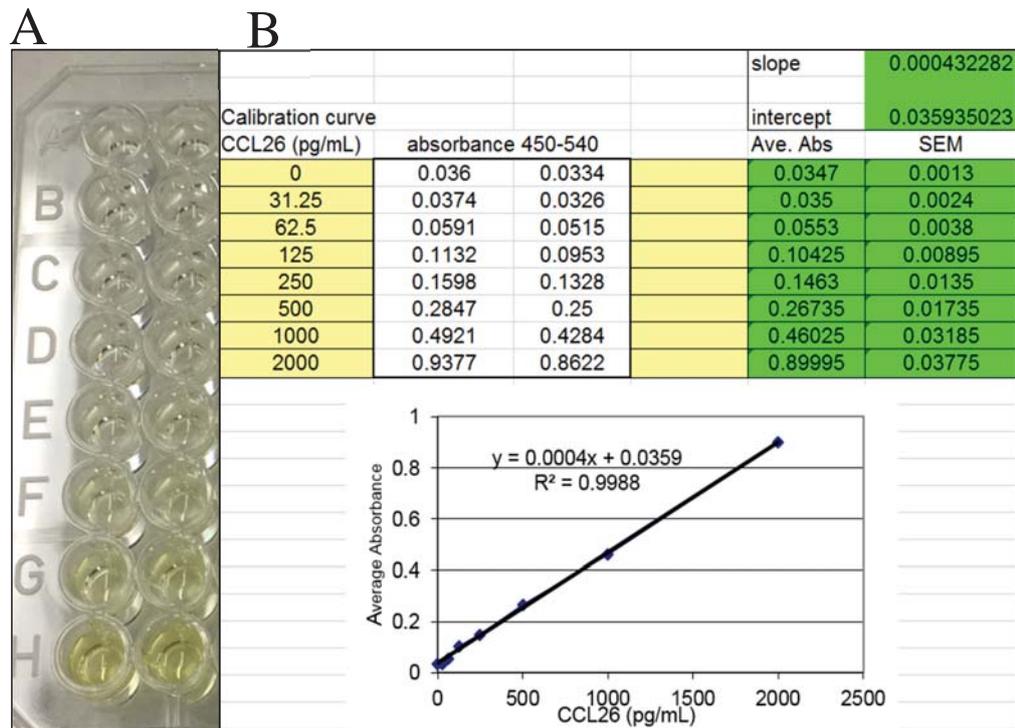


Figure 4-2: An ELISA standard curve.

A) A representative standard curve included on each ELISA plate. B) The graph used for creating the standard curve y-intercept and slope.

horseradish peroxidase (HRP) enzyme. Biotin and streptavidin are two proteins that have considerable affinity for each other, a relationship that has been exploited for the development of many biological assays. Step six was the addition of a substrate solution containing TMB. The HRP enzyme can catalyse the conversion of TMB into a coloured product (blue) with hydrogen peroxide as the oxidizing agent. A stop solution of sulfuric acid stops the reaction (yellow) (Figure 4-2). Absorbance was then measured at 450 nm and 540 nm. The absorbance at 540 nm was subtracted from the absorbance at 450 nm to account for the absorbance of the assay buffer and polystyrene plate.

A standard curve was used to quantify the amount of target antigen. A 7-point serial dilution of a known concentration of the target antigen, termed ‘the standard’, is included on each ELISA plate in conjunction with experimental samples. Each

commercially purchased ELISA kit contained standards and the manufacturer provided a suggested serial dilution that determines the range of detection for their product. Figure 4-2A shows a representative standard curve. Wells H1 and H2 demonstrate the colour change for 2000 pg/mL of target antigen. A serial dilution was created down to 31.25 pg/mL (wells B1/B2), with PBS alone representing 0 pg/mL (wells A1/A2). The duplicate absorbance measurements for each standard curve point are averaged and graphed (Figure 4-2B); thus, allowing the concentration of target antigen in experimental samples to be calculated with the slope and y-intercept of the standard curve (Figure 4-3). To avoid calculated experimental sample values being negative, each experiment included baseline samples in triplicate. These baseline samples were the extracellular media from cells alone with no treatments or stimulation. The absorbance readings were averaged for the baseline samples and set as the y-intercept. This represented that routine culture of cells had 0 pg/mL of investigated target antigens.

$$\text{Sample } \frac{\text{pg}}{\text{mL}} = \frac{(\text{Avg. Sample Abs.}) - (\text{y intercept set as baseline abs.})}{\text{Slope of Standard Curve}}$$

Figure 4-3: Equation for calculating sample pg/mL of target antigen

4.4.5 Developing controls for cytotoxicity assays

Cell viability was measured by a water soluble tetrazolium-1 (WST-1) assay and the lactate dehydrogenase (LDH) assay to ensure experimental conditions were not toxic to cell cultures (see chapters 6 and 7). During the WST-1 assay, the rate at which tetrazolium salts are cleaved to formazan by cellular mitochondrial dehydrogenases correlates with the number of viable cells in the culture, thus WST-1 assay measures metabolic activity of cells as an indicator of viability (Figure 4-4). For the LDH assay, measuring the presence of LDH in the extracellular media is an indirect measure of

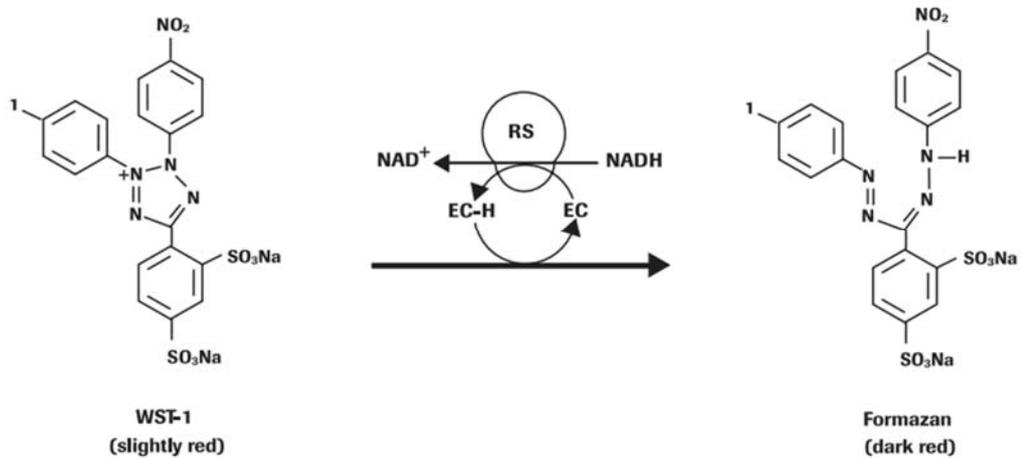


Figure 4-4: The reaction involved in the WST-1 cell viability assay

The WST-1 reagent is a tetrazolium salt which is cleaved by cellular dehydrogenases into formazan. Product information copyright of Roche.

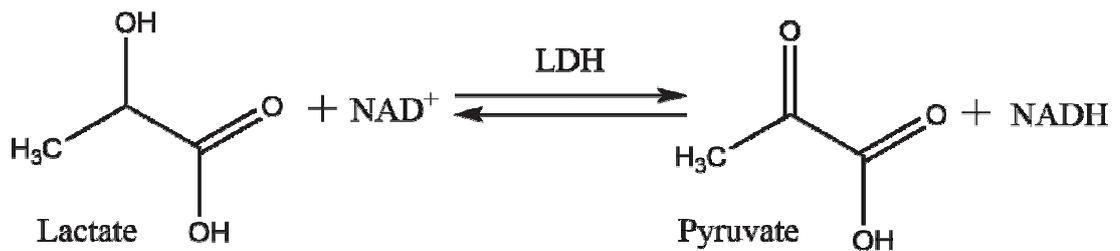


Figure 4-5: The LDH enzyme reaction

The intracellular lactate dehydrogenase (LDH) enzyme catalyses the interconversion of pyruvate and lactate accompanied by the oxidation of NADH to NAD⁺. This is a reaction that is associated with membrane integrity.

cellular plasma membrane integrity. LDH catalyses the interconversion of pyruvate and lactate with the oxidation of NADH to NAD⁺ and NAD⁺ to NADH respectively (Figure 4-5) The LDH assay measures absorbance of NADH at 340 nm over time. The change in NADH concentration is then used as a measure to demonstrate the concentration of LDH in the extracellular media. The presence of LDH in a sample would be evidence that the treatment was damaging to the cell plasma membrane integrity allowing LDH enzyme to leak out of the cell. Assay conditions and positive controls were developed for both of these cytotoxicity assays.

4.4.5.1 WST-1 assay conditions

For the WST-1 positive control, A549 cells were seeded at 5×10^4 per well in a 96-well plate, serum starved for 24 h and then incubated with a range of hydrogen peroxide (H₂O₂) concentrations or media alone for 6 h. After a wash with PBS, WST-1 assay reagent (10 µl) was added to each well containing fresh PBS with Ca²⁺Mg²⁺ (100 µl), according to the manufacturer's directions. WST-1 reagent incubation time is advised to be optimized for each cell type. Four identical experimental plates were seeded then incubated for 30, 60, 90, or 120 min. Plates incubated with WST-1 reagent for 30, 90, and 120 min showed similar results (Figure 4-6). H₂O₂ concentration decreased A549 cell viability in a concentration dependent manner. Cell viability was approximately at 90%, 65%, 50%, and 25% of baseline for 1.25, 2.5, 5, and 10 mM H₂O₂. Concentrations higher than 10 mM H₂O₂ showed no further reduction in viability beyond 25%. The plate incubated with WST-1 for 60 minutes showed a higher percentage of viability, which I believe is an anomaly due to insufficient shaking prior to absorbance reading. The 60 min assay plate was shaken with a bench top shaker prior to reading absorbance. The 30, 90, and 120 min plates were shaken vigorously by the plate reader's own shaking settings. Collectively, these data demonstrated that vigorous

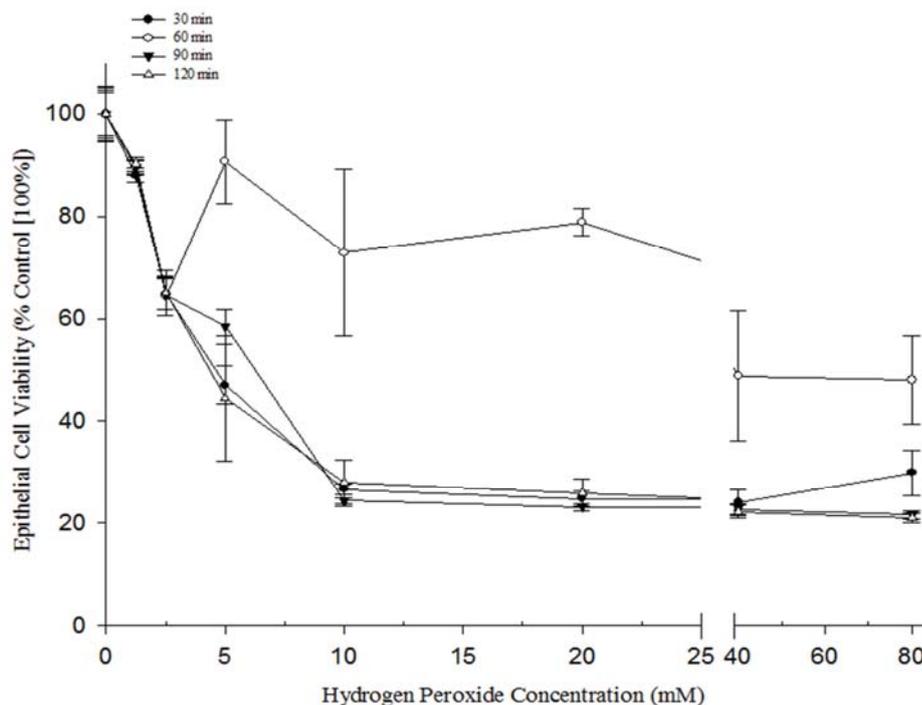


Figure 4-6: Determining appropriate positive control for WST-1 assay

A549 cells were seeded at 5×10^4 per well in 96-well plates, serum starved for 24 h, and incubated with a range (1.25 to 80 mM) of H_2O_2 concentrations, or control media for 6 h. Cells were washed and incubated with WST-1 reagent for 30, 60, 90, or 120 mins and then absorbance measured at 450 and 620 nm. Results are means expressed relative to baseline (0 mM H_2O_2 = 100% viability) \pm SEM, which were from 1 experiment and 4 individual measurements.

shaking is necessary for proper absorbance readings, that 30 min incubation with WST-1 reagent is sufficient for the tetrazolium salts to be cleaved to create formazan, and that 2.5 mM H_2O_2 is an appropriate positive control for the WST-1 assay when using A549 cells.

4.4.5.2 LDH assay conditions

The LDH assay is a method for measuring cell viability complementary to the WST-1 assay, as they measure different cellular parameters. LDH assay conditions were optimized in preliminary experiments. Figure 4-7 shows the change in NADH concentration over time (10 min) for a range of LDH standard concentrations as each converts the substrate solution consisting of 3.8 mM sodium pyruvate and 0.8 mM

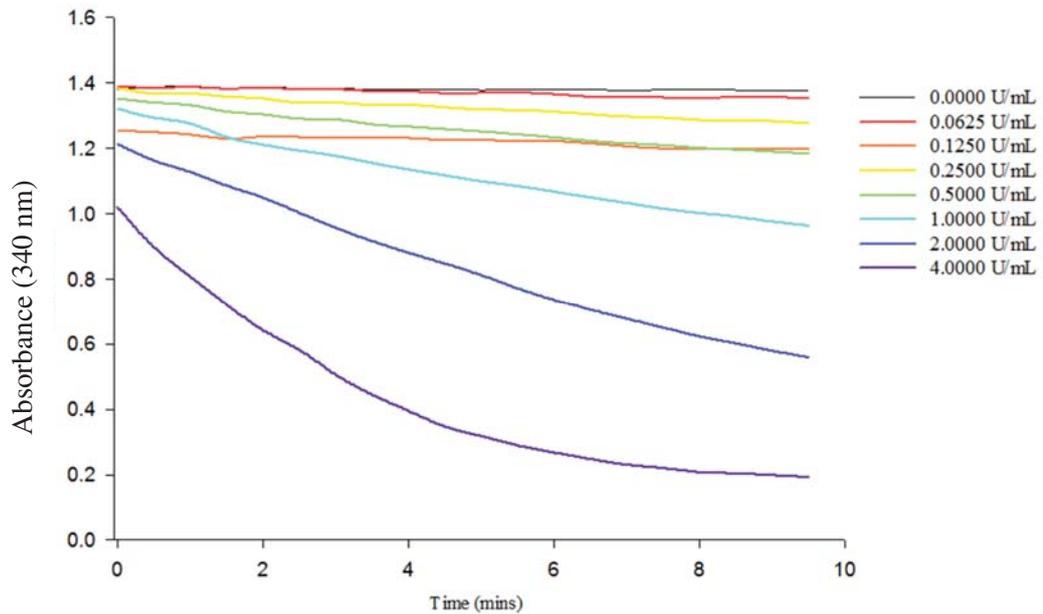


Figure 4-7: LDH assay using standards

LDH enzyme standards in a range of concentrations were combined with 21 mM sodium pyruvate and 4.8 mM NADH. Absorbance of NADH was measured at 340 nm over 10 mins. Results are from 1 experiment with single enzyme dilutions for each concentration.

NADH. LDH enzyme at concentrations ranging from 0.0625 – 0.1250 U/mL were not enough to see a noticeable decrease in NADH (i.e. flat lines). The NADH oxidation over time began to increase with 0.2500 U/mL LDH, created a linear reaction with 1 and 2 U/mL (i.e. sloped line) and increased to saturation of the reaction at 4.0 U/mL (i.e. curved line). These data using standards established the technical setup was sufficient for measuring changes in NADH concentration and that the experimental substrate and BMG Polarstar Omega plate reader (Alphatech) settings were appropriate. Next, I investigated the optimal cell density. A549 cells were seeded at different densities, serum starved for 24 h, and then exposed to 1% Triton-X 100 for 10 min at room temperature. Collected supernatant samples were centrifuged at 12,000 x g for 15 min and immediately assayed for the presence of LDH. In a 96-well plate, 100 μ L of each sample was pipetted into a well, and using automated microplate injectors of the

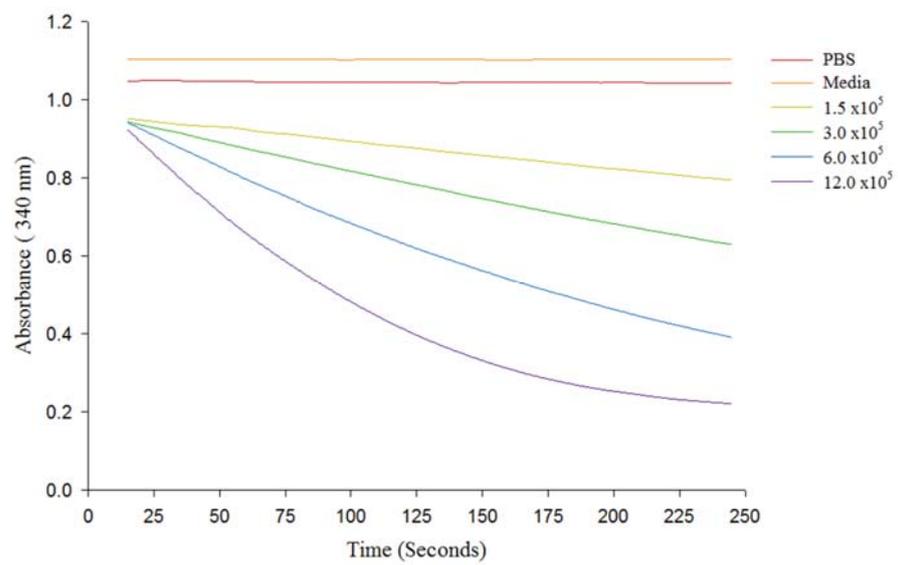


Figure 4-8: LDH assay of 100 % cell death from varied A549 cell densities.

A549 cells were seeded at different densities, serum starved for 24 h, and then exposed to 1% Triton-X 100 for 10 min at room temperature. Collected supernatant samples were centrifuged at 12,000 x g for 15 min and immediately assayed for the presence of LDH. Results are from 1 experiment with one well for each cell density.

POLARstar Omega Plate Reader LDH substrate was added to one well at a time and absorbance of NADH read at 340 nm for approximately 4 min (245 seconds). Figure 4-8 shows the maximal NADH oxidation for cell densities ranging from 0.15 – 1.2 million A549 cells. All investigated cell densities produced linear reactions with the exception of 1.2 million cells which saturated the reaction. From these data, 0.9 million cells was the chosen cell density in order to achieve the largest linear slope without saturation. These data also show that 2 min (120 seconds) is sufficient time to measure the slope of NADH oxidation.

For the development of a positive control, A549 cells were plated at 9×10^5 in 6-well plates, serum starved for 24 h, and then incubated with 25–400 mM H_2O_2 for 6 h.

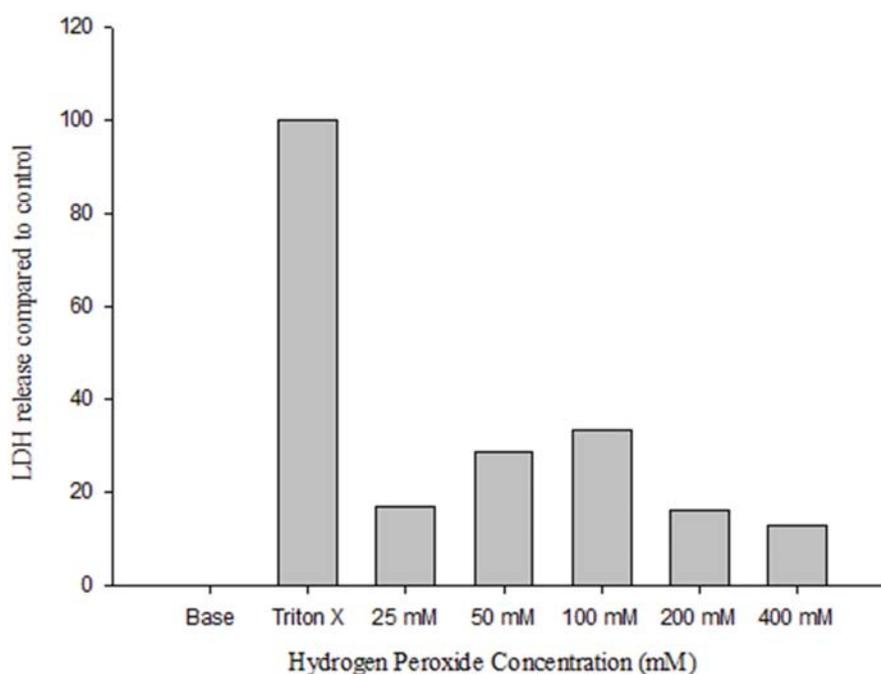


Figure 4-9: Development of positive control of LDH assay

A549 cells were seeded at 9×10^5 in 6-well plates, serum starved for 24 h and incubated with a range of H_2O_2 concentrations or media only (baseline) for 6 h. Supernatants were collected and assayed for lactate dehydrogenase (LDH). Results are the mean Δ absorbance reading (245 sec) of three replicates from one experiment expressed relative to baseline (0% cell death) and 1% Triton X-100 (100% cell death).

Supernatant was collected, centrifuged at 19,800 rpm for 15 mins. Samples were pipetted into a 96 well plate and absorbance of NADH read at 340 nm for 2 min. Hydrogen peroxide concentrations from 25 mM to 100 mM created a dose dependent increase in NADH oxidation up to 100 mM (Figure 4-9). LDH release was 17%, 29%, and 33% of control for 25 mM, 50 mM, and 100 mM respectively. The LDH production for 200 mM and 400 mM showed lower LDH release than 100 mM which was possibly due to these concentrations completely killing the cell cultures and the LDH released degrading during the 6 h incubation; though, the exact reason for the lower presence of LDH is unknown, it was clear the higher concentrations of hydrogen peroxide would not be suitable for use as a positive control. From these data, 100 mM was determined to be a sufficient positive control for the LDH assay and was included with each experiment investigating procyanidin cytotoxicity.

4.4.6 Flow cytometry

Flow cytometry is laser-based technology used to analyse cellular characteristics of individual cells. It utilizes fluorescently tagged antibodies in conjunction with different colour lasers to allow for measuring target antigen in a heterogeneous mixture. In this project, cytokine and chemokine receptors on the surface of epithelial cells were



Figure 4-10: BD Fluorescent Activated Cell Sorter (FACSVerse™) system

measured using a Becton Dickinson (BD) Fluorescent Activated Cell Sorting (FACSVerse™) system with data analysis using FlowJo software (Figure 4-10). Fluorescently labelled antibodies are incubated with cells to allow for labelling of target antigen on the cells. The machine used had 3 lasers (blue 488 nm, red 640 nm, and violet 405 nm) allowing for an 8 color configuration. The FACSVerse™ system has two main systems: fluidics and optics.

4.4.6.1 FACSVerse™ Fluidics

Proper flow cytometric analysis depends on single cells passing in front of a laser. Single file lines of cells is achieved through hydrodynamic focusing of the cell samples. It is difficult to create tunnels with a small enough diameter to manage single

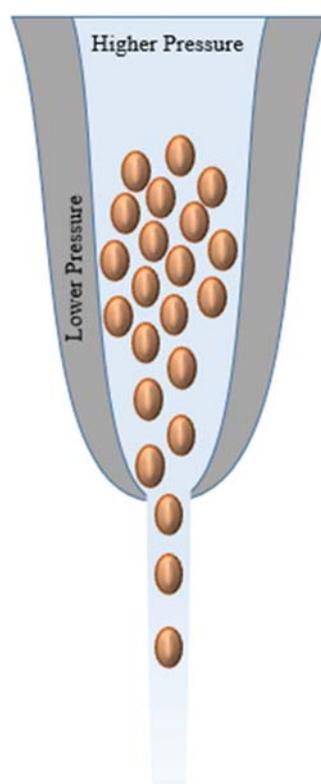


Figure 4-11: Hydrodynamic focusing of cell suspensions

The fluidics system within the FACSVerse™ uses hydrodynamic focusing of cell samples in order to create a single file line of cells. This is achieved through sheath fluid [grey] having lower pressure than the higher pressured sample fluid [blue].

file cells, thus cell samples are injected into a stream of sheath fluid. The sample pressure is higher than the sheath fluid surrounding it. The differential pressure ensures fluid streams do not mix (Figure 4-11).

4.4.6.2 FACSVerse™ Optics

The cells that pass through the flow chamber cross in front of one or more laser beams with specific wavelengths (Figure 4-12). The light that strikes the cell in the lasers forward path is collected as forward scatter (FSC) and is an indication of cell size. The light that strikes the cell and is collected at 90° from the laser beam is side scatter

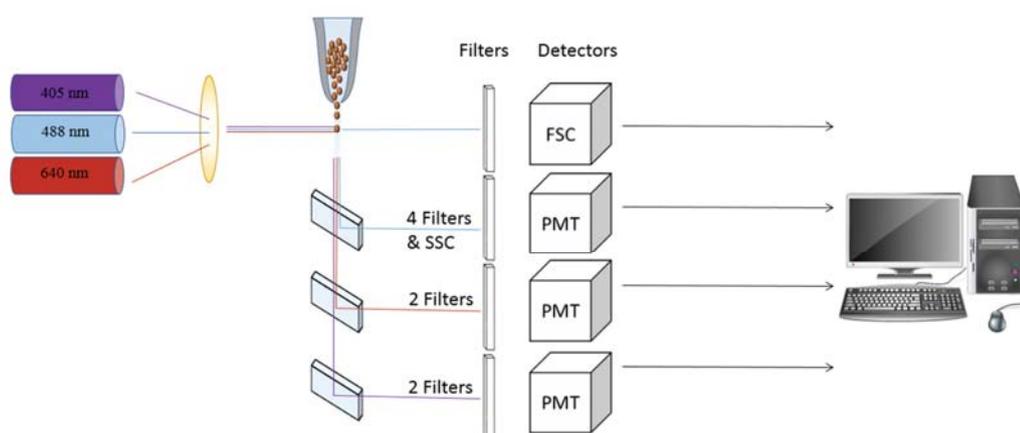


Figure 4-12: Schematic of FACSVerse™ flow cytometer optics system

Light from the three lasers (blue, red, violet) hit single cells in the flow chamber. Forward scatter (FSC) is collected parallel to the laser beams. Side scatter (SCC) is collected perpendicular to the laser beam. The filter wavelengths are configured to all for 4 parameters to be detected by the blue laser, 2 parameters to be detected by the red laser, and 2 parameters to be detected by the violet laser. Light is collected by photomultiplier tubes (PMT).

(SSC) and is an indicator of the granularity of a cell. The FACSVerse™ is equipped with 3 lasers and a specific set of filters which determine which fluorophores can be used for labelling target cellular antigens. The blue (488 nm) laser can excite the Alexa Fluor®488, PE, and PE-Cy™7 fluorophores among others. The red (640 nm) laser can

excite the APC, and APC-Cy™7 fluorophores among others. The violet (405 nm) laser can excite the Brilliant Violet 421 fluorophore among others (Figure 4-13A). The specifics of deciding which fluorophores to use for labelling target antigen is termed ‘panel design’.

4.4.6.3 Panel design

The FACSVerse™ has the ability to measure 8 parameters simultaneously on a single cell if a panel of fluorophores is appropriately chosen such that each is measured by only one of the 8 different filters in the machine. These are referred to as fluorescence channels (Figure 4-13A). Choosing two fluorophores which fall in the same channel would cause the light signals to be indistinguishable from each other, referred to as ‘spill-over’. The logic for panel design is to start with the most limiting

A)

Excitation Laser	Fluorescence Channel	Designed Panel
488 nm	BD Horizon™ BB515, FITC, Alexa Fluor®488	TNFR1
	PE	CgC
	PerCP, PerCP-Cy™5.5, Pe-Cy™5	IL-4Rα1
	PE-Cy™7	-
640 nm	APC, Alexa Fluor®647	IL-13Rα1
	BD™ APC-H7, APC-Cy™7	Zombie NIR™ Live/Dead Marker
405 nm	BD Horizon™ BV421, V450, VPD450	CCR3
	BD Horizon™ BV510, V500	-

B)

	CD124: Human IL-4Rα	CD213a1: Human IL-13Rα	CD120a:Human TNF R1	CD193: Human CCR3	CD132: Human yc common chain (Negative Control receptor)
BioLegend	APC	APC		Alex Fluor 647	APC
	PE	PE		APC/ Cy7	PE
	PE/Cy7			Brilliant Violet 421	
				PE	
				PerCP/Cy5.5	
R&D	PE	PE	PE	PE	PE
	APC	APC	APC	APC	APC
	Fluorescein	Fluorescein	Alexa Fluor 488	Fluorescein	Fluorescein
	Alexa Fluor 700	Alex Fluoro 700	PerCP	PerCP	
	PerCP	PerCP		Alex Fluor 700	
BD	PE		Biotin		

Figure 4-13: Panel Design for target antigens

A) The fluorescent channels available on the FACSVerse™ and chosen antibodies which fall within each channel (shaded). B) The strategic designing of an antibody panel is based around availability of fluorophores bound to target antigen antibodies. BioLegend, R&D, and BD are three companies with available products.

factor and work toward greatest availability in terms of fluorophores if purchasing commercially labelled antibodies which I did for this project. The commercially available products for the 6 receptors of interest, IL-4R α , IL-13R α 1, TNF R1, CCR3, and the common γ chain (CgC) (See chapter 8) are listed in figure 4-13B.

For this work, the APC-CyTM7 channel was filled first to include a live-dead marker. The Zombie NIRTM dye is a fluorescent dye that will not label living cells, but can permeate cells with compromised membranes (dead cells). For antibodies, I picked an Alexa Fluor 488[®]-labelled TNF R1 and Brilliant Violet 421TM-labelled CCR3 first because these fluorophores were not available for any of the other receptors. Next, I chose PE-labelled CgC because there were fewer available options (5 options compared with 7 and 9 for IL-13R α 1 and IL-4R α respectively). Then, PerCP-labelled IL-4R α was chosen because PE is already taken, PE-CyTM7 runs the risk of having spill over into the negative control channel which is the PE-CgC, the Alexa Fluor 488TM is already taking up the channel that measures fluorescein (FITC), and Alexa Fluora 700TM can't be measured on the FACSVerseTM; thus of the commercially available option, only PerCP or APC are options so one was given to IL-4R α and the other to IL-13R α . Fluorophore brightness can come into play if your target antigen has low expression, it would be advantageous to label it with a bright fluorophore. A fluorophore brightness index is available through BioLegend[®] (i.e. Brilliant Violet 421 is a level 5 brightness; whereas Alexa Fluor[®] 488 is a level 3 brightness)[188]. For this project, brightness of fluorophores was not a priority due to use of the A549 cell-line which is not a truly heterogeneous cell population which needed to be sorted.

4.4.7 Data analysis with FlowJo, LLC software – Gating

The data generated from flow cytometry was analysed with the FlowJo LLC software. In order to determine the amount of target antigen measured in a cell population it can be necessary to define a specific subset of those cells. This can be achieved through a ‘gate’. Minimal gating was necessary for this project because experiments used a more homogenous cell-line. I did gate in order to discriminate doublet cells (cells that did not pass through the flow chamber as a single cell) and to eliminate dead cells (Figure 4-14). By starting with an ungated cell population (Figure 4-14A), doublet discrimination is achieved by plotting the height against the area for FSC (Figure 4-14B) and SSC (Figure 4-14C) and then gating along the 45° plane. Single cells should have height and area values equal to each other thus, should fall

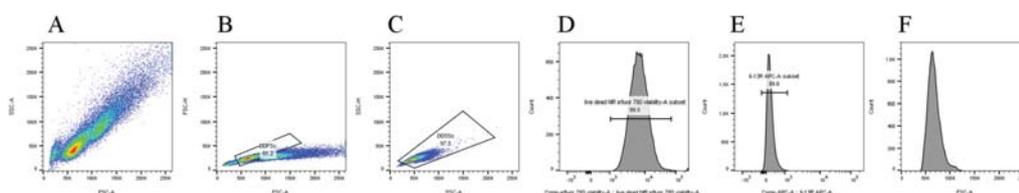


Figure 4-14: Gating used for flow cytometry data analysis

An ungated population of cells [A] is narrowed to remove the doublet cells which do not fall long the 45° plane of the FSC [B] and SSC [C]. Dead cells are removed through gating the Zombie NIR™ dye [D] and then target antigen can be gated [E]. This leaves a final population of singlet, living cells with the target antigen [F].

along the 45° angle. Gating for cells that fall along the 45° plane will remove doublet cells from the population of cells being analysed. Next, I used the fluorescence signal from the Zombie NIR™ dye to gate for live cells (Figure 4-14D). This narrowed population can then be gated for the fluorophore of interest measuring your target antigen (i.e.IL-13Rα1; Figure 4-14E). Altogether, this left the fluorescence of only singlet, living cells with IL-13Rα1 (Figure 4-14F).

4.5 Statistics

Statistical analysis of all data was performed using GraphPad Prism 5 (San Diego, CA, USA), GenStat: 14th Edition (VSN International, London, UK), or SigmaPlot 12.5 (Systat Software Inc., London, UK). Experiments that did not reach a $p < 0.05$ threshold are listed as not significant (NS). Statistical significance in figures is denoted as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Chapter Five

Inducing TSLP production in human lung epithelial cells

5.1 Inducing TSLP production in human lung epithelial cells

Contribution Declaration

This thesis chapter is not published elsewhere.

The content of this chapter was written by Sara Coleman (100%).

5.2 Abstract

BACKGROUND: The allergic asthma inflammatory cascade is initiated by secretion of TSLP, among other cytokines, from a hyperreactive airway epithelium. TSLP is responsible for dendritic cells promoting the maturation of Th2 lymphocytes. Its modulation could represent a means to assist the management of airway inflammation by reducing downstream development of innate effector cells.

OBJECTIVE: Here I ventured to develop an *in vitro* protocol for inducing TSLP production in airway epithelial cells and to utilize this model to investigate procyanidins as possible modulators of TSLP production.

METHODS: Human lung epithelial cell lines A549, BEAS-2B, and Calu-3 were investigated in an attempt to induce TSLP production *in vitro*. Cells were exposed to cytokines including IL-4, TNF α , IL-1 β or a protease (trypsin) at varied concentrations and incubation times; optimal culture conditions including cell density and culture volume were also investigated. TSLP production in the extracellular media was measured by ELISA.

RESULTS: The use of cytokines TNF α , IL-1 β , and/or IL-4 did not robustly induce the production of TSLP in A549 cell, or BEAS-2B cells. The use of trypsin in conjunction with IL-4 was ineffective to induce TSLP from BEAS-2B cells. Modifications in incubation time, cell culture density, or cell culture volume did not improve the inconsistent production patterns of TSLP from lung epithelial cell lines. The Calu-3 cell line was unrecoverable from liquid nitrogen storage, and was not viable for culturing.

CONCLUSIONS: The development of a cell culture protocol to robustly induce TSLP secretion was not achieved. Experiments to determine effects of procyanidins on TSLP were not performed. This line of experiments was abandoned for more promising work exploring procyanidin inhibition of CCL11 and CCL26.

5.3 Introduction

Allergic asthma is perpetuated predominantly by Th2 cell mechanisms. Naïve T cells are primed to differentiate into Th2 effector cells by the microenvironment found within the lymph nodes and their interactions with dendritic cells that have migrated into the lymph nodes from the peripheral tissues. TSLP is released from the epithelium after allergen exposure [189]. The presence of TSLP modulates receptor expression on lung DCs, and skews them to promote a Th2 response from naïve T cells [190]. The maturation of Th2 cells is a significant event in the allergic cascade and subsequently a promising target for reducing downstream inflammatory events [191,192]. The potential for procyanidins to significantly affect airway inflammation in a whole-body system would be supported by demonstrating modulation of multiple components in the allergic airway inflammatory cascade. I sought to develop an airway epithelial cell culture model system that would produce TSLP following a stimulatory event, and then utilize the optimized *in vitro* model to investigate dimeric procyanidins as modulators of cytokine-induced TSLP production.

5.4 Materials and methods

5.4.1 Materials

Three lung epithelial cell lines were investigated, A549, BEAS-2B, and Calu-3 for inducing TSLP production. All cell culture methodology is consistent with materials and methods listed in chapter four. The human lung epithelial cell-lines were all purchased from the American Tissue Cell Collection (c/o Cryosite Lane Cove, New South Wales, Australia). The TSLP DuoSet® ELISA kit and IL-4 were purchased from R&D systems (In Vitro Technologies, Auckland, New Zealand). Trypsin (2.5%) was purchased from Life Technologies (Auckland, New Zealand). All other chemicals not specifically listed were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.4.2 Cell culture

Cells were grown under standard tissue culture conditions of 37°C in a 95% humidified atmosphere at 5% CO₂ in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin B, and 10% FBS. The cell cultures were grown to form an 80% monolayer and then growth arrested for 24 h in the absence of FBS before conducting experiments. Experiments used A549 cells from passages 89-100. Experiments used BEAS-2B cells from passages 9-20. Cells were seeded at various densities in 6, 12, or 24-well plates as described below. Cytokines and trypsin were dissolved to the described concentrations in serum-free media and incubated with epithelial cells for various lengths of time as described below. Extracellular media were collected and frozen at -80 °C until analysis for TSLP using a commercially available sandwich ELISA kit following the manufacturer's instructions.

5.5 Results

5.5.1 Validating TSLP ELISA Kit

Initial validation of the TSLP DuoSet® ELISA kit was accomplished by performing the assay on the standards (see section 4.4.4) that are included with the ELISA kit. The kit has a detection range of 31.25 pg/mL up to 2000 pg/mL TSLP indicated by the manufacturer. The ELISA of the standards demonstrated (via appropriate colour change) that the kit and supplies were effective for measuring TSLP. Additionally, the ELISA of the standards showed the capture antibody, detection antibody, and standards had been dissolved appropriately.

5.5.2 ELISA complications

Following the initial validation of the TSLP ELISA kit, experiments began to present inconsistent results in measuring both assay standards and experimental samples. The manufacturer was contacted, and provided with data and specifics of how the ELISA kit was faulty. A new TSLP ELISA kit was provided along with an accessory pack with all necessary items to run the assay. With the use of the accessory pack supplies, it was established that the ELISA standards were satisfactory, however there were difficulties in achieving reproducible results when using supplies from our own lab. During a 4 month period, there were difficulties identifying the cause of the inconsistencies experienced by multiple scientists within the Food and Wellness group. After much trouble shooting and collaborated data analysis, it came to light that it was necessary to run the R&D Duoset® ELISAs on a specific brand of 96-well ELISA plate. Greiner products produced the most consistent ELISA reactions. Furthermore, it was demonstrated that the in-house mixed substrate for the photoluminescence reaction needed thorough mixing that was previously being overlooked. All ELISAs from this point were only run on Greiner brand plates and attention was given to thoroughly mixing the photoluminescence reaction substrate before pipetting into 96 well-plates.

5.5.3 Inducing TSLP production from lung epithelial cell lines

It was reported in the literature that A549 cells could be stimulated to produce TSLP [193]. Lee et al. provided data that demonstrated normal human bronchial epithelial cells could be induced to produce TSLP with cytokines, IL-1 β and TNF α . In the text of their publication, they described without data that a similar result pattern was obtained with both mRNA and protein in two other human airway epithelial cell lines, 16HBE14o⁻ and A549. I contacted the authors for clarification on their experience with inducing A549 to produce TSLP and no reply was given. Nevertheless, experiments

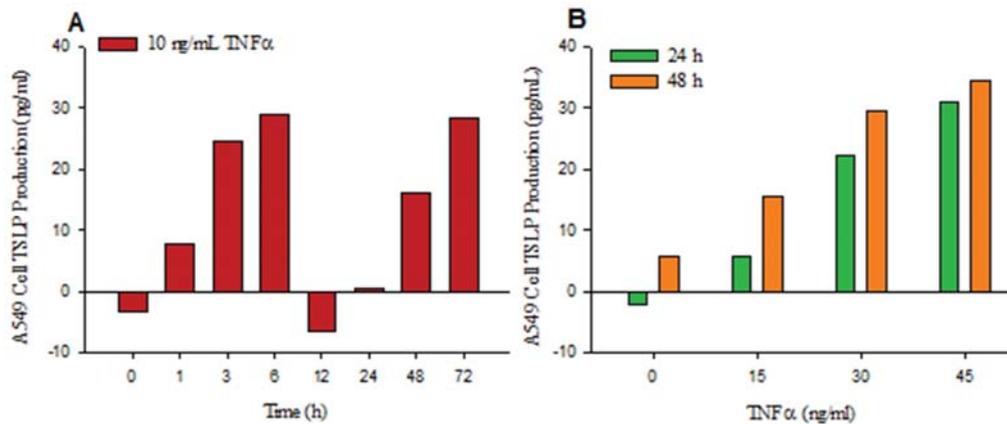


Figure 5-1: Inducing TSLP with TNF α in A549 cells

A549 cells were seeded at 3×10^5 in 12-well plates (1 mL volume), serum starved for 24 h and incubated with (A) 10 ng/ml TNF α for 1-72 h (red) or (B) 0-45 ng/mL TNF α for 24 (green) or 48 h (orange). Collected supernatants were measured for TSLP by ELISA. The results are expressed as one individual measurement from one experiment.

were designed to induce A549 cells to produce TSLP. A TNF α exposure time course was investigated in A549 cells stimulated with 10 ng/mL TNF α for 0 – 72 h (Figure 5-1A). Despite the apparent dose and time profile response, TSLP production was below detectable limits for all times investigated. The 6 h max was 29 pg/mL TSLP, which fell just below the reliable limits of the 7-point standard curve (2000, 1000, 500, 250, 125, 62.5, 31.25 pg/mL). A dose response ranging from 0-45 ng/mL TNF α incubated on A549 cells for 24 or 48 h also demonstrated TSLP production below detectable limits (45 ng/mL TNF α for 48 h max of 34 ng/mL TSLP; Figure 5-1B).

Experiments were next performed investigating the potential of IL-1 β to induce TSLP production in A549 cells. A549 cells were exposed to 0.5, 1, 2, or 5 ng/ml IL-1 β for 1 to 24 h. TSLP production reached 204 pg/ml and 248 pg/ml respectively for 0.5 ng/ml and 1 ng/ml IL-1 β when incubated for 2 h, with both 2 and 5 ng/mL showing minimal induction (Figure 5-2). These data prompted a modification in culture conditions in order to concentrate TSLP production in the culture media and give

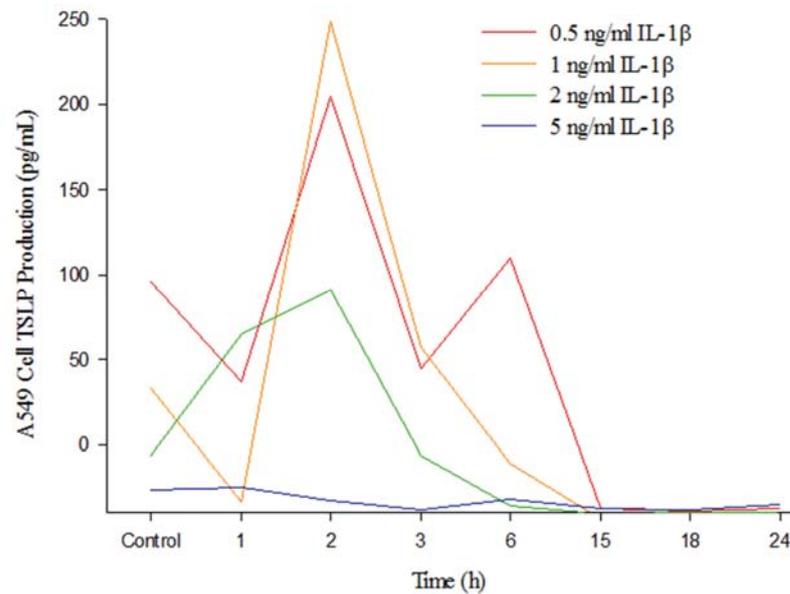


Figure 5-2: Inducing TSLP with IL-1 β in A549 cells

A549 cells were seeded at 3×10^5 in 12-well plates (1 mL volume), serum starved for 24 h and incubated with 0.5 (red), 1 (orange), 2 (green), or 5 ng/mL IL-1 β (blue) for 1- 24 h. Collected supernatants were measured for TSLP by ELISA. The results are expressed as one individual measurement in one experiment.

greater detectability. The cell count was doubled while keeping the cell culture volume consistent. Experiments investigating TSLP production from 6×10^5 A549 cells/well in a 1 ml volume exposed to IL-1 β for 1-6 h had less detectable TSLP in the extracellular media than the previous design (Figure 5-3). All conditions gave TSLP values below 10 ng/mL TSLP, well below the reliable detection limits of the ELISA kit. It was possible that the higher cell density resulted in cells that were too crowded and did not allow for IL-1 β and TNF α access to their appropriate receptors on A549 cells. To investigate this possibility, 0.3 million A549 cells were plated in 12-well plates in a cell culture volume of 0.5 ml as opposed to 1 ml in an attempt to concentrate TSLP in the extracellular media without crowding cells. BEAS-2B bronchial cells were also used in the similar set up. Both cell lines were then stimulated with 0.5 or 1 ng/ml IL-1 β , 5 ng/ml TNF α with 0.5 IL-1 β , or 5ng/ml TNF α with 0.5 ng/ml IL-1 β , and 5 ng/ml IL-4 for 1, 2, 3, or 6

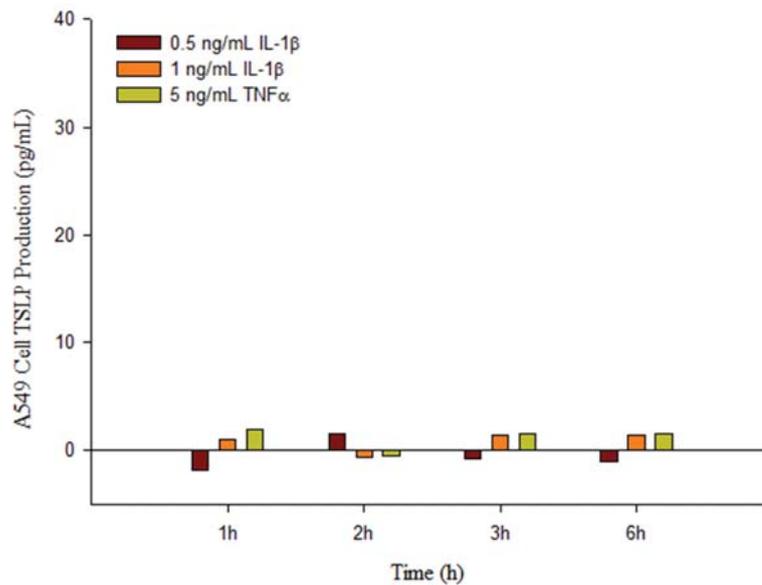


Figure 5-3: Concentrating cytokine-induced TSLP production in A549 cells

A549 cells were seeded at 6×10^5 in 12-well plates (1 mL volume), serum starved for 24 h and incubated with 0.5 ng/mL IL-1 β (red), 1 ng/mL IL-1 β (orange), or 5 ng/mL TNF α (yellow) for 1- 6 h. Collected supernatants were measured for TSLP by ELISA. The results are expressed as one individual measurement in one experiment.

h (Figure 5-4). The mixing of cytokines was an additional attempt to increase TSLP production. Figure 5-4 shows that neither A549 nor BEAS-2B cells produced TSLP for any of the cytokine mixtures or time points. Experiments shifted to investigate BEAS-2B specifically because it was reported in the literature that BEAS-2B cell could be stimulated to produce TSLP with proteases such as trypsin. Kouzaki et al. reported TSLP production from BEAS-2B cells seeded at 5×10^4 cells/well in a 24-well plate, growing 2 days to confluence, and then exposing them to 10 nM trypsin with or without 100 ng/ml IL-4 for 6 or 24 h. Their data demonstrated roughly 175 pg/ml TSLP production with trypsin and IL-4 together [194]. This experimental design was the template for performed experiments here investigating BEAS-2B cells plated at 9×10^5 cells per well (3 ml volume) in 6-well plates, 3×10^5 cells per well (500 μ L volume) in 12-well plates, and 1×10^5 cells per well (500 μ L volume) in 24-well plates. Cells were

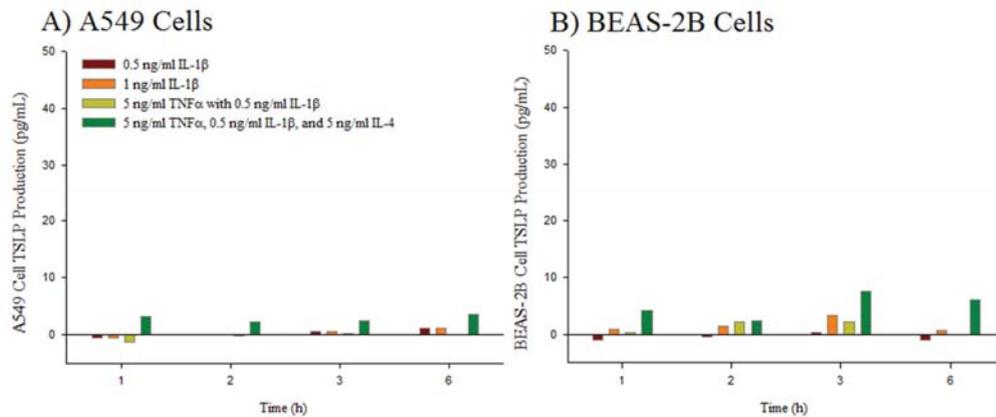


Figure 5-5: Inducing TSLP with mixed cytokines in A549 and BEAS-2B cells

A) A549 cells or B) BEAS-2B cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h and incubated with either 0.5 ng/mL IL-1 β alone (red), 1 ng/mL IL-1 β alone (orange), 0.5 ng/mL IL-1 β with 5 ng/mL TNF α (yellow), or 0.5 ng/mL IL-1 β with 5 ng/mL TNF α and 5 ng/mL IL-4 (green) for 1- 6 h. Collected supernatants were measured for TSLP by ELISA. The results are expressed as the mean of 3 individual measurements in one experiment.

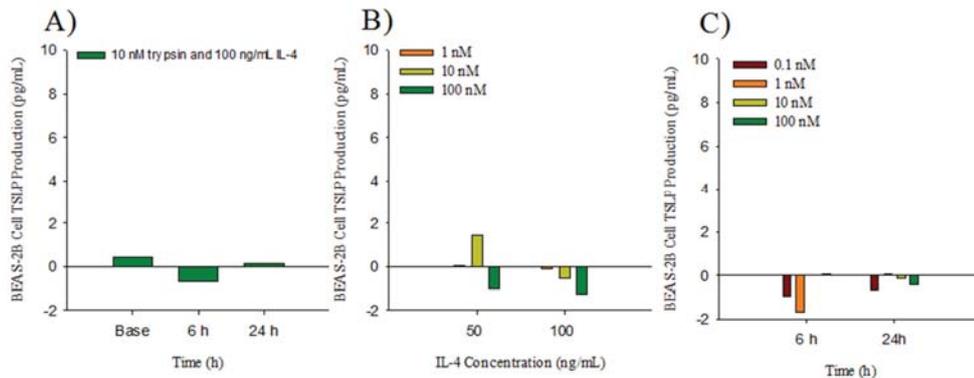


Figure 5-4: Inducing TSLP with trypsin and IL-4 in BEAS-2B cells.

BEAS-2B cells seeded at A) 9×10^5 in 6-well plates B) 3×10^5 in 12-well plates, or C) 1×10^5 in 24-well. Cells were serum starved for 24 h and then exposed to A) 10 nM trypsin with 100 ng/mL IL-4, or B) 1–10 nM trypsin with 50–100 ng/mL IL-4, or C) 0.1– 100 nM trypsin with 100 ng/mL IL-4 for either 6 or 24 h. Collected supernatants were measured for TSLP by ELISA. The results are expressed as 1 individual measurements from one experiment.

exposed to varied doses of trypsin (10 –100 nM) with either 50 or 100 ng/ml IL-4 for 6 or 24 h. None of the configurations allowed for a measureable amount of TSLP in the extracellular media (Figure 5-5). The enzymatic activity of the trypsin used here could not be compared to the trypsin used in the literature as the exact product's enzymatic activity was not described and the company from which it was purchased (Sigma) carried multiple trypsin products. To investigate the highest level of trypsin the BEAS-2B cells could tolerate before cell death, a dose response was performed and images taken to visualize cell integrity. Control A549 cells formed an intact squamous cell monolayer. Cells exposed to 97, 195, and 390 nM trypsin retained their elongated oval shape. Adjacent cells were in contact with each other and the cell monolayer was attached to the cell culture well surface. Trypsin concentrations below 781 nM did not appear to disrupt the BEAS-2B cell monolayer when incubated for 6 h (Figure 5-6). Cells incubated with trypsin at 781, 1562, and 3125 nM did disrupted the cell monolayer (Figure 5-6). Cells were lifting from the bottom of the cell culture plate and where no longer in contact with adjacent cells leading to a scaffolding effect. Cells incubated with 6250 or 12,500 nM trypsin were completely rounded with extensive cell debris. There was no longer a monolayer of any kind and all cells appeared to be dead (Figure 5-6). When BEAS-2B cells plated at 2×10^5 cells per well in 24-well plates in a 500 μ l volume were exposed to all the above trypsin concentrations along with 100 ng/ml IL-4 and assayed for TSLP by ELISA, there was no detectable TSLP in the extra cellular media (data not shown).

Inducing TSLP from A549 and BEAS-2B was proving to be a challenge. Thus, other available cell lines were investigated as a possible solution. The Food and Wellness lab had 6 vials of Calu-3 cells, and thus an attempt was made to grow this cell line as it had a reputation for being quite difficult. The first attempt used one 1 mL vial

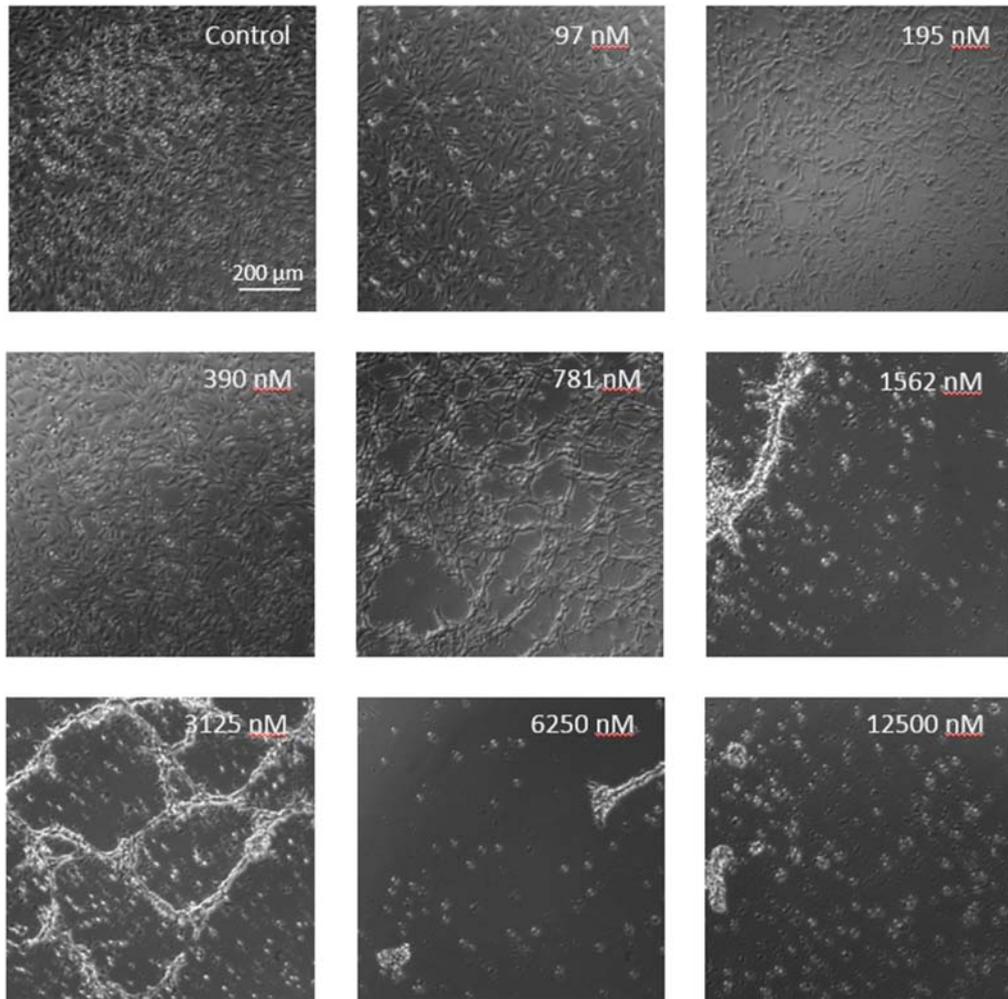


Figure 5-6: BEAS-2B cell integrity following exposure to trypsin

BEAS-2B cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h and incubated with 0 – 12,500 nM trypsin. Cells were visualized with a Zeiss Axiovert 10 inverted microscope with a Macrotive monochrome camera by Optronics. Magnification 10x. Results are from 1 well for each condition from one experiment.

pipetted into 10 mL of my standard DMEM cell culture media with serum and incubated at standard cell culture conditions for 24 h. One day after introducing cells into a cell culture flask, no cells had attached to the plastic. The second attempt, used one-1 mL vial pipetted into 10 mL of complete DMEM cell culture media with serum and incubated at standard conditions for 3 h to allow for attachment to plastic. The media was then gently replaced in order to remove the DMSO that the cells where

frozen in and incubated for an additional 18 h. Again, no cells attached. In a last ditch effort, the remaining 4- 1 mL vials were pipetted into 10 mL complete DMEM, incubated at standard cell culture conditions for 3 h, and then replaced with fresh media. A very small amount of cells attached to plastic. I left cells to recover with no disturbances for 3 days and no growth or cell division was seen from attached cells. The Calu-3 cell-line was concluded to be an unviable option for further experimentation.

5.6 Discussion

Allergic asthma and other atopic diseases such as atopic dermatitis, and allergic rhinitis have a genetic predisposition to develop hyperreactive immune responses to benign environmental components. It was demonstrated in animal models that TSLP acts as an initiating cytokine at the top of the atopic inflammatory cascade [189]. Moreover, the overexpression of the TSLP gene specifically in airway epithelial cells led to asthma in mice and TSLP expression in the airways of human asthmatics was demonstrated to be significantly higher than healthy controls [195,196]. TSLP was thus investigated in this project as a biomarker of interest for which dimeric procyanidin bioactivity could be studied *in vitro*. A cell culture model was needed in which TSLP production could be induced. Multiple accounts were found in the literature for inducible TSLP production from airway epithelial cells; the majority utilized primary bronchial epithelial cells as opposed to cell-lines [193,194,197]. The singular report found in the literature that included work on A549 cells mentioned them without data, and there were no ELISA data provided from their normal human bronchial cells from which to gauge an estimated protein secretion range. Experiments were performed attempting to stimulate the production of TSLP from A549 cells, however, none of the conditions had detectable levels of TSLP production (Figures 5-1, 5-2, 5- 3, 5-4). After a thorough literature search, TSLP secretion in the extra cellular media was found to

range between 50 and 200 pg/ml depending on cell type and conditions [189,194,197]. The highest TSLP production reported in the literature were from BEAS-2B cells stimulated with the protease trypsin in addition to 100 ng/ml IL-4 [194]. The experiments here attempted to replicate the published design in terms of cell density, incubation time, and stimulatory events, and also investigated further cytokine combinations and cell seeding variations but were unsuccessful in producing measurable TSLP from the extracellular media (Figure 5-4 and 5-5)

The commercially purchased ELISA kit used in this work had a suggested standard curve serial dilution range of 31.25 pg/ml up to 2000 pg/ml TSLP. This range would not allow for the anticipated TSLP production range to be accurately and reliably measured as it did not fall within the linear portions of the standard curve which would be 250–1000 pg/ml TSLP. Furthermore the work by Kato et al., listed the ELISA kit in their work had a minimum detection limit of 31.25 pg/ml and presented data with TSLP production between 50-75 pg/ml [197]. Assaying a biological sample by ELISA requires the use of a standard curve of known concentrations in which the unknown concentration is then calculated. This calculation can only be deemed accurate if the unknown absorbance value falls within the linear portion of the standard curve and it is my concern that much of the work published utilized standard curves in their ELISAs that would not allow for accurate measurement of TSLP production.

The primary goal of developing a cell culture model to produce TSLP was to utilize the model to investigate possible modulation by dimeric procyanidins. Without robust induction of the TSLP it would be quite challenging to see a measurable effect by procyanidins in the event there were one to measure. It was concluded that the options for developing a successful TSLP model system, which would require purchasing a

primary cell line, purchasing another ELISA kit with greater sensitivity, or measuring TSLP with qPCR, would still not allow for the effective evaluation of procyanidins as a means of modulating TSLP as the induced levels of TSLP were not robust enough for measurable difference with procyanidins. Thus, the possibilities in this area were regarded as moot and the project moved to investigate other biomarkers of airway inflammation.

Chapter Six

Modulation of IL-4-induced CCL11 production by dimeric procyanidins

6.1 Modulation of IL-4-induced CCL11 production by dimeric procyanidins

Contribution Declaration

This thesis chapter was published in whole as a journal article and is reprinted with permission from IOS Press:

Coleman, S.L.; Hurst, R.D.; Sawyer, G.M.; Kruger, M.C.; The in vitro evaluation of isolated procyanidins as modulators of cytokine-induced eotaxin production in human alveolar epithelial cells. *J. Berry Res.* 2016; 5: 115-124; DOI: 10.3233/JBR-160121

The original manuscript of this chapter was written by Sara Coleman (90%), with content and grammatical editing from Roger Hurst, Greg Sawyer, and Marlena Kruger (10%). The revised manuscript was then internally reviewed by Dr Birgit Ha (Food and Wellness, PFR) and Dr Tony McGhie (Phytochemistry, PFR) as is standard practice at PFR. The internally reviewed manuscript was entered into SPTS at PFR, which includes professional format and grammar editing. The final manuscript was published as cited above.

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6.2 Abstract

BACKGROUND: Populations that consume procyanidin-rich diets are less susceptible to inflammatory disease. Allergic asthma is an inflammatory lung disease perpetuated by a hyperreactive airway epithelium and eosinophil infiltration into the lung. Eotaxin-1 (CCL11) mediates eosinophil migration into tissues and its modulation could represent a means to assist the management of airway inflammation.

OBJECTIVE: Here I evaluated procyanidins as a means of modulating CCL11 production *in vitro*.

METHODS: I used human lung epithelial cells (A549) and optimized the conditions to induce CCL11 production *in vitro*. Cells were exposed to procyanidins for 6 h prior to an inflammatory insult of 5 ng/mL IL-4 with 5 ng/mL TNF α for 48 h. An enzyme-linked immunosorbent assay was used to measure CCL11 production.

RESULTS: Cells exposed to 5 μ M procyanidin A2 prior to the inflammatory challenge showed significantly inhibited (36%) CCL11 production. Under the same conditions, procyanidins B1 and B2 elicited no effect. Furthermore, combinations of procyanidins A2 and B2 (5 μ M total) demonstrated no evidence of a synergistic interaction.

CONCLUSIONS: These data demonstrate that the regulation of CCL11 by lung epithelial cells is not ubiquitous among the three investigated procyanidins. I demonstrate a differential inhibition of CCL11 by A-type and B-type procyanidins. This evidence supports further studies into procyanidins, specifically A-type, for managing inappropriate airway inflammation.

6.3 Introduction

Epidemiological evidence has revealed populations that consume procyanidin-rich diets are less susceptible to inflammatory disease, such as asthma [163,198]. Asthma is an inflammatory lung disease that involves inappropriate airway inflammation, bronchiole constriction, and lung tissue remodelling that collectively restrict airflow [8]. In allergic asthma these pathophysiological features are perpetuated by Th2 cell driven mechanisms and the infiltration of immune cells into lung tissue [199]. During homeostasis, eosinophil migration into the lung tissue would be tightly controlled; however, the hyperreactive airway epithelium of an asthmatic produces the chemokine eotaxin (CCL11 and its associated isoforms CCL24 and CCL26) in excess, which along with other cytokines instigates the allergic cascade [200].

Procyanidins are polyphenolic secondary plant metabolites that are thought to be biologically active in the context of human health. They are the product of polymerization of monomeric flavan-3-ol units by a currently unknown mechanism within plants [2]. The monomeric units can be bonded together by either A-type or B-type linkages. The differences in structure that result due to linkage are suspected to play a role in the biological activity of procyanidins. In the western diet, fruit are the predominate source of dimeric procyanidins, compounds that consist of two flavan-3-ol units [82]. Cranberries and lingonberries are high in A-type bonded procyanidins such as structurally-defined, procyanidin A2, whereas, apples are a high source of B-type bonded procyanidins such as structurally-defined procyanidin B1 and procyanidin B2 [29]. Polyphenolic metabolites are thought to accumulate in plants as a consequence of stress or pathogenic infiltration [201]. Modern agriculture limits these stresses; Joven et al. suggested that in a western diet it would be difficult to consume enough polyphenols from fruit and vegetables to influence health as a result of reduced polyphenolic profiles

due to modern agricultural practices [14,202]. Furthermore, the mean consumption of fruit and vegetable servings per person in the US is half of the recommended portions at 1.5-2 cups and 2-3 cups, respectively, per day [203]. Thus, concentrated extracts or isolated compounds could be incorporated into foods to supplement the typical western diet for improved health and wellbeing.

In this study, I investigated commercially sourced, structurally-defined, pure dimeric procyanidin compounds for *in vitro* efficacy of modulating one key biomarker, CCL11, relevant in allergic asthma. Understanding the role of procyanidins in managing inflammation could allow for their use in fresh or processed foods as a natural means to prevent and manage inflammatory illness, limit the use of pharmaceutical interventions, and assist with improving human health.

6.4 Materials and methods

All materials are previously listed in section 4.2.

6.4.1 Isolated procyanidins

Procyanidins were dissolved in DMSO and stored at -80°C until experimental use. Procyanidins were then dissolved in serum-free media to desired concentration and added immediately to cell cultures. In synergy experiments concentrations of each procyanidin were prepared separately and added together in the cell culture well. The final DMSO concentration was 0.03% and 0.06% (v/v) DMSO respectively for cultures exposed to 5 µM and 10 µM procyanidins and were used in the negative controls.

6.4.2 Cell culture conditions

Cells were grown under standard tissue culture conditions of 37°C in a 95% humidified atmosphere at 5% CO₂ in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 2 mM L-glutamine, 50 µg/mL penicillin, 50

$\mu\text{g/mL}$ streptomycin, $100 \mu\text{g/mL}$ neomycin, and 10% FBS. The cell cultures were grown to form a monolayer (80% confluence) and then growth arrested for 24 h in the absence of FBS before conducting experiments. Experiments used A549 cells of passage 89-107.

6.4.3 Cytotoxicity

Cell metabolism (viability/ cytotoxicity) after 6 h procyanidin exposure was measured using water soluble tetrazolium-1 (WST-1) reagent to ensure isolated procyanidins were not cytotoxic to cell cultures [204]. During the WST-1 assay, the rate at which tetrazolium salts are cleaved to formazan by cellular mitochondrial dehydrogenases correlates with the number of viable cells in the culture. Changes in the formation of formazan in response to procyanidins is extrapolated to cellular viability/cytotoxicity. A549 cells were plated at 5×10^4 per well in 96-well plates with media changes to serum-free media 24 h before starting the experiment. Cells were incubated with control media, positive control 2.5 mM H_2O_2 , or procyanidin B1, B2, or A2 at concentrations ranging from $1 \mu\text{M}$ to $20 \mu\text{M}$ for 6 h. After a wash with PBS, WST-1 assay reagent was added to each well and cellular viability was measured according to the manufacturer's directions.

6.4.4 Inducing the production of CCL11

The production of CCL11 can be induced in A549 airway epithelial cells *in vitro* to model the inappropriate inflammation that occurs during allergic asthma. Production of CCL11 is both concentration- and time-dependent. Other work by our group characterized the concentration- and time-dependant profile of IL-4 on the production of CCL26, another isoform of eotaxin, generated by A549 cells [1]. IL-4 and $\text{TNF}\alpha$ exposure alone were insufficient to substantially induce CCL11 (data not shown). Thus,

I evaluated CCL11 production when exposed to 5 ng/mL IL-4 with a range of TNF α concentrations from 0.75 ng/mL to 40 ng/mL for 48 h as well as determined the time course of CCL11 production after exposure to 5 ng/mL IL-4 with 5 ng/mL TNF α for times ranging from 1 -72 h. Experiments used A549 cells plated at 5×10^5 per well in 12-well plates with media changes as described above. Cells were then exposed to serum-free media containing the cytokines. Extracellular media was collected and frozen at -80°C until analysed for CCL11 by ELISA. The R&D Duoset ELISA kit has a detection range of 31.25 pg/mL up to 2000 pg/mL as indicated by the manufacturer.

6.4.5 Enquiry into procyanidin efficacy

In experiments examining effects of procyanidins on inflammation, A549 cells were plated at 5×10^5 per well in 12-well plates with media changes as described above. Cells were then incubated with media containing procyanidins at concentrations ranging from 1 μ M to 10 μ M, carrier control DMSO, or positive control 5.8 μ M IFN γ for 6 h, washed with PBS, and then stimulated with 5 ng/mL IL-4 and 5 ng/mL TNF α for 48 h to induce CCL11 production. Extracellular media was collected and frozen at -80°C until analysed for CCL11 by ELISA. Synergy experiments utilised A549 cells plated and set up as described above. Combinations of procyanidin A2 and procyanidin B2 (5 μ M total) were added to cultures for 4 h prior to the inflammatory insult as described above. Extracellular media was collected and frozen at -80°C until analysed for CCL11 by ELISA.

6.4.6 Statistics

Statistical analysis of data was performed using GraphPad Prism 5 (San Diego, CA, USA) or GenStat: 14th Edition (VSN International, London, UK). Data from CCL11 induction time course and TNF α dose response experiments used GraphPad

Prism and were analysed with a 1-way ANOVA with Dunnett's multiple comparison test against baseline with statistical significance set at $P < 0.05$. All other experiments used GenStat and were analysed with a randomized block design ANOVA using a Fisher's least square difference test with statistical significance set at $P < 0.05$. Experiments that did not reach the $P < 0.05$ threshold are listed as not significant (NS). Statistical significance in figures is denoted as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

6.5 Results

6.5.1 Optimization of airway epithelial cell bioassay conditions

The dose response profile of CCL11 production is shown in Figure 6-1.

Production of CCL11 increased rapidly and reached statistical significance at 2.5 ng/mL TNF α (178 ± 23 pg/mL CCL11, $P < 0.01$), then the profile of production slowed from 5 ng/mL TNF α (315 ± 39 pg/mL CCL11), and reached a maximum with 40 ng/mL TNF α (515 ± 6 pg/mL CCL11) (Fig. 6-1A). The time course of CCL11 production ranging from 1 - 72 h is also shown in Figure 6-1. The profile reached a modest statistically insignificant plateau between 18 - 24 h (298 ± 24 pg/mL CCL11 at 18 h), increased to

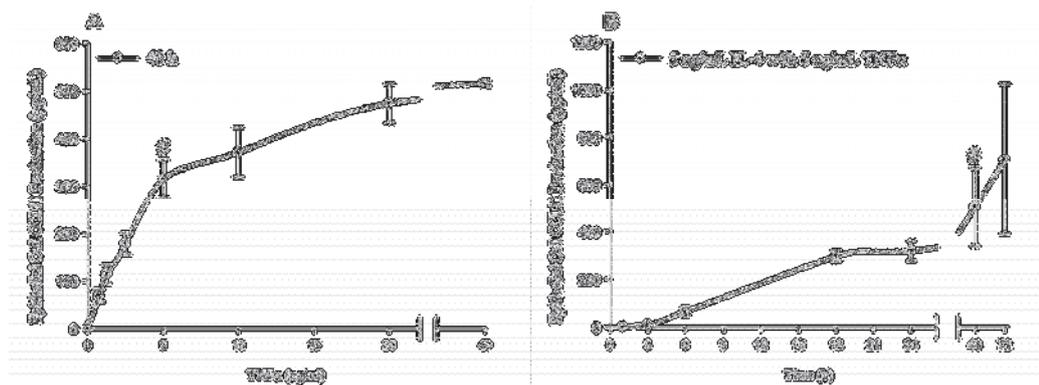


Figure 6-1: Cell culture model optimized for inducing CCL11 in A549 cells

A549 cells were incubated with A) 5 ng/mL IL-4 with 0.75 – 40 ng/mL TNF α for 48 h or B) 5 ng/mL IL-4 with 5 ng/mL TNF α for 1 – 72 h. The culture media supernatants were collected and measured for CCL11 by ELISA. Results are expressed as means \pm SEM, n=3 separate experiments. # indicates the optimal time or dose for inducing CCL11 production.

become statistically significant at 48 h (505 ± 166 pg/mL CCL11, $P < 0.05$), and continued to 706 ± 316 pg/mL CCL11 at the last time point of 72 h (Fig. 6-1B). From these data I determined that 5 ng/mL IL-4 with 5 ng/mL TNF α for 48 h provided the optimal conditions to induce CCL11 production and additionally allow for the evaluation of effects of procyanidins.

6.5.2 Cytotoxicity of procyanidins

Our primary focus in this study was to investigate modulation of CCL11 by procyanidins. To ensure changes observed after exposure were due to procyanidins and

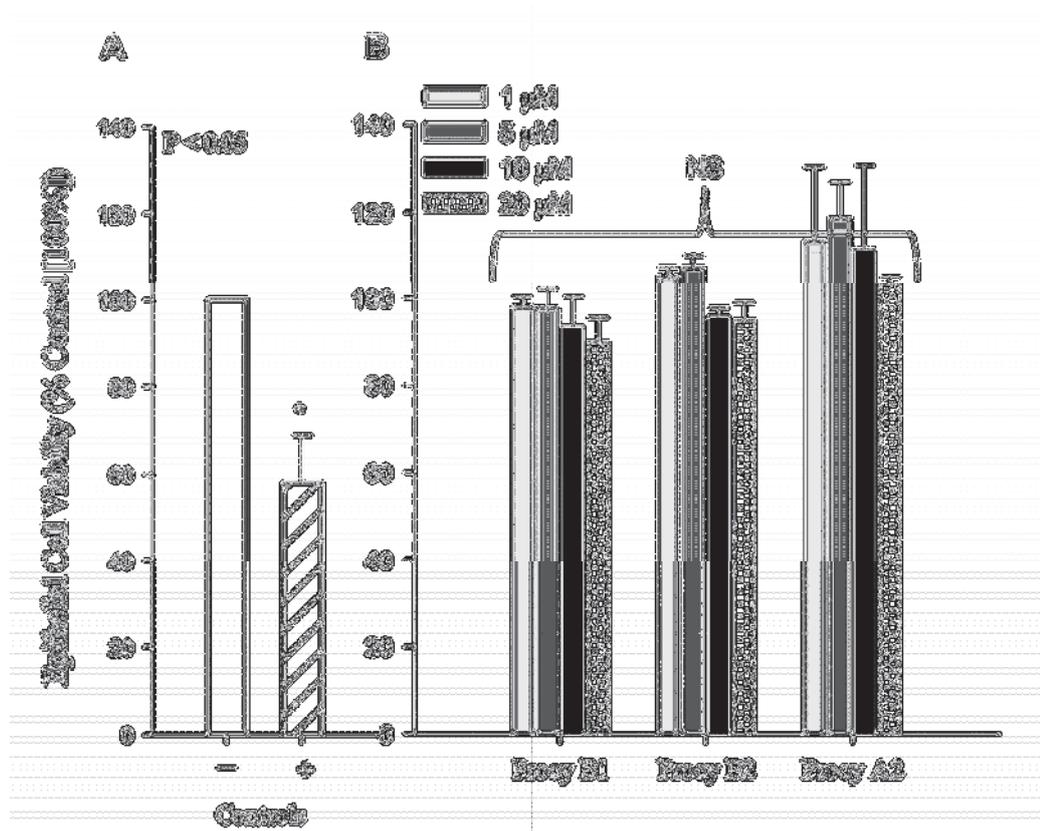


Figure 6-2: Effect of procyanidins on viability using the WST-1 assay

A549 cells were incubated with A) negative (-) control media, or positive (+) control 2.5 mM H₂O₂ or B) procyanidin (Procy) B1, B2, or A2 for 6 h at 1 μ M (light grey), 5 μ M (grey), 10 μ M (black), and 20 μ M (speckled), washed with PBS and then assayed with WST-1 reagent for cellular viability. Results are expressed as means \pm SEM as a percentage of the negative control [100%], n=3 separate experiments. NS = not statistically significant. * = $P < 0.05$

not an artefact of cytotoxicity, I utilized the WST-1 assay to investigate changes in cell metabolism (viability) after exposure to a range of procyanidin concentrations. A positive control of 2.5 mM H₂O₂ was established (Figure 6-2A) through preliminary experiments (section 4.4.5) that investigated a range (1.25–100 mM) of H₂O₂ concentrations and demonstrated a dose-dependent reduction in viability. Exposure to procyanidins B1, B2, and A2 at 1, 5, 10, and 20 μM had no statistically significant effect (Figure 6-2B) on cell viability compared to control media (i.e. not cytotoxic). Thus, any changes in CCL11 production following procyanidin exposure at these doses is attributed to an effect of the procyanidins and not as a result of toxicity.

6.5.3 Evaluation of the effects of Procyanidins

Experiments evaluating the effects of exposure to procyanidins prior to the inflammatory insult revealed distinct differences in efficacy between the three investigated compounds. Experiments included the use of a positive control IFN γ (5.8 μM), a known inhibitor of eotaxin, that demonstrated a measurable inhibition of CCL11 production (Figure 6-3, P < 0.0001). The procyanidin range utilised in our experiments was selected based on previous work in the literature, the physiological relevance, and demonstrated efficacy on other biomarkers evaluated in other unpublished research by us [40]. I observed that 1, 5 and 10 μM procyanidin A2 had a statistically significant (P < 0.01) inhibitory effect on CCL11 production (17.0 ± 3.3 , 36 ± 9.0 , and $41.9 \pm 9.0\%$ inhibition respectively) in a dose-dependent manner when incubated (6 h) prior to the inflammatory challenge (Figure 6-3). Conversely, procyanidin B1 and B2 had no inhibitory effect on CCL11 production at 1, 5, and 10 μM (1.4 ± 2.8 , 9.2 ± 2.5 , and $5.6 \pm 2.2\%$ inhibition for procyanidin B1 and 6.0 ± 1.3 , 4.5 ± 5.3 , $9.5 \pm 2.4\%$ inhibition for procyanidin B2 respectively) (Figure 6-3).

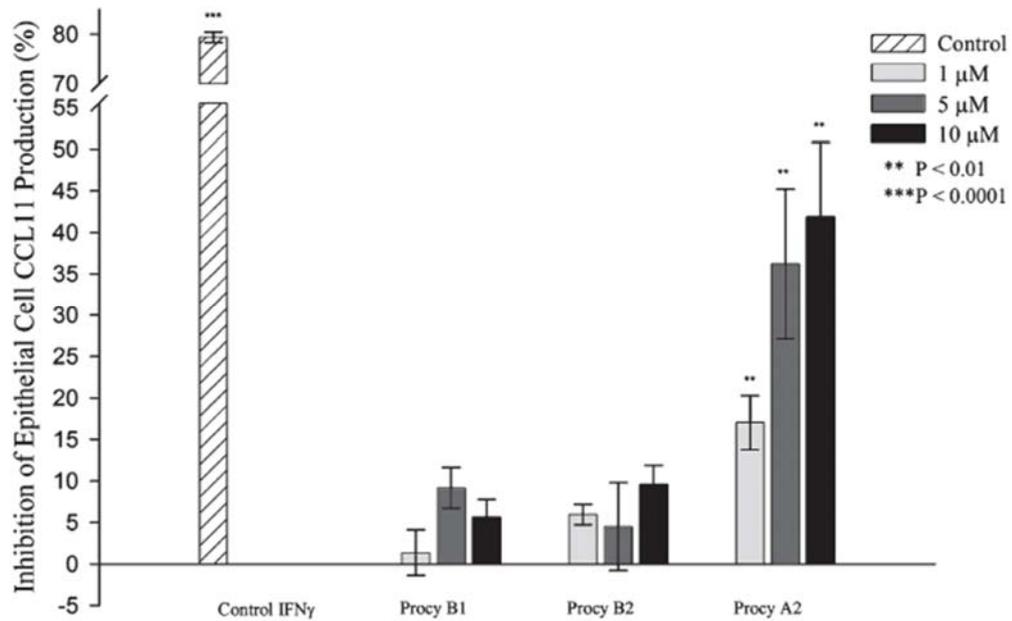


Figure 6-3: Inhibition of CCL11 production in A549 cells by procyanidins

A549 cells were exposed to positive control 5.8 μM IFN γ , or procyanidins (Procy) B1, B2, or A2 for 6 h prior to an inflammatory insult of 5 ng/mL IL-4 with 5 ng/mL TNF α for 48 h. The culture media supernatants were collected and analysed for CCL11 by ELISA. Results are expressed as mean percent change from DMSO control \pm SEM, n=3-5 separate experiments. ** = P < 0.01, *** = P < 0.001

Furthermore, I investigated procyanidins A2 and B2 for possible synergistic relationships. I evaluated the CCL11 inhibitory capacity for a range of concentrations of procyanidins A2 and B2 that together would total 5 μM (e.g. 4 μM A2 with 1 μM B2). I decided to evaluate procyanidin combinations at a total concentration of 5 μM because at this dose an inhibitory capacity is likely to be detected (A2 alone inhibition detected, Figure 6-3) and this total dose is physiologically relevant [40]. I chose procyanidin B2 as a representative of B-type procyanidins as I had observed it as a moderate inhibitor of another eotaxin isoform (CCL26, see chapter 7). Concentrations of each procyanidin were mixed separately and added together in the cell culture well. For all combinations of procyanidin A2 and B2 that totalled 5 μM , I observed no statistically significant additional inhibition versus procyanidin A2 alone in the utilized model (Table 6-1).

Table 6-1: Investigating synergies between procyanidin A2 and procyanidin B2

Procy A2 (μ M)	5	4	3	2.5	2	1	0
Procy B2 (μ M)	0	1	2	2.5	3	4	5
CCL 11 production (pg/mL)	446.30 \pm 17.30	459.53 \pm 24.15 ^{NS}	465.28 \pm 21.56 ^{NS}	474.76 \pm 10.97 ^{NS}	514.04 \pm 16.32 ^{NS}	514.64 \pm 25.31 ^{NS}	480.54 \pm 37.24 ^{NS}

A549 cells were exposed to combinations of procyanidin (procy) A2 and B2 at a range of doses with each combination totalling 5 μ M for 4 h prior to an inflammatory insult of 5 ng/mL IL-4 with 5 ng/mL TNF α for 48 h. The culture media supernatants were collected and analysed for CCL11 by ELISA. Results are expressed as epithelial cell CCL11 production (pg/mL) \pm SEM, $n = 3$ separate experiments. NS= not statistically significant vs 5 μ M A2. Control 5 μ M DMSO = 549.03 \pm 48.66 pg/mL CCL11 production which gave 5 μ M Procy A2 alone a 17.66 \pm 6.5 % inhibition from DMSO.

6.6 Discussion

Evidence supporting the use of plant polyphenolics to improve human health is strong. Scientists are now working toward defining the bioactive molecules that are eliciting effects and through which mechanisms they modulate change [205-207]. There is substantial evidence that procyanidins are capable of modulating key inflammatory pathways in various tissues which has been reviewed elsewhere [82]. Briefly, procyanidins have been demonstrated to modulate signalling pathways that include signal transducers and activators of transcription (STAT), nuclear factor- κ B (NF- κ B), as well as transcription factors in the mitogen-activated protein kinases (MAPK) pathway.

Here, I established an epithelial cell culture model of airway inflammation, and evaluated three different commercially sourced, structurally-defined, pure dimeric procyanidin compounds for their ability to modulate cytokine-induced CCL11 production. This study provided evidence that procyanidin A2, but not procyanidins of the B-type, inhibits cytokine-induced production of CCL11, a chemokine that facilitates the early-stage infiltration of eosinophils into lung tissue [208]. I further demonstrated that effects were not due to cytotoxicity and my model demonstrated no synergistic interactions between procyanidins A2 and B2 in terms of inhibition of CCL11 production. My data contribute evidence in support of the continued investigation of A-type procyanidins, and more work in this area with actual A-type procyanidin rich foods

(e.g. cranberries) would be a possible next step and could provide further evidence to support possible dietary intervention studies for assisting with the prevention/management of airway inflammation.

Previous work from the Food and Wellness group proposed that proanthocyanidins may be the bioactive components responsible for blackcurrant extract-mediated modulation of the production of CCL26 (an isoform of eotaxin) *in vitro*, using a similar lung epithelial cell culture model of airway inflammation [1]. Furthermore, the Food and Wellness group identified that the ratio of specific anthocyanins, another type of polyphenolic compound, in blackcurrant cultivars was an important determinate for influencing the suppression of CCL26 in these cells [164]. Blackcurrants and most other fruits contain procyanidins of the B-type linkage; the present study however, revealed no evidence of modulation of CCL11 by procyanidin B1 or B2. This would suggest that B-type procyanidins are not effective alone but may work in tandem with other bioactives when in a complex polyphenolic mixture such as fruit extracts to elicit a change in airway inflammation. However, our knowledge is limited on the exact role, if any, B-type procyanidins play in assisting the inhibition of airway inflammation.

My evidence is promising, but should not be extrapolated at this time as further investigation is needed to determine if A-type procyanidins would have an effect on airway inflammation *in vivo*. The model system utilized in this work was designed to emulate distribution of procyanidins to the airway epithelium through systemic circulation; another interesting approach would be to investigate A-type procyanidins applied as aerosols, mimicking inhalation. This could be modelled experimentally by procyanidin exposure directly to the apical surface of an air-liquid interface epithelial

cell culture. The cell culture model used here in this study attempted to mimic Th2 cytokine mediated responses, which are relevant to the allergic asthma endo-type [8]. Allergic asthma is now understood to be a disease that can be caused by multiple biological mechanisms, which present in the clinic as differences in immunological features, histology, morphological characteristic of the tissue as well as differences in responses to treatment [8]. The prominent Th2 cytokines in allergic asthma responses are IL-4 and IL-13, both of which play roles in asthma pathogenesis [199]. IL-4 signalling can utilize two interleukin receptors, the type I IL-4R which can only bind IL-4 and the type II IL-4R, which can also bind IL-13. In the present work I used IL-4 to induce epithelial cell CCL11 production as it has been suggested that the type I IL-4R is necessary for eosinophilia [209]. It could be useful in future work to investigate the modulation of other biomarkers due to an inflammatory insult by IL-13, as IL-13 binding to its own unique receptor chain, IL-13R α 2, may have a distinct role in airway inflammation [210]. Our model did not include the potentially large impact of the gut microbial community. There is currently considerable controversy on the bioavailability of dimeric procyanidins. Some evidence suggests that dimeric proanthocyanidin compounds found in foods, referred to as the parent compounds, would be able to enter systemic circulation and reach peripheral tissues in the original form [15], whereas other research suggests it is unlikely procyanidins of any size could reach tissues, and that any polyphenolic compounds available at tissues would be metabolites of the gut microbial community and/or subject to phase two conjugation [14,211]. Epithelial cells of both the gastrointestinal tract and the lung are capable of drug metabolism and in our *in vitro* model it is unknown if the bioactivity measured is due to an epithelial cell metabolite or the parent compound, procyanidin A2. Our studies warrant further investigation of the effects of procyanidins in an *in vitro* model system of inflammation to decipher

mechanism of action. Furthermore, investigation is needed in a whole body system of either animal or human to determine if the use of A-type procyanidins could support the management of inappropriate airway inflammation found in allergic asthma.

In our study, only procyanidin A2 was able to inhibit CCL11 production in the cell culture system, procyanidins B1 and B2 were not able to modulate CCL11 production significantly. Other work by our group has suggested that B-type procyanidins, in a complex mixture from fruit extracts, may inhibit other isoforms of eotaxin and thus I investigated the possibility of a synergistic relationship between A-type and B-type procyanidins for CCL11 inhibition. However, there were no significant differences observed in my experimental model when inhibition of CCL11 production by a combination of A- and B-type procyanidins was compared with inhibition of CCL11 production by procyanidin A2 alone. In plants, procyanidin compounds are suggested to play a role in mitigating stresses, such as drought, excess UV exposure, and/or pathogen invasion, and yet there isn't a fruit identified with high concentrations of both A-type and B-type procyanidins [14]. Thus it could be speculated that these two different types of procyanidins may result in the same terminal protection of the plant from stress but through different pathway. It has been shown that B-type procyanidins are capable of forming structurally unique hydrogen bonds with subunits of NF- κ B and are thus capable of inhibiting transcription of genes controlled by the NF- κ B transcription factor [12]. Furthermore, Matsukura et al. demonstrated that the mechanism for CCL11 expression, when induced by TNF α and IL-4, is via the activation of the transcription factors, NF- κ B and STAT6 in BEAS-2B cells [110]; though, A549 cells have been suggested to be completely insensitive to TNF α activation of NF- κ B [212]. As A-type procyanidins do not bind to NF- κ B in the same manner as

B-type and TNF α inducing CCL11 is likely not via NF- κ B, it can be speculated that A-type procyanidin inhibition of CCL11 could be mediated through STAT6.

The greatest potential for a dietary intervention to improve asthma outcomes in people would be to modulate multiple aspects of the condition, as asthma aetiology is not only characterised by inappropriate airway inflammation and there are data to suggest a dissociation between airway hyper-responsiveness and the other pathophysiological features of asthma [213]. The *in vitro* data presented here support the hypothesis that procyanidins may be able to assist with the prevention of airway inflammation. More research is warranted to investigate the effect of procyanidins on other biomarkers of airway inflammation, such as eotaxin isoform CCL26 as well as biomarkers that are involved in the other aspects of asthma progression such as hyper-responsive bronchiole constrictions, or lung tissue remodelling. Understanding the potential capabilities of each procyanidin would allow for the focused use of single or multiple procyanidins in fresh and processed foods as a natural means to prevent inflammatory illness, limit the use of pharmaceutical intervention, and improve human health.

Chapter Seven

Modulation of IL-4–induced CCL26
production by dimeric procyanidins

7.1 Modulation of IL-4-induced CCL26 production by dimeric procyanidins

Contribution Declaration

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Coleman, S.L.; Kruger, M.C.; Sawyer, G.M.; Hurst, R.D. Procyanidin A2 Modulates IL-4-Induced CCL26 Production in Human Alveolar Epithelial Cells. *Int. J. Mol. Sci.* 2016, 17, 1888; doi:10.3390/ijms17111888

The original manuscript was written by Sara Coleman (80%), with content and grammatical editing from Roger Hurst, Greg Sawyer, and Marlena Kruger (20%). The revised manuscript was then internally reviewed by Dr. Harry Martin (Biological Chemistry & Bioactives, PFR) and Dr. Jeff Greenwood (Food and Wellness, PFR) as is standard practice at PFR. The internally reviewed manuscript was entered into SPTS at PFR, which includes professional formatting and grammar editing. The final manuscript was published as cited above.

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Data from experiments investigating repeated incubations with procyanidin A2 are unpublished. The results pertaining to these data were written by Sara Coleman (100%).

7.2 Abstract

BACKGROUND: Allergic asthma is an inflammatory lung disease that is partly sustained by the chemokine eotaxin-3 (CCL26), which extends eosinophil migration into tissues long after allergen exposure. Modulation of CCL26 could represent a means to mitigate airway inflammation.

OBJECTIVE: Here I evaluated procyanidin A2 as a means of modulating CCL26 production and investigated interactions with the known inflammation modulator, interferon γ (IFN γ).

METHODS: I used the human lung epithelial cell line A549 and optimized the conditions for inducing CCL26. Cells were exposed to a range of procyanidin A2 or IFN γ concentrations for varied lengths of time prior to an inflammatory insult of interleukin-4 (IL-4) for 24 h. An enzyme-linked immunosorbent assay was used to measure CCL26 production.

RESULTS: Exposing cells to 5 μ M procyanidin A2 (prior to IL-4) reduced CCL26 production by 35% compared with control. Greatest inhibition by procyanidin A2 was seen with a 2 h exposure prior to IL-4, whereas IFN γ demonstrated greatest inhibition at 24 h. Neither concomitant incubation of procyanidin A2 and IFN γ nor repeated incubations with procyanidin A2 alone extended the inhibition efficacy.

CONCLUSIONS: These data provide evidence that procyanidin A2 can modulate IL-4-induced CCL26 production by A549 lung epithelial cells and that it does so in a manner that is different from IFN γ .

7.3 Introduction

Procyanidins are polyphenolic secondary plant metabolites that are recognized as biologically active in the context of human health. They are the product of polymerization of monomeric flavan-3-ol units by a currently undefined mechanism in plants [2]. The monomeric units can be linked together in various patterns, which can then dictate their ability to influence specific biological pathways. In plants, procyanidin compounds are suggested to play a role in mitigating stresses, such as drought, excess UV exposure, and/or pathogen invasion [14]. Furthermore, there is accumulating evidence that plant polyphenols may have a role in managing inflammatory stress in the human body [55,82]. Unfortunately, the average consumption of fruit and vegetables per person in the US is half of the USDA recommended daily intake of 1.5–2 cups and 2–3 cups, respectively [203]. Thus, concentrated extracts or isolated bioactive plant compounds could supplement the typical western diet for improved health and wellbeing.

Inflammatory diseases, such as allergic asthma, are less prevalent in populations that consume procyanidin-rich diets [163,198]. The lung epithelium is responsible for regulating pulmonary inflammation and secretes a range of cytokines, which coordinate physiological responses that restrict airflow. Eosinophil migration into the lung tissue is a defining feature of the inflammation that perpetuates allergic asthma [8]. The eosinophil chemokine eotaxin-3 (CCL26) is responsible for sustaining eosinophil migration in the lung tissue for at least 24 h after allergen exposure [111,214].

Procyanidin A2 is one structurally defined A-type procyanidin (Figure 7-1) that is predominately found in cranberries and lingonberries. In this study, I investigated a commercially sourced, procyanidin A2 for *in vitro* efficacy in modulating interleukin-4 (IL-4)-induced CCL26 production relevant to allergic asthma. I characterized the concentration and temporal patterns of CCL26 inhibition by procyanidin A2. Interferon

γ (IFN γ) is a cytokine with dichotomous roles as both an innate and adaptive immune modulator. It is pro-inflammatory in regard to autoimmune disease [215,216], and anti-inflammatory in regard to Th2 perpetuated disease, such as allergic asthma [217-219]. Investigation of IFN γ (100 ng/mL; 5.8 nM) *in vitro* has demonstrated ability to inhibit IL-4-induced CCL26 production from lung epithelial cells [217,220]; furthermore, IFN γ administered via the airways has demonstrated efficacy in mice at reducing airway eosinophilia [221]. Thus, I also evaluated the temporal profile of CCL26 inhibition by IFN γ as a tool for comparison and investigated possible interactions between IFN γ and procyanidin A2 seeking insights into how procyanidin A2 might modulate *in vitro* airway inflammation.

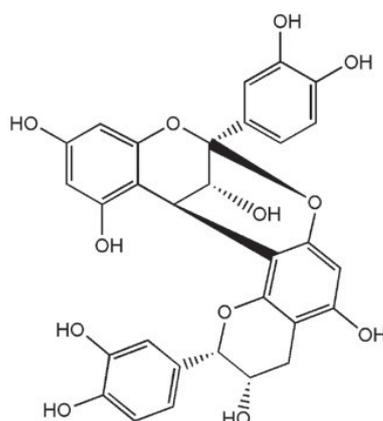


Figure 7-1: Procyanidin A2: epicatechin-(4 β -8, 2 β -O-7)-epicatechin.

7.4 Material and methods

7.4.1 Materials

All materials consistent with section 4.2.

7.4.2 Cell culture conditions

Cells were grown under standard tissue culture conditions as previously described [165] (see also section 4.3 and 4.4). Briefly, A549 cells were kept at 37 °C in a 95% humidified atmosphere at 5% CO₂ in Dulbecco's Modified Eagle Medium: Nutrient

Mixture F-12 (DMEM/F-12) containing 2 mM L-glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL neomycin, and 10% FBS. The cell cultures were grown to form a monolayer (80% confluence) and then growth arrested for 24 h in the absence of FBS before conducting experiments. Experiments used A549 cells of passage 89–107.

7.4.3 Optimizing the production of CCL26

The production of CCL26 can be induced by IL-4 in A549 airway epithelial cells *in vitro* to model the inappropriate inflammation that occurs during allergic airway inflammation. In order to optimize a bioassay for CCL26 production, I characterized both the concentration- and time-dependent profiles of CCL26 production after incubation with IL-4. I measured CCL26 production in A549 cells exposed to IL-4 at a range of concentrations from 0.25 to 50 ng/mL for 24 h as well as determined the time course of CCL26 production after exposure to 5 ng/mL IL-4 for times ranging from 1 to 72 h. Experiments used A549 cells seeded at 5×10^5 per well in 12-well plates with culturing and growth arrest as previously described. Cells were then exposed to serum-free media containing IL-4. Extracellular media were collected and frozen at $-80\text{ }^{\circ}\text{C}$ until analysis for CCL26 using a commercially available sandwich ELISA kit following the manufacturer's instructions.

7.4.4 Procyanidin preparation

Procyanidin A2, B1, and B2 were dissolved in 100% DMSO to 10 mg/mL and stored in aliquots at $-80\text{ }^{\circ}\text{C}$ until experimental use. Procyanidins were then brought to the desired concentration in serum-free media and added immediately to cell cultures. Final DMSO concentrations were 0.03% and 0.06% (v/v) DMSO, respectively, for cultures exposed to 5 µM (2.88 µg/mL) and 10 µM (5.765 µg/mL) procyanidin A2, B1, and B2. DMSO concentrations were used in the negative controls.

7.4.5 Cytotoxicity

The potential cytotoxicity of procyanidin A2 was evaluated by determining effects on cellular plasma membrane integrity through the measurement of the release of the cytosolic enzyme LDH. Experiments used A549 cells seeded at 9×10^5 in 6-well plates in a 3 mL volume with culture and growth arrest as described above. Cells were incubated with procyanidin A2 at concentrations from 1 to 20 μM , negative control media, or a positive control of 100 mM H_2O_2 for 6 h. A representation of 100% release of cellular LDH was obtained by exposing untreated cells to 1% Triton X-100 for 10 min at room temperature. Collected supernatant samples were centrifuged at $12,000 \times g$ for 15 min and immediately assayed for the presence of LDH. In a 96-well plate, 100 μL of each sample was pipetted into a well, and using automated microplate injectors of a BMG Polarstar Omega plate reader (Alphatech), LDH substrate consisting of 3.8 mM pyruvate and 0.8 mM NADH was added to one well at a time and absorbance of NADH read at 340 nm for 2 min.

7.4.6 Modulation of CCL26

In experiments examining effects of procyanidins on inflammation, A549 cells were seeded at 5×10^5 per well in 12-well plates with culture and growth arrest as described above. Cells were then incubated with media containing procyanidin A2, B1, or B2 at final concentrations ranging from 0.0001 to 10 μM , or corresponding DMSO concentrations (max 0.06%) for 6 h, washed briefly with PBS, fresh media replaced and then stimulated with 5 ng/mL IL-4 for 24 h to induce CCL26 production. Extracellular media were collected and frozen at -80°C until analysis for CCL26 production by ELISA. A similar protocol was used for experiments using low (0.5 nM) and high (5.8 nM) IFN γ . The R&D human CCL26 DuoSet ELISA kit has a detection range of 62.5 pg/mL up to 4000 pg/mL as indicated by the manufacturer.

A time course of CCL26 inhibition was determined for 5 μ M procyanidin A2, low concentration (0.5 nM) and high concentration (5.8 nM) IFN γ , by exposing A549 cells for 1–24 h prior to the inflammatory insult of 5 ng/mL IL-4. CCL26 production was also investigated following a 6 h concomitant exposure to 5 μ M procyanidin A2 added to low (0.5 nM) and high (5.8 nM) IFN γ . Procyanidin A2 and IFN γ concentrations were prepared separately and added together in the cell culture well. Additional characterization of procyanidin A2 inhibition was performed using a similar experimental set-up followed by incubation with procyanidin A2 (5 μ M) or equivalent DMSO (0.03% v/v) for 3 h or repeatedly at 1, 3, and 6 h prior to IL-4. For repeated incubations with procyanidin A2 at the times indicated the cells were washed with PBS (to remove any residual procyanidin A2), and media containing fresh procyanidin A2 was added back. A549 cells were then stimulated with IL-4 as described above.

7.4.7 Statistics

Statistical analysis of data was performed using GraphPad Prism 5 (San Diego, CA, USA) or GenStat: 14th Edition (VSN International, London, UK). Data in the IL-4 concentration response (Figure 7-2A) and time course (Figure 7-2B) were analyzed with a one-way ANOVA and post-hoc Dunnett's multiple comparison tests against control 0 ng/mL IL-4 or time 0 h, respectively. Cytotoxicity data (Figure 7-3) were analyzed with a one-way ANOVA and post-hoc Tukey's multiple comparison tests between all experimental conditions. Data for CCL26 inhibition by procyanidin A2 (Figure 7-4), procyanidin B1 and B2 (Figure 7-5) as well as data for concomitant incubation of procyanidin A2 and IFN γ (Figure 7-6) were analyzed with a mixed linear model and post-hoc Fisher's unprotected least significant difference test (5%) because the data were unbalanced. Time points of interest for data in Figure 7-5 were analyzed with one-way unpaired *t*-tests with a Welch's correction. The Δ inhibition following one-off or repeated

incubations was analyzed with an unpaired t-test. Data that did not reach a $p < 0.05$ threshold were listed as not significant (NS). Statistical significance in the figures is denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

7.5 Results

7.5.1 Optimization of airway epithelial cell bioassay conditions

The concentration response characteristics for CCL26 production following IL-4 exposure (24 h) are shown in Figure 7-2A. Production of CCL26 increased steeply with increasing IL-4 concentration and reached a plateau at 10 ng/mL IL-4 (2423 ± 109 pg/mL CCL26). The CCL26 response was statistically different from control at IL-4 concentrations of 1.25 ng/mL (1134 ± 54 pg/mL CCL26, $p < 0.001$) and above. The time course for CCL26 production following 5 ng/mL IL-4 ranging from 1 to 72 h is shown in Figure 7-2B. Production of CCL26 became statistically different from baseline at 24 h (1280 ± 276 pg/mL CCL26, $p < 0.01$) and continued to increase in a linear manner until the final measured time point of 72 h (4865 ± 373 pg/mL CCL26, $p < 0.001$). From these data I determined that 5 ng/mL IL-4 for 24 h provided a sufficiently robust induction of

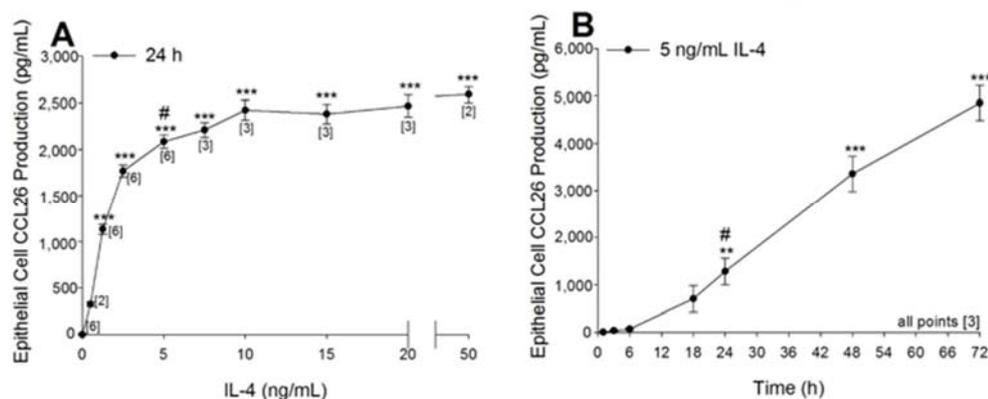


Figure 7-2: IL-4 conditions for inducing CCL26 production in A549

A549 cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h and incubated with (A) 0.5–50 ng/mL IL-4 for 24 h or (B) 5 ng/mL IL-4 for 1–72 h. Collected supernatants were measured for CCL26 by ELISA. The results are expressed as mean \pm SEM, which were from 2–6 separate experiments and 6–18 individual measurements. Brackets, [n], indicate number of experiments for each data point. # indicates the chosen optimized dose or time for inducing CCL26 production. ** $p < 0.01$, *** $p < 0.001$.

CCL26, which enabled us to progress into utilising this model for the evaluation of procyanidins.

7.5.2 Cytotoxicity assessment

To ensure any changes observed were due to procyanidin A2 and not an artefact of cytotoxicity, I evaluated the release of the cytosolic enzyme lactate dehydrogenase (LDH) from procyanidin A2-exposed epithelial cells as an indicator of the breakdown of the cellular plasma membrane. A positive control of 100 mM H₂O₂ was used, and demonstrated a measurable release of LDH ($p < 0.001$ compared with baseline control). Exposure to procyanidin A2 at 1, 5, 10 and 20 μ M did not mediate any significant change in LDH release from the baseline control (Figure 7-3). Procyanidin A2 was therefore regarded as not cytotoxic to the epithelial cells at the concentrations and times evaluated.

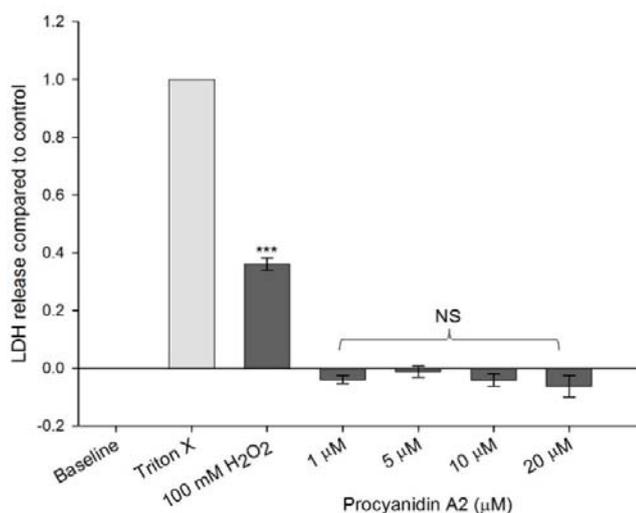


Figure 7-3: Effect of procyanidin A2 on cell viability investigated by the LDH assay

A549 cells were seeded at 9×10^5 in 6-well plates, serum starved for 24 h and incubated with procyanidin A2 from 1 to 20 μ M, control media, or positive control 100 mM H₂O₂ for 6 h. Supernatants were collected and assayed for lactate dehydrogenase (LDH). Results are expressed relative to baseline (0% cell death) and Triton X-100 (100% cell death). The results are expressed as mean \pm SEM, which were from 3 separate experiments and 9 individual measurements. NS = not significant, *** $p < 0.001$.

7.5.3 Evaluation of procyanidins

For experiments evaluating the potential modulation of CCL26 generation, I selected a procyanidin concentration range of 0.001–10 μM . This procyanidin range was selected based on previous work from the literature, physiological relevance, and reported efficacy at inhibiting the secretion of another eotaxin isoform, CCL11 [12,40,165,222]. Procyanidin A2 at 5 and 10 μM was demonstrated to have significant inhibitory effects (891 ± 260 and 988 ± 242 pg/mL CCL26, respectively compared with the DMSO control

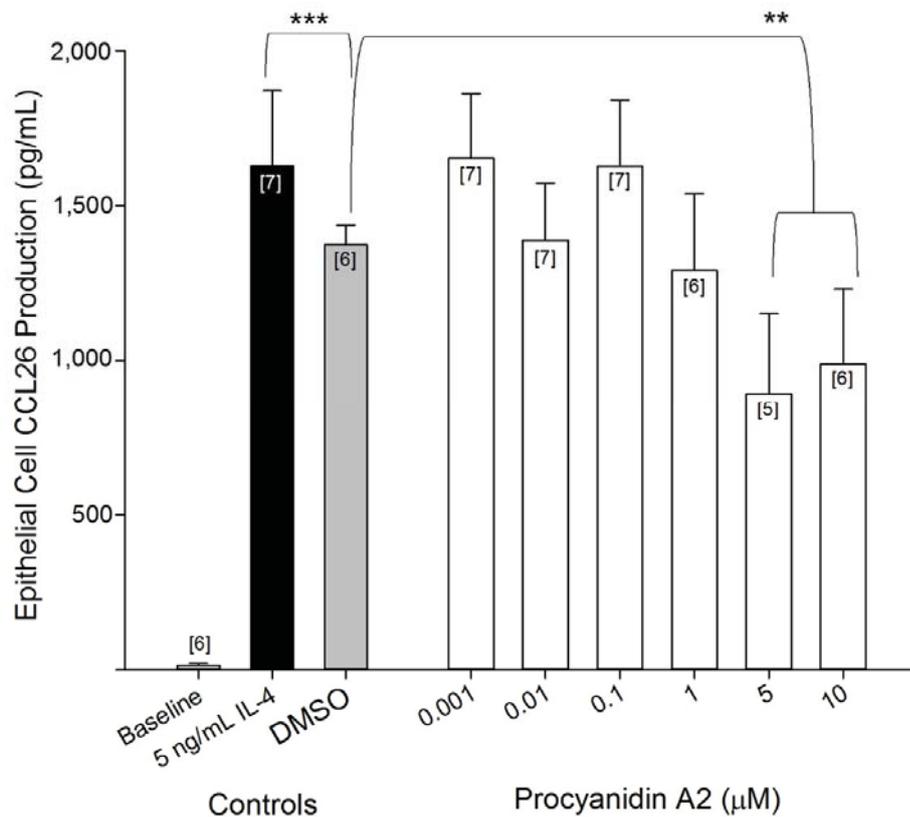


Figure 7-4: Procyanidin A2 inhibits IL-4-stimulated CCL26 production.

A549 cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h and incubated with control DMSO or a range of procyanidin A2 concentrations for 6 h, washed and then stimulated with 5 ng/mL IL-4 for 24 h. Collected supernatants were measured for CCL26 by ELISA. The results are expressed as mean \pm SEM, which were from 5–7 separate experiments and 15–21 individual measurements. Brackets, [n], indicate number of experiments for each data point. ** $p < 0.01$, *** $p < 0.001$.

1375 ± 62 pg/mL CCL26 ; $p < 0.01$) on IL-4–induced CCL26 production when incubated for 6 h prior to an inflammatory insult of 5 ng/mL IL-4 for 24 h (Figure 7-4). These data corresponded to 35% and 28% inhibition of CCL26 production beyond DMSO by 5 and 10 µM procyanidin A2, respectively. Procyanidin A2 was dissolved in DMSO, which created different concentrations of DMSO in each investigated procyanidin A2 concentration. Control experiments were performed on the range of DMSO concentrations (to a maximum of 0.06%) corresponding to the anticipated doses of procyanidin A2. Only at the highest concentration evaluated was there a significant inhibitory effect of DMSO on CCL26 production from alveolar cells (1375 ± 62 pg/mL CCL26) compared with IL-4 stimulation only (1629 ± 242 pg/mL CCL26; $p < 0.001$; Figure 7-4). These data correspond to a 16% inhibition of CCL26 production by DMSO. Procyanidins with a B-type link did not demonstrate as potent inhibition of CCL26. Procyanidin B1 had no inhibitory effect on IL-4 induced CCL26 compared to DMSO control (Figure 7-5). Procyanidin B2 (5 µM) reduced CCL26 production 12 % compared to DMSO control, which was roughly half of the inhibition demonstrated by procyanidin A2 (Figure 7-5).

7.5.4 Time-dependent inhibition of CCL26 by procyanidin A2 and IFN γ

The investigation of plant-based compounds as a means to reduce the reliance on pharmaceutical interventions and maintain human health is currently a very active research area. With evidence to support procyanidin A2 as a modulator of CCL26 production, I sought to explore the temporal pattern of CCL26 inhibition by procyanidin A2 and compared this with the known CCL26 secretion inhibitor, IFN γ . A time course of inhibition was conducted for 5 µM procyanidin A2 and compared against two concentrations of IFN γ : 0.5 nM (low) and 5.8 nM (high). The time courses for procyanidin A2, low IFN γ , and high IFN γ inhibition of CCL26 are shown in Figure 7-6.

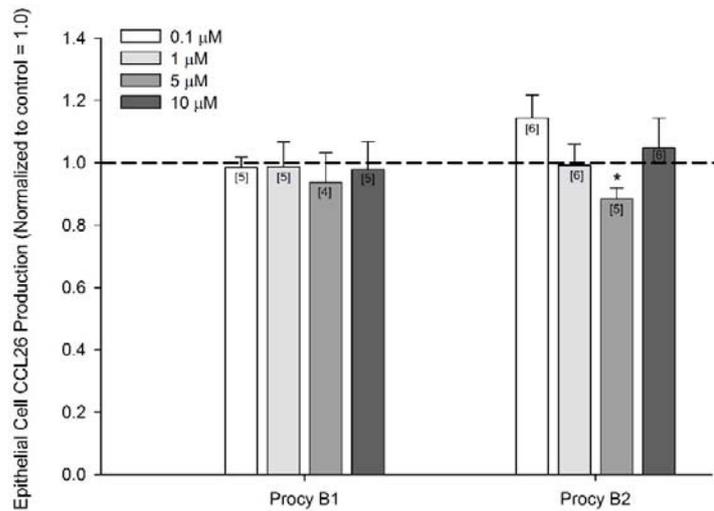
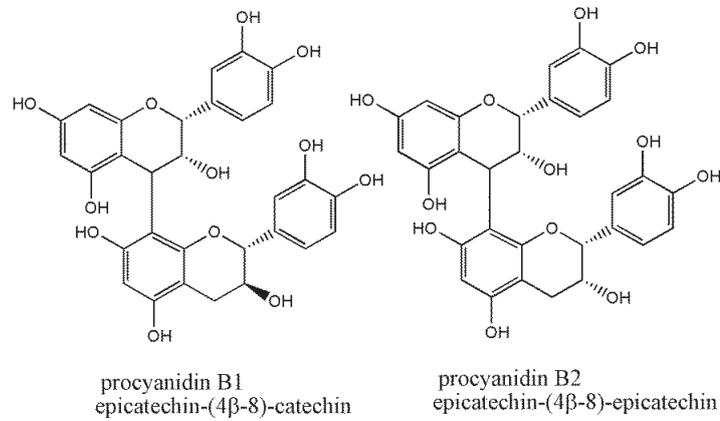


Figure 7-5: Procyanidin B1 and B2 effects on IL-4-stimulated CCL26 production.

A549 cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h and incubated with control DMSO or a range of procyanidin B1 (procy B1) and procyanidin B2 (procy B2) concentrations for 6 h, washed and then stimulated with 5 ng/mL IL-4 for 24 h. Collected supernatants were measured for CCL26 by ELISA. Results are expressed as mean \pm SEM normalized to control = 1.0, $n = 4-6$ separate experiments. Brackets, [n] indicate number of experiments for each data point. * $p < 0.05$. 5 μ M procyanidin B2 reduced CCL26 production 12% compared to control.

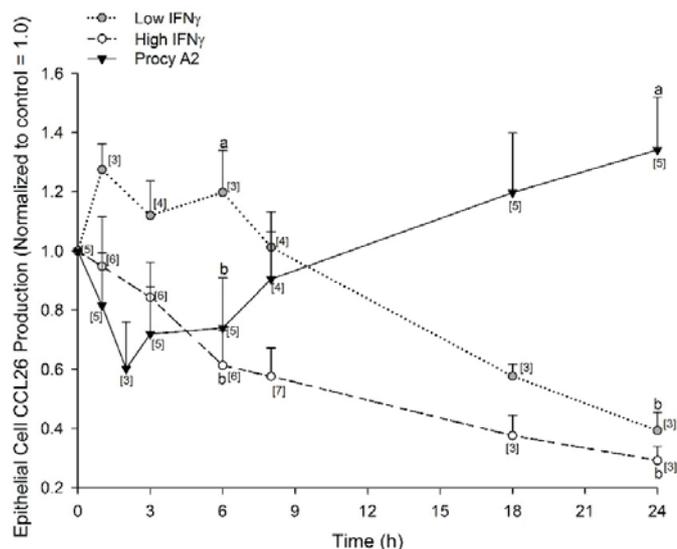


Figure 7-6: IFN γ and procyanidin A2 inhibit IL-4- stimulated CCL26 production in a time-dependent manner.

A549 cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h and incubated for 1–24 h with low IFN γ (0.5 nM, grey dots), high IFN γ (5.8 nM, open dots) or procyanidin A2 (Procy A2; 5 μ M, black triangles), washed with PBS and then stimulated with 5 ng/mL IL-4 for 24 h. Collected supernatants were measured for CCL26 by ELISA. Results expressed as mean \pm SEM normalized to control = 1.0, which were from 3–7 separate experiments and 9–21 individual measurements. Brackets, [n], indicate number of experiments for each data point. Different letters “a” and “b” at 6 h or 24 h represent statistical difference, $p < 0.05$.

Procyanidin A2 data are expressed relative to control wells incubated with 0.03% DMSO for 24 h, or media only for IFN γ . Procyanidin A2 demonstrated peak inhibition of CCL26 production when incubated for 2 h (0.60 ± 0.16 normalized to control = 1.0) prior to an inflammatory insult. The inhibition was no longer observed at 8 h (0.90 ± 0.16 ; 24 h: 1.3 ± 0.18 normalized to control = 1.0; Figure 7-6). Low and high IFN γ demonstrated the greatest inhibition of CCL26 production when incubated for 24 h prior to an inflammatory insult with both concentrations significantly inhibiting to a similar degree (0.5 nM: 0.39 ± 0.06 , 5.8 nM: 0.29 ± 0.05 normalized to control = 1.0; Figure 7-6). CCL26 production measured after 6 h exposure to the higher concentration of IFN γ was significantly different from that mediated in the presence of the lower concentration of IFN γ (0.5 nM: 1.20 ± 0.14 , 5.8 nM: 0.61 ± 0.12 normalized to control = 1.0; $p < 0.05$; Figure 7-6). The low concentration of IFN γ did not inhibit CCL26 production to below control levels until after 8 h of exposure prior to the inflammatory insult. Visual inspection of A549 cells after incubation with 5.8 nM IFN γ (100 ng/mL) showed no disruption of the cell monolayer; the observed IFN γ -induced inhibition to 30% of control was consistent with reports in the literature [217,220].

A revealing feature of the CCL26 inhibition time courses is seen from the comparison of 5 μ M procyanidin A2 with IFN γ concentrations at 6 h prior to an inflammatory insult. Procyanidin A2 inhibited CCL26 production to 74% of the control after 6 h, while low concentration IFN γ inhibited to 120% of the control, and high concentration IFN γ demonstrated an inhibition to 61% of the control. Thus 5 μ M procyanidin A2 was a more potent inhibitor of CCL26 production at 6 h compared with low concentration IFN γ (procyanidin A2: 0.74 ± 0.16 , 0.5 nM IFN γ : 1.20 ± 0.14 normalized to control = 1.0, $p < 0.05$). Procyanidin A2 had a similar inhibition of CCL26 production at 6 h when compared with high concentration IFN γ (5.8 nM IFN γ : 0.61 ± 0.12 normalized to control

= 1.0; Figure 7-6). The concentration and time-dependent differences observed with procyanidin A2 and IFN γ concentrations led me to consider possible cooperative interactions between procyanidin A2 and IFN γ .

7.5.5 Procyanidin A2 impedes IFN γ -mediated CCL26 inhibition

The distinct temporal patterns of CCL26 inhibition by procyanidin A2 and IFN γ prompted us to next evaluate the possibility that concomitant 5 μ M procyanidin A2 and IFN γ may assist in maintaining peak procyanidin A2 inhibition (observed at 2 h) to a later (6 h) time point or improve inhibitory efficacy beyond that of procyanidin A2 alone (Figure 7-7). Investigating cooperative inhibition with a 6 h exposure prior to an inflammatory insult was chosen as both procyanidin A2 and IFN γ display suboptimal inhibition with this length of time. At 6 h the inhibition of CCL26 by 5 μ M procyanidin

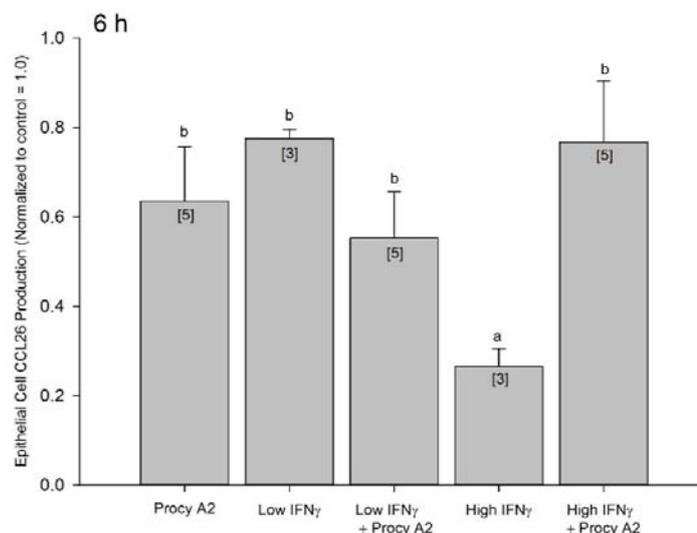


Figure 7-7: Concomitant incubation (6 h) of procyanidin A2 and IFN γ does not improve inhibition of CCL26 production.

A549 cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h and incubated for 6 h with either 5 μ M procyanidin A2 (Procy A2), low (0.5 nM), high (5.8 nM) IFN γ alone, or concomitantly IFN γ plus 5 μ M procyanidin A2. Cells were washed and then stimulated with 5 ng/mL IL-4 for 24 h. Collected supernatants were measured for CCL26 by ELISA. Results are expressed as mean \pm SEM normalized to control = 1.0, which were from 3–5 separate experiments and 9–15 individual measurements. Brackets, [n], indicate number of experiments for each data point. Different letters “a” and “b” represent statistical difference, $p < 0.05$.

A2 alone, low (0.5 nM) IFN γ , or the concomitant incubation of 5 μ M procyanidin A2 with low IFN γ showed no statistical difference from each other (0.64 ± 0.12 , 0.78 ± 0.02 , 0.55 ± 0.10 respectively normalized to control = 1.0; Figure 7-7). High IFN γ alone significantly inhibited CCL26 production beyond that of procyanidin A2 (0.27 ± 0.04 normalized to control = 1.0; $p < 0.05$; Figure 7-7). When 5 μ M procyanidin A2 was concomitantly added with high (5.8 nM) IFN γ , CCL26 production was no longer statistically different from procyanidin A2 alone (0.77 ± 0.14 normalized to control = 1.0; Figure 7-7). These data suggests procyanidin A2 does not have cooperative inhibition with IFN γ , and in fact interferes with high dose IFN γ -mediated inhibition of CCL26.

7.5.6 Repeated incubations with procyanidin A2 does not further reduce CCL26

The management of inflammation by procyanidin A2 was also characterised further by exploring the effect of repeated incubation with procyanidin A2 (5 μ M). I chose incubations at 1 h, 3 h, and 6 h prior to IL-4 stimulation as they fell within the demonstrated efficacy window and also straddled the strongest inhibition time point of 2 h. CCL26 production after incubation with procyanidin A2 for 3 h or repeatedly at 1, 3, and 6 h prior to IL-4 stimulation was 50 % and 60 % respectively compared to their DMSO matched control (0.50 ± 0.15 , and 0.60 ± 0.32 normalized to control = 1.0; Figure 7-8). These data resulted in changes between DMSO and procyanidin A2 for cells incubated once (Δ 3 h, black bar) or repeatedly (Δ 1, 3, 6 h, grey bar) of 50 % and 40 % respectively (0.50 ± 0.15 and 0.40 ± 0.32 ; Figure 7-8). Thus, there was no detectable change in the effectiveness of procyanidin A2 on CCL26 production with repeated incubation of procyanidin A2 prior to cytokine stimulation in A549 cells.

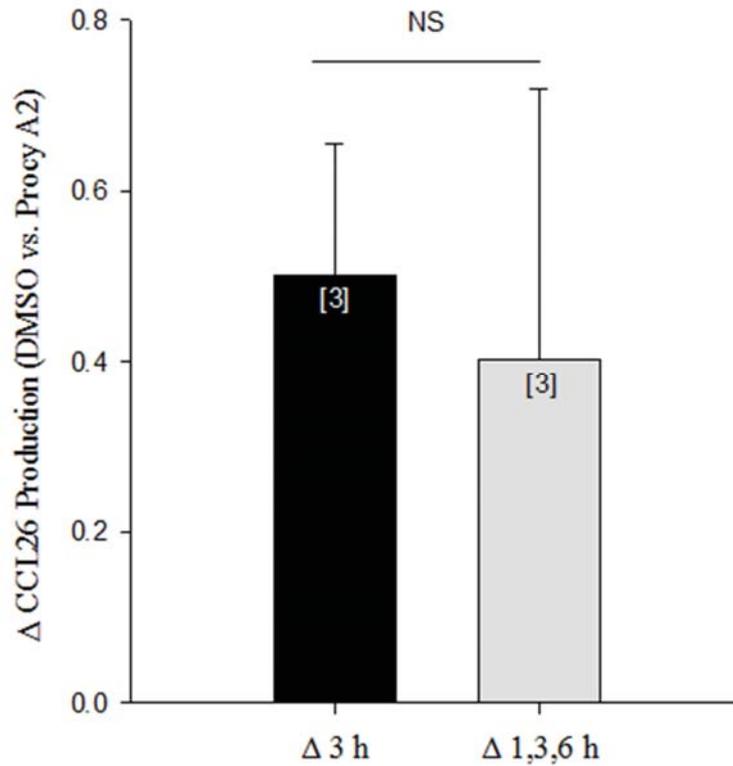


Figure 7-8: Repeated incubations does not affect inhibition by procyanidin A2

A549 cells were seeded at 5×10^5 in 12-well plates, serum starved for 24 h, incubated with DMSO (0.03 % v/v) or procyanidin A2 (5 μ M) for 3 h or repeatedly at 1,3, and 6 h, washed with PBS and then stimulated with 5 ng/mL IL-4 for 24 h. Collected supernatants were measured for CCL26 by ELISA. The mean change (Δ) in CCL26 production between DMSO and procyanidin A2 (Δ 3 h) treated cells is compared to the mean difference in CCL26 production between repeated incubations with DMSO or procyanidin A2 (Δ 1, 3, 6). Results expressed as mean change \pm SEM normalized to corresponding DMSO control = 1.0, which were from 3 separate experiments and 9 individual measurements. Brackets, [n], indicate number of experiments for each data point. NS = not significant.

7.6 Discussion

This study sought to evaluate the potential modulation of cytokine-induced CCL26 by a commercially sourced, structurally-defined, pure dimeric procyanidins. I confirmed IL-4 exposure induces CCL26 production in A549 cells, thereby modelling an aspect of airway inflammation of relevance to allergic asthma. Exposure time and IL-4 concentration were optimized for CCL26 production. These experiments demonstrated that 5 ng/mL IL-4 for 24 h allowed for a robust induction of CCL26 production in A549 cells. Using these parameters I demonstrated that both 5 and 10 μ M procyanidin A2 when incubated 6 h prior to an inflammatory insult had an inhibitory effect on CCL26 production, with much less inhibition by B-type procyanidins. Experiments determining cytotoxicity by measuring LDH, along with previous work in our group using the WST-1 assay [165], demonstrate that procyanidins at the concentrations investigated in this study were not detrimental to cell membrane integrity or cellular metabolism; thus, the inhibitory effect observed on the inflammatory biomarker CCL26 was attributed to an action of procyanidin A2 on CCL26 production and not as a consequence of cytotoxicity. I then expanded our investigation of procyanidin A2 by measuring the temporal profile of inhibition for 5 μ M procyanidin A2 and used the known CCL26 secretion inhibitor IFN γ as a tool for comparison. I investigated possible cooperative inhibition of CCL26 secretion by procyanidin A2 and IFN γ as well as an effect with repeated incubations of procyanidin A2.

There is a growing pool of evidence that supports the ability of polyphenolic compounds, specifically procyanidins, to modulate key inflammatory events (see reviews) [55,82]. Aberrant inflammatory responses at the airway epithelium of an asthmatic prolongs immune cell presence in the lung leading to tissue damage. The eosinophil chemokine CCL26, which facilitates the late-stage infiltration of eosinophils into lung tissue, is one target for procyanidin modulation that could be useful for

improving lung health [223]. I contribute evidence with our work here that the plant polyphenol procyanidin A2 can indeed act as a modulator of inflammation. I provide evidence that procyanidin A2 inhibits IL-4-induced production of CCL26 *in vitro* and its efficacy is the greatest when exposed to cell cultures 2–3 h prior to an inflammatory insult. This temporal profile is distinct from that of the CCL26 inhibitor IFN γ , which demonstrated greatest inhibition in our model system at 24 h. IFN γ is a known effector of CCL26 production and can act on the epithelium to inhibit airway inflammation [221]. There are several mechanisms by which IFN γ has been demonstrated to inhibit CCL26 production, including through intracellular effects on transcription factors [224], up-regulation of endogenous inhibitors [225], increasing the rate of decay for CCL26 messenger RNA [217], increasing the degree of methylation in the promoter region of the *CCL26* gene [226], and increasing the expression of the IL-4 receptor component, IL-4R α [220]. The temporal pattern of inhibition for procyanidin A2 reported here does not match that of IFN γ . The temporal profile of 5 μ M procyanidin A2 inhibition was strongest at 2 h, reduced by 8 h, and was no longer present by 24 h (Figure 7-6). When I investigated possible coordinated interactions between procyanidin A2 and IFN γ , I found no evidence of cooperative inhibition at 6 h. In contrast, procyanidin A2 interfered with high-concentration IFN γ -mediated inhibition of CCL26 when simultaneously incubated together prior to an inflammatory insult. These data allude to a more transient mechanism of inhibition by procyanidin A2 and likely via a mechanism of action independent from IFN γ .

To date the demonstrated biological effects of A-type linked procyanidins include activity as an antioxidant, which is attributed to the catechol group in the B-ring of many flavonoids that can trap free radicals [227-229], and in the prevention of oral and urogenital epithelial infections due to the anti-adherence properties [230-232]. The

inclination of procyanidins to bind proteins and form insoluble complexes [233] leads to the possibility of direct cell receptor masking. A recent study has demonstrated that cyanidin, a monomeric polyphenol ion structurally similar to catechin, elicited biological activity through direct blocking of cytokine with receptor [234]. The experiments presented here were performed with the intention of evaluating procyanidin efficacy for modulating IL-4-induced CCL26, with less emphasis on determining the underlying mechanism. However, major signal transduction pathways such as signal transducers and activators of transcription (STAT), transcription factors in the mitogen-activated protein kinases (MAPK), as well as the NF- κ B pathway have all been identified as being affected by the presence of procyanidins [82]; furthermore, controlling signal transduction pathways may not be the only mechanism by which procyanidins could influence inflammatory events. A more recent, and still developing, theory is that procyanidins could mediate a physical interaction at the cell plasma membrane that modulates cellular events [235]. There is work in the literature that demonstrates other flavonoids and the flavan-3-ol monomeric units catechins, which bind to form procyanidins, can insert themselves in plasma membranes as a mechanism for eliciting biological activity [236-238]. Interestingly, experiments with liposomes suggested that procyanidins can protect against lipid oxidation by affecting membrane surface potential and integrity [227,239]. Extensive work by Verstraeten, Fraga, & Oteiza investigating A-type procyanidin effects on the gastrointestinal epithelium have demonstrated that they indeed can impact biological processes via interactions with the apical membrane of the enterocyte *in vitro* [235]. Procyanidins are implicated as mitigating oxidative stress and the activation of pro-inflammatory events [235]. Thus, it is suggested that A-type procyanidins may favourably affect gastrointestinal health, though there has been no work to date investigating A-type procyanidins and gut inflammation *in vivo*. Similarly, recent experiments by Zhu et al.

have demonstrated membrane perturbations as a possible mechanism for the ability of A-type procyanidins to inhibit 3T3-L1 preadipocyte differentiation [240,241]. Our findings here, whereby procyanidin A2 interferes with an intracellular inhibitor $\text{IFN}\gamma$, is preliminary evidence that A-type procyanidins could interact with cellular membranes as a mechanism to reduce inappropriate inflammation and promote lung health.

Previous work from our group in a similar cell culture model suggested proanthocyanidins as the bioactive components responsible for the ability of blackcurrant extracts to modulate CCL26 production *in vitro* [1]. The study provided evidence to support the theory that a proanthocyanidin-enriched blackcurrant extract potentiated $\text{IFN}\gamma$ -induced suppression of IL-4 stimulated CCL26 secretion, and the authors put forth the idea that proanthocyanidin metabolites, particularly epigallocatechin, may modulate similar cellular events and complement the inhibitory action of $\text{IFN}\gamma$ on eosinophilic inflammation [1]. At first consideration, this appears to be in conflict with the data presented here; however the blackcurrant study used 1 ng/mL $\text{IFN}\gamma$ (0.058 nM), which is consistent with our data for low (0.5 nM) $\text{IFN}\gamma$ data presented here in which I show that procyanidin A2 trends (but did not reach significance) toward assisting low concentration $\text{IFN}\gamma$ at inhibiting CCL26 production (Figure 7-7). The two studies differed in the duration of inhibition after exposing cells to polyphenolic compounds suggesting the blackcurrant extracts bioactive compounds and procyanidin A2 may inhibit CCL26 through different mechanisms. The blackcurrant proanthocyanidin-enriched extract evaluated in the previous study displayed inhibition of CCL26 starting at 6 h, which remained evident at 24 h, whereas our work here demonstrated the strongest inhibition around 2 h and did not remain evident beyond 8 h. It may also be important to note that the blackcurrant work incubated cells with the proanthocyanidin-enriched extract prior to an inflammatory insult of $\text{IFN}\gamma$ and IL-4 together, and our work here exposed cell to

procyanidin A2 and IFN γ prior to an inflammatory insult of IL-4 alone. Furthermore, previous work in our group identified that the ratio of specific anthocyanins, another class of phytochemicals, in blackcurrant cultivars was an important determinant for influencing the suppression of cytokine-induced CCL26 in A549 cells [164]. Collectively, these studies and our data demonstrate that the biological activity of polyphenols, and specifically procyanidins, has repeatedly indicated that structural specificity is paramount to bioactivity and it cannot be extrapolated across a phytochemical classification. For example, A-type linked and B-type linked procyanidins by virtue of structural differences demonstrate distinct interactions with the transcription factor nuclear factor- κ B (NF- κ B) [12]. Differences in bioactivity were also reported by our group between A-type and B-type procyanidins for inhibition of another isoform of eotaxin, CCL11 [165] (see chapter six). Furthermore, B-type procyanidins were investigated here for their ability to modulate IL-4-induced CCL26. Procyanidin B1 had no inhibitory effect, whereas procyanidin B2 had much less inhibition compared to the effects shown by procyanidin A2 (Figure 7-5). Thus, the stronger inhibitory activity demonstrated by procyanidin A2 in the context here could be structure specific and/or restricted to polyphenolic compounds with A-type bonds between the flavan-3-ol monomeric units.

In order to progress our understanding of the role procyanidins may have in mitigating airway inflammation, future work could involve, among others, investigation of procyanidin A2 by utilizing an air-liquid interface epithelial cell culture system. This would mimic and evaluate an application via inhalation as opposed to oral ingestion. Studies investigating the efficacy of IFN γ applied through inhalation have suggested its use in eosinophilic asthma, with effects limited to the respiratory tract [218]. This approach could also be applied to procyanidins. Furthermore, while I recognize more research is needed, our work here could suggest the potential for procyanidin A2 as a

preventative measure for lung inflammation when exposure to known allergens is anticipated such as seasonal hay fever allergies, or air pollution.

Our data provide evidence that pre-exposure to procyanidin A2 is a prompt yet transitory method to inhibit CCL26 production in A549 human lung epithelial cells. The temporal profile of inhibition is observed to be different from that of IFN γ . These data are encouraging, but further investigation is needed in an animal model or whole body system to determine if procyanidin A2 could be utilized as a preventative approach to help manage airway inflammation.

Chapter Eight

Evaluating procyanidin–induced alterations in cytokine receptor expression

8.1 Evaluating procyanidin–induced alterations in cytokine receptor expression

Contribution Declaration

This thesis chapter is not published elsewhere.

The content of this chapter was written by Sara Coleman (100%).

8.2 Abstract

BACKGROUND: Cytokine-induced CCL11 and CCL26 production are reduced in A549 cells when incubated with procyanidin A2 prior to an inflammatory insult. The cellular mechanism for this inhibition is unclear. One mechanism by which procyanidin A2 could attenuate CCL11 and CCL26 production is by modification of cytokine receptor expression on the surface of A549 cells.

OBJECTIVE: I sought to measure the effect of procyanidin A2 exposure on the expression of four cytokine receptors: IL-4R α , TNF R1, IL-13R α 1, and CCR3 on the surface of A549 cells with the common γ chain (CgC) used as a negative control.

METHODS: Commercially-purchased fluorescently labelled monoclonal antibodies for the receptors of interest were titrated using human lung epithelial cells (A549). Cells were exposed to procyanidin A2 or DMSO for 2 h and then receptor expression was analysed by flow cytometry with a BD FACSVerserTM flow cytometer.

RESULTS: Of the four investigated receptors, IL-13R α 1 was the sole detectable receptor on A549 cells and demonstrated no measurable change in expression due to procyanidin A2 exposure. Variations to experimental protocols did not improve detection of IL-4R α , TNF R1, or CCR3 receptors on A549 cells. Complementary experiments using the IM9 human B lymphoblast cell line detected IL-13R α 1 and the CgC indicating that the protocol for antibody staining was likely appropriate and not a source of the technical difficulties here.

CONCLUSIONS: These data provide no evidence to support the hypothesis that procyanidin A2 could attenuate CCL11 and CCL26 production by modifying cytokine receptor expression on the surface of A549 cells.

8.3 Introduction

Previous work in this project demonstrated that A549 cells exposed to procyanidin A2 prior to an inflammatory insult displayed reduced production of CCL11 and CCL26 [165,166]. The mechanism by which procyanidin exposure leads to this inhibition is unclear. There are numerous ways by which procyanidins could be affecting eotaxin production including direct steric interference at the plasma membrane, modulation of: receptor expression, plasma membrane properties, signal transduction, transcription, translation, post-translational modifications, or upregulation/downregulation of inhibitors/promoters (Figure 8-1). I have previously described in detail (chapter two) evidence for procyanidins controlling prominent inflammatory signal transduction pathways [82]. I sought to investigate other possible mechanisms by which procyanidins could be inhibiting CCL11 and CCL26 production. I turned my attention to investigating procyanidin effects on extracellular cell surface receptors because of the uncertainty in the literature over procyanidin intracellular accessibility. Cytokine receptor expression was investigated here as it was technically

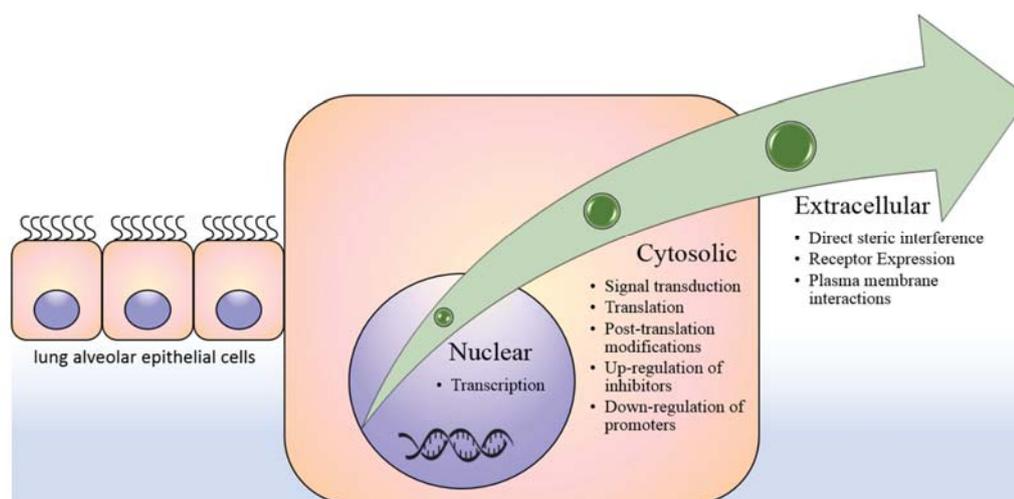


Figure 8-1: Mechanistic possibilities for procyanidin inhibition of cytokine-induced CCL11 and CCL26 production

feasible in this project. Recently, direct steric interference was demonstrated as a mechanism by which the polyphenol ion cyaniding alleviates inflammation in vivo [234].

There were three specific aims for this work. First, I wanted to directly demonstrate the presence of the type II IL-4 receptor (see below) and TNF R1 on A549 cells surface to support previous work using IL-4 and TNF α to induce the production of CCL11 and CCL26. Second, I sought to investigate modulation of cytokine receptor expression by procyanidins. Lastly, I sought to develop my skills in the technique of flow cytometry in order to progress this project to investigate the effect of procyanidins on other parameters of airway inflammation or in other cell types.

Cytokine signalling, and specifically IL-4 signalling, uses transmembrane receptor proteins that bind ligand extracellularly and physical changes in the receptor relay the message intracellularly. This occurs through association with one of the Janus kinase (JAK) proteins, which couple ligand binding to tyrosine phosphorylation of various signal transducers and activators and transcription (STAT; chapter two) signalling proteins [61,242]. IL-4 signalling has been the subject of substantial scientific inquiry due to its similarities with IL-13 signalling [210,243-247]. These two cytokines share the type II IL-4 receptor which is a transmembrane heterodimer of the IL-4R α and IL-13R α 1 subunits. IL-4 can also bind to the type I IL-4 receptor, a heterodimer of IL-4R α and the common γ chain (C γ C) subunits; whereas, IL-13 can also bind IL-13R α 2. Interestingly, IL-13R α 2 binding IL-13 has been suggested to have its own distinct roles in allergic asthma airway inflammation [248]. The type II receptor subunits IL-4R α and IL-13R α 1 were both chosen as part of the investigated receptors for this work because they are the receptor subunits responsible for IL-4 signalling on non-hemopoietic cells

including A549 cells. The TNF R1 receptor is responsible for TNF α signalling, and was investigated because TNF α was used for inducing the production of CCL11 (see chapter six). The CCR3 receptor (see section 3.3) is found on A549 epithelial cells [249]; though, it was investigated as part of this study here as a connection to lead to possible future experiments involving co-cultured epithelial and immune cells. It was demonstrated that expression levels of CCR3 is upregulated in inflamed asthmatic airways and correlates with allergic asthma disease severity [250-252]. Collectively, I sought to measure the presence of these four cytokine receptors of interest: IL-4R α , TNF R1, IL-13R α 1, and CCR3 on the surface of A549 cells with common γ chain (CgC) as a negative control and investigate receptor modulation by procyanidin A2.

8.4 Materials and methods

8.4.1 Materials

Materials for cell culture of A549 human lung epithelial cells and cytokine stimulation with IL-4/ TNF α or exposure to procyanidin A2 are consistent with sections 4.4 and 7.4. The human B lymphoblast cell line (IM9) was purchased from the American Type Culture Collection (ATCC® CCL-159™), (c/o Cryosite, New South Wales, Australia). Roswell Park Memorial Institute cell culture media (RPMI), 2.5% trypsin, and EDTA were purchased from Life Technologies (Auckland, New Zealand). TrypLE™ was from ThermoFisher Scientific (Auckland, New Zealand). VectorShield™ Hard Set mounting media was purchased through Abacus ALS (Auckland, New Zealand). The IL-13 α R1, CCR3, and CgC flow cytometry antibodies (Table 8-1), Zombie NIR™ viability kit (Table- 8-1), Human TruStain FcX™ Fc receptor blocking solution, along with the cell staining buffer were from BioLegend and purchased through Medi'Ray New Zealand (Auckland, New Zealand). The IL-4R α and TNF R1 antibodies (Table 8-1) were from R&D systems (In Vitro Technologies,

Table 8-1: Flow cytometry antibodies with corresponding fluorophores

Excitation Laser	Fluorescence Channel	Designed Panel
488 nm	BD Horizon™ BB515, FITC, Alexa Fluor®488	TNFR1
	PE	CgC
	PerCP, PerCP-Cy™5.5, Pe-Cy™5	IL-4Rα1
	PE-Cy™7	-
640 nm	APC, Alexa Fluor®647	IL-13Rα1
	BD™ APC-H7, APC-Cy™7	Live-Dead Marker
405 nm	BD Horizon™ BC421, V450, VPD450	CCR3
	BD Horizon™ BV510, V500	-

Auckland, New Zealand). The anti-mouse and anti-rat Ig, κ negative control compensation particles sets were purchased through BD (Auckland, New Zealand). Coverslips, slides, flow cytometry tubes (FACS tubes) and all other materials not specifically listed were purchased from Sigma-Aldrich (St. Louis, MO, USA).

8.4.2 Cell culture conditions

The A549 human lung epithelial cells were cultured as previously described in sections 4.4.1, 4.4.2, and 7.4.2. The IM9 human B lymphoblast cell line was grown in suspension under standard tissue culture conditions of 37°C in a 95% humidified atmosphere at 5% CO₂ in RPMI cell culture media supplemented with 2 mM L-glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL neomycin, and 10% FBS. Titration of the antibodies (experiment 1 and 2, Table 8-2) used A549 or IM9 cells cultured in T75 flasks, detached using either Tryple™ or trypsin if applicable (as described below), counted, aliquoted into a 96-well plate at 5 x 10⁵ cells in 200 µL, and stained with single antibodies (described below) for IL-4Rα, TNF R1, IL-13Rα1, CCR3, or CgC at dilutions of 1:20, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, or left unstained. Cells were analysed by flow cytometry using a BD FACSVerser™ (section 4.4.6 and 4.4.7). Experiments labelled as involving ‘differentiated A549’ used A549 cells grown to confluence in a T75 flask which remained in culture for 14 days with

media replaced every second day (experiment 3, Table 8-2). These cells were potentially more differentiated; however, no parameters were investigated to assess differentiation state.

Table 8-2: Overview of the six flow cytometry experiments performed

Experiment	1	2	3	4	5	6
Description	Basic titration	Basic titration w/ cytokine stimulation	Cytokine stimulation and differentiation of cells	Fixation and detachment variations	Investigating Procyandin A2	Investigating Procyandin A2 (repeat)
Antibody Dilutions	varied titrations	varied titrations	1:50	1:50	1:50	1:50
Receptors measured on A549 Cells	IL-4R α , TNFR1, IL-13R α 1, CCR3, CgC	IL-4R α , TNFR1, IL-13R α 1, CCR3, CgC	IL-4R α , TNFR1, IL-13R α 1, CCR3, CgC	IL-4R α , TNFR1, CCR3	IL-13R α 1 & Zombie NIR™	IL-13R α 1 & Zombie NIR™
Receptors measured on IM9 Cells	CgC	CgC	IL-4R α , IL-13R α 1, CgC	none	none	none
Cytokine stimulation	none	5 ng/mL IL-4 & TNF α	20 ng/mL IL-4 & TNF α	none	5 ng/mL IL-4	5 ng/mL IL-4
Cell differentiation	undifferentiated	undifferentiated	undifferentiated & differentiated	undifferentiated	undifferentiated	undifferentiated
Procyandin exposure	no	no	no	no	yes	yes
Detachment	Trypsin & TrypLE™	TrypLE™	TrypLE™	TrypLE™	TrypLE™	TrypLE™
Fixation	no	no	no	yes	no	no
Use of comp beads	no	no	no	yes	no	no

8.4.3 Cytokine stimulation of A549 cells

Cytokine receptors can have limited baseline expression on the surface and may require their cytokine-ligand to be present in order to induce expression; thus, cytokine stimulation with IL-4 and TNF α was used to induce detectable levels of IL-4R α , TNFR1, and CCR3. The basic titration with cytokine stimulation (experiment 2, table 8-2) used A549 or IM9 cells set up as described above with the addition of 5 ng/mL IL-4 and 5 ng/mL TNF α in the cell media for 24 h prior to detachment. The 3rd experiment used an identical cell set-up with 20 ng/mL IL-4 and TNF α for 48 h prior to various detachment and fixation methods (Table 8-2).

8.4.4 Detachment methods

TrypLE™ and trypsin were two methods used to detach adherent A549 cells. It was standard practice by the Food and Wellness group to detach cells with trypsin. Cell culture media was removed, cells rinsed with PBS without Ca^{+2} Mg^{+2} , and then incubated with 0.5% trypsin at 37°C for 5-10 min. Trypsinized cells were diluted in media, spun down, supernatant removed, and resuspended in fresh media. TrypLE™ is a non-animal based milder version of trypsin which is promoted as being gentle on cell surface proteins. It was directly substituted for trypsin without any protocol changes.

8.4.5 Fixation of A549 cells

Detachment of A549 cells from cell culture had the possibility of disrupting cell surface proteins. In an attempt to avoid this and improve receptor detection, A549 cells were fixed as a monolayer before flow cytometry. Cells were fixed through exposure to 0.5% formaldehyde for 5 min using a slow drip technique. They were then either antibody stained in well and then detached into single cell suspension, or detached first and then stained in a flow cytometry tube.

8.4.6 Antibody staining

For antibody staining, cells were washed in cell staining buffer (CSB) (BioLegend), spun, supernatant removed, and resuspended in antibody/ blocking co-incubation solution for 30 min at 4°C. The antibody/blocking solution was an appropriate dilution of antibodies of interest (Table 8-2) in CSB with 1:100 TruStain FcX Fc receptor blocking solution or unstained with blocking solution alone. Cells were then spun, washed with CSB, resuspended in fresh CSB and stored at 4°C until analysis by flow cytometry. Fluorescence of cell preparations was measured with a BD FACSVerser™ equipped with violet (405 nm), blue (488 nm), and red (640 nm) lasers.

Data were analysed using FlowJo V10 software (FlowJo, LLC, Ashland, OR, USA). Specifics of using the FACSVerse™, antibody panel design, gating, and data analysis are described in detail in materials and methods sections 4.4.6 and 4.4.7.

8.4.7 Compensation beads for multicolour staining

Using multiple antibodies to stain a single sample is the basis of multicolour flow cytometry and was used in experiment 4 (Table 8-2). During multicolour flow cytometry, it is possible that a specific fluorophore's emission light will be detected in an off-target channel; this concept is referred to as 'spill-over'. The spill over can be controlled for with the use of commercially purchased compensation beads like BD anti-mouse Ig, κ / Negative control compensation particles set. This product is two distinct populations of polystyrene microparticles, one which will bind any mouse κ light chain-bearing antibody and a second population which has no binding capacity. The ability of the antibodies to bind the immunoglobulin κ chain as well as fluorescence spill-over can be determined with the use of compensation beads. The BD compensation beads were used according to the manufacturer's directions.

8.4.8 Fluorescence microscopy

Fluorescence microscopy was used to visually inspect the antibody staining efficiency for the IL-4R α , IL-13R α 1, and TNF R1 cytokine receptors. Experiments used A549 cells seeded at 3×10^5 per well in 6-well plates with a 18 x 18 mm ½ oz cover slip in the bottom of the well. Cells were grown to form a monolayer (80%) and then the media was replaced with either fresh serum media or serum-free media for an additional 24 h. Cells were washed in PBS (3x), and then fixed in ice-cold methanol (100%) for 15 min. Cells were washed in PBS (3x), and then permeabilized with 0.1% Triton X-100 for 10 min. A permeabilization step was included in an attempt to visualize receptors of

interest within the cell in addition to the cell surface. Cells were washed (3x) again in PBS and blocked with 5% BSA for 30 min. The coverslips were then removed from the wells, excess buffer wicked away, and stained with antibodies diluted in CSB to appropriate concentration for 1 h on ice. The antibody dilution for IL-4R α , IL-13R α 1, and TNF R1 were 1:20 (1.25 μ g/mL), 1:200 (1 μ g/mL), and 1:100 (1 μ g/mL) respectively. Coverslips were washed (3x) in PBS and mounted on slides using VectorShield hard set mounting media. Slides were then imaged on a Leica SP5 DM6000B scanning confocal microscope with the assistance of Manawatu Microscopy & Imaging Centre.

8.4.9 Modulation of receptor expression by procyanidin A2

Experiments investigating procyanidin effects on cytokine receptor expression used A549 cells seeded at 5×10^5 per well in 12-well plates, serum starved for 24 h, exposed to procyanidin A2 (5 μ M) or DMSO control for 2 h (see procyanidin A2 inhibition time course chapter 7), and then stimulated with 5 ng/mL IL-4 for 24 h or left unstimulated. Cells were detached with TrypLE™ and stained (1:50) with a co-stain of the Zombie NIR™ viability kit and IL-13R α 1 Cells were analysed by flow cytometry using a BD FACSVerser™.

8.4.10 Statistics

Statistical analysis of data was performed using SigmaPlot 12.5 (Systat Software Inc., London, UK). The median fluorescence intensity values for the effect of procyanidin A2 on IL-13R α 1 expression on A549 cells was analysed with a one-way ANOVA.

8.5 Results

8.5.1 Antibody titration

It is best practice when planning experiments with new antibodies to optimize the staining concentration through titrations on target cells. Receptor expression across cells and cell-lines can vary greatly; the titration allows for choosing a staining concentration with strong enough fluorescence to detect the receptor of interest while also mitigating against saturation which risks high levels of non-specific binding. Titrations also serve the dual function of promoting conservation of antibody reagents by not using more antibody than is necessary to sufficiently stain receptors of interest. Figure 8-2 shows the titration of IL-4R α (8-2A,F), TNF R1 (8-2B,G), IL-13R α 1 (8-2C,H), CCR3 (8-2D, I), and CgC (8-2E, J) antibodies at concentrations ranging from 1:20 –1:1600 on A549 cells detached with either TrypLE™ (8-2A,B,C,D,E) or Trypsin (8-2F,G,H,I,J)(Experiment 1, Table 8-2). The CgC should not be detectable on A549 cells, thus the IM9 human B lymphoblast cell line, which should express CgC, was used to titrate the CgC antibody as an addition in order to verify the staining protocol. Unfortunately, IL-4R α , TNF R1, and CCR3 were not detectable at any of the investigated antibody concentrations (Figure 8-2A, B, D, F, G, I) on A549 cells. The titration of IL-13R α 1 demonstrated 1:20, 1:50, and 1:100 antibody dilutions could detect receptor presence on A549 cells. The titrations were similar for cells removed with TrypLE™ and trypsin and demonstrate that IL-13R α 1 is likely not sensitive to cleavage during detachment. The negative control CgC was not detectable on A549 cells as expected, and was detected on IM9 cells (Figure 8-2E, J, K). Hoping to promote receptor expression of the three undetectable receptors (IL-4R α , TNF R1, and CCR3), a second titration was performed for all receptors of interest following cytokine stimulation of A549 cells with 5 ng/mL IL-4 and 5 ng/mL TNF α (Experiment 2, Table

8-2). Figure 8-3 shows titrations of IL-4R α (8-3A), TNF R1 (8-3B), IL-13R α 1 (8-3C), CCR3 (8-3D), and CgC (8-3E) antibodies at dilutions ranging from 1:20 –1:1600 on A549 cells detached with TrypLE™. A titration of CgC on IM9 cells was included for comparison (8-3F). The second titration was consistent with the first in that IL-4R α 1, TNF R1, and CCR3 were not detected. Additionally, the negative control CgC was not detectable on A549 cells as expected, and was detected on IM9 cells (Figure 8-3E, F). IL-13R α 1 demonstrated a similar titration spread as the previous titration, with 1:20, 1:50, and 1:100 antibody dilutions demonstrating positive detection for IL-13R α 1. From these data 1:50 was determined to be an adequate staining concentration for IL-13R α 1 and was used for all other antibodies as well in the absence of an appropriate titration.

8.5.2 Protocol modifications to promote detection of IL-4R α , TNF R1, and CCR3

After two antibody titrations IL-4R α , TNF R1, and CCR3 were yet to be detectable with current protocols. Thus, A549 cells were cultured for 14 days, as opposed to the normal 5 days, to investigate the possibility that a more differentiated state may be required to allow for detection of IL-4R α , TNF R1, and CCR3 (Experiment 3, Table 8-2). Furthermore, A549 cells were also stimulated with an increased cytokine concentration (20 ng/mL IL-4 and 20 ng/mL TNF α for 48 h) because cytokine receptor expression can be ligand dependant and may not have reached the threshold for increased expression with 5 ng/mL IL-4 and TNF α . Figure 8-4 shows receptor expression for IL-4R α (8-4A), TNF R1 (8-4B), IL-13R α 1 (8-4C), CCR3 (8-4D) or CgC (8-4E) on cytokine stimulated undifferentiated and the potentially more differentiated A549 cells. Increased cytokine stimulation and cell differentiation did not allow for detection of IL-4R α , TNF R1, or CCR3 (Figure 8-4A, B, D). Interestingly, the undifferentiated A549 cell samples used in this experiment demonstrated a new IL-

13R α 1 high population that was not seen previously, which was absent when the cells were cultured for 14 days (Figure 8-4C).

8.5.3 Investigating effects of detachment solutions on receptor expression of IM9 cells

It was demonstrated that IL-13R α 1 receptor expression on A549 cells was not sensitive to TrypLE™ or trypsin detachment methods. However, it was still possible that the undetectable receptors (IL-4R α , TNF1, and CCR3) were being affected by detachment solutions. IM9 cells were used to investigate the effect of detachment solutions on IL-4R α , IL-13R α 1, and CgC. Figure 8-5 shows IL-4R α , IL-13R α 1, and CgC receptor expression on IM9 cells stimulated with 20 ng/mL IL-4 and 20 ng/mL TNF α and then exposed to trypsin, TrypLE™, or EDTA solutions equivalent to those used to detach the adhered A549 cells. IL-4R α was not detectable on IM9 cells (Figure 8-5A). IL-13R α 1 had no change in expression due to detachment solutions which was consistent with A549 cells (Figure 8-5B). CgC expression was reduced by IL-4 and TNF α stimulation of IM9 cells, though exposure to the detachment solutions did not have any further reduction on CgC expression (Figure 8-5C).

8.5.4 Effect of fixation on cytokine receptor expression on A549 cells

In a final attempt to detect the IL-4R α , TNF R1, and CCR3 receptors on A549 cells, fixation of the cell monolayer was included into the sample preparation protocol. Cells were fixed with 0.5% formaldehyde for 5 min then either detached with TrypLE™ followed by antibody staining, or antibody stained in well and then detached with TrypLE™ (Experiment 4, Table 8-2). Neither fixation nor antibody staining prior to detachment allowed for the detection of IL-4R α , TNF R1, and CCR3 receptors on A549 cells (Figure 8-6). The cell samples in experiment 4 were multicolour stained; thus, compensation beads were used during this experiment. The compensation bead data

demonstrated that the IL-4R α , IL-13R α 1, TNF R1, CCR3, and CgC antibodies were all capable of binding the beads (Figure 8-7, red line). It was also demonstrated that the PE fluorophore spills into the PerCP channel, the APC fluorophore spills into the PerCP channel, and the Alexa Fluor™ 488 fluorophore spills into the PE channel (Figure 8-7, blue circles). The spill over level are adjusted for by the BD FACSVerser™ machine's software.

8.5.5 Investigating IL-4R α and TNF R1 antibody staining by fluorescence microscopy

The inability to detect IL-4R α , TNF R1, or CCR3 prompted the use of fluorescence microscopy to determine if I could visualize cytokine staining. Figure 8-8 shows A549 cells stained with IL-4R α , IL-13R α 1, and TNF R antibodies under serum or serum free conditions. Unfortunately, fluorescent microscopy did not enable the visual detection of receptor staining. DAPI staining of DNA included in the mounting media was visible in the nucleus of pictured A459 cells (Figure 8-8). It was a concern that IL-13R α 1 was not visually confirmed, as this receptor had titrated well and been consistently detected by flow cytometry.

8.5.6 Procyanidin A2 effect on IL-13R α 1 expression on A549 cells

The effects of procyanidin A2 exposure on cytokine receptor expression was explored for the one receptor detectable on A549 cells, IL-13R α 1 (Experiment 5 & 6, Table 8-2). A549 cells were exposed to procyanidin A2 (5 μ M) or DMSO control for 2 h and then stimulated with 5 ng/mL IL-4 for 24 h to remain consistent with previous work or left unstimulated. Cells were detached with TrypLE™ and stained (1:50) with a co-stain of the Zombie NIR™ viability kit and IL-13R α 1. The Zombie NIR™ viability kit allowed for the gating of live cells (Figure 8-9). In the representative sample, 94% of cells were gated as living. Exposure to procyanidin A2 had no measurable effect on

basal or cytokine-stimulated IL-13R α 1 expression on A549 cells (Figure 8-10A, B). The median fluorescence intensity values for unstimulated basal cells, DMSO exposed, and procyanidin A2 exposed cells were 214 ± 13 , 201 ± 15 , and 216 ± 12 , respectively ($p = 0.714$). The median fluorescence intensity values for basal cells, IL-4 stimulated, stimulated DMSO exposed, and stimulated procyanidin A2 exposed were 219 ± 14 , 234 ± 12 , 217 ± 11 , and 213 ± 14 , respectively ($p = 0.624$).

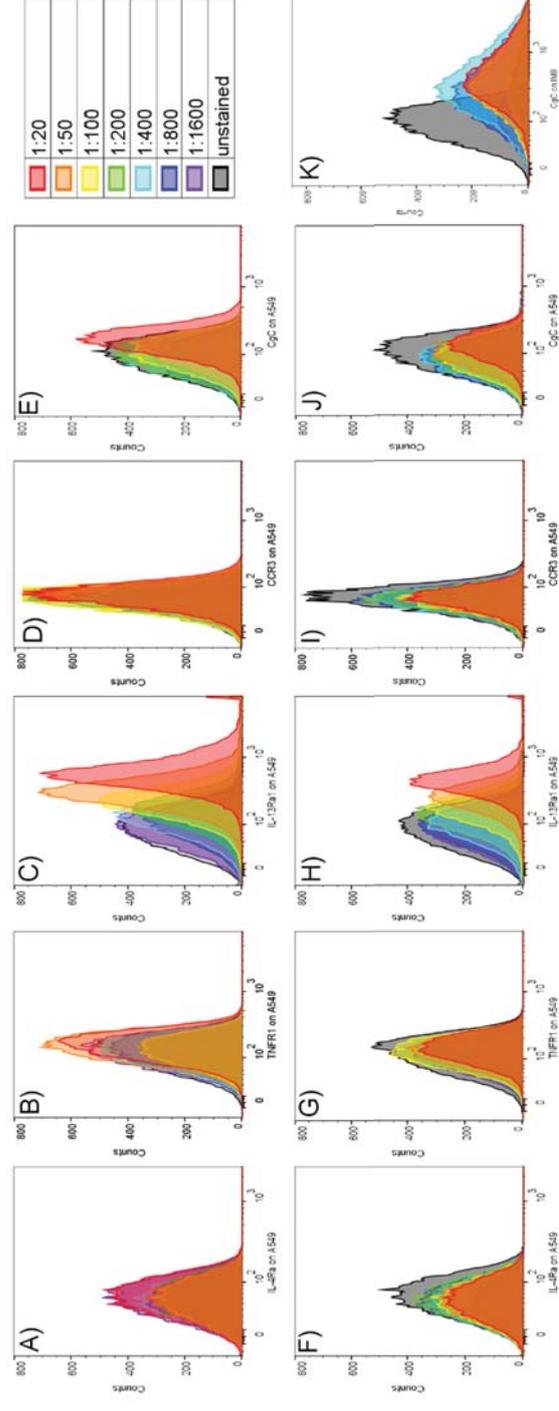


Figure 8-2: Titration for IL-4R α , TNF R1, IL-13R α 1, CCR3, and CgC antibodies on A549 and IM9 cells

A549 (A-J) or IM9 (K) cells were cultured in T75 flasks and detached using either TrypLE™ (A,B,C,D,E) or Trypsin (F,G,H,I,J). Cells were counted, aliquoted into a 96-well plate at 5×10^5 in 200 μ L, and stained with single antibodies for IL-4R α (A,F), TNF R1 (B,G), IL-13R α 1 (C,H), CCR3 (D, I) or CgC (E, J, K) as described in the materials and methods section at concentrations of 1:20 (red), 1:50 (orange), 1:100 (yellow), 1:200 (green), 1:400 (light blue), 1:800 (dark blue), 1:1600 (purple), or left unstained (black). Cells were analysed by flow cytometry using a BD FACSVerser™. Results are one sample per dilution from one experiment.

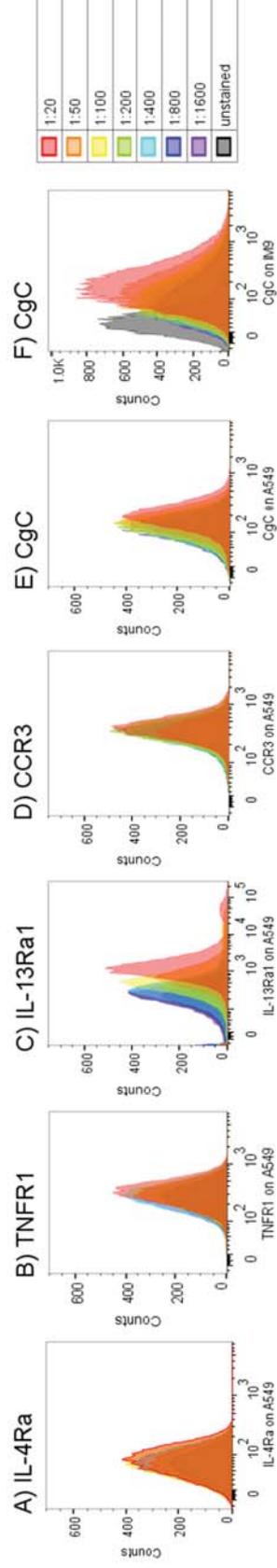


Figure 8-3: Titration for IL-4R α , TNF α , IL-13R α , CCR3, and CgC antibodies on A549 and IM9 cells following cytokine stimulation

A549 (A-E) or IM9 (F) cells were cultured in T75 flasks and stimulated with 5 ng/mL IL-4 and 5 ng/mL TNF α for 24 h before being detached using TrypLE™. Cells were counted, aliquoted into a 96-well plate at 5×10^5 in 200 μ L, and stained with single antibodies for IL-4R α (A), TNFR1 (B), IL-13R α 1 (C), CCR3 (D) or CgC (E, F) as described in the materials and methods section at concentrations of 1:20 (red), 1:50 (orange), 1:100 (yellow), 1:200 (green), 1:400 (light blue), 1:800 (dark blue), 1:1600 (purple), or left unstained (black). Cells were analysed by flow cytometry using a BD FACSVerser™. Results are one sample per dilution from one experiment.

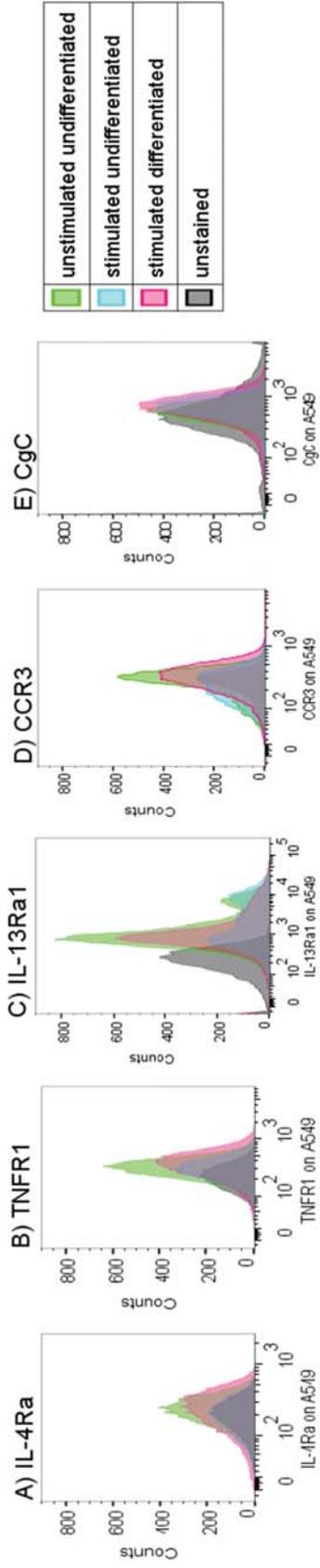


Figure 8-4: Stimulation and differentiation of A549 cells for improving cytokine receptor detection

Undifferentiated A549 (cultured 5 days) and differentiated (cultured 14 days) were prepared in T75 flasks and stimulated with 20 ng/mL IL-4 and 20 ng/mL TNF α for 48 h or left unstimulated before being detached using TrypLE™. Cells were counted, aliquoted into a 96-well plate at 5×10^5 in 200 μ L, and stained (1:50) with single antibodies for IL-4Ra (A), TNFR1 (B), IL-13Ra1 (C), CCR3 (D) or CgC (E) as described in the materials and methods section or left unstained (black). Cells were analysed by flow cytometry using a BD FACSVerser™. Each histogram compares unstimulated undifferentiated (green), stimulated undifferentiated (light blue), stimulated differentiated (pink), and unstained A549 cells (black). Results are one sample per condition from one experiment.

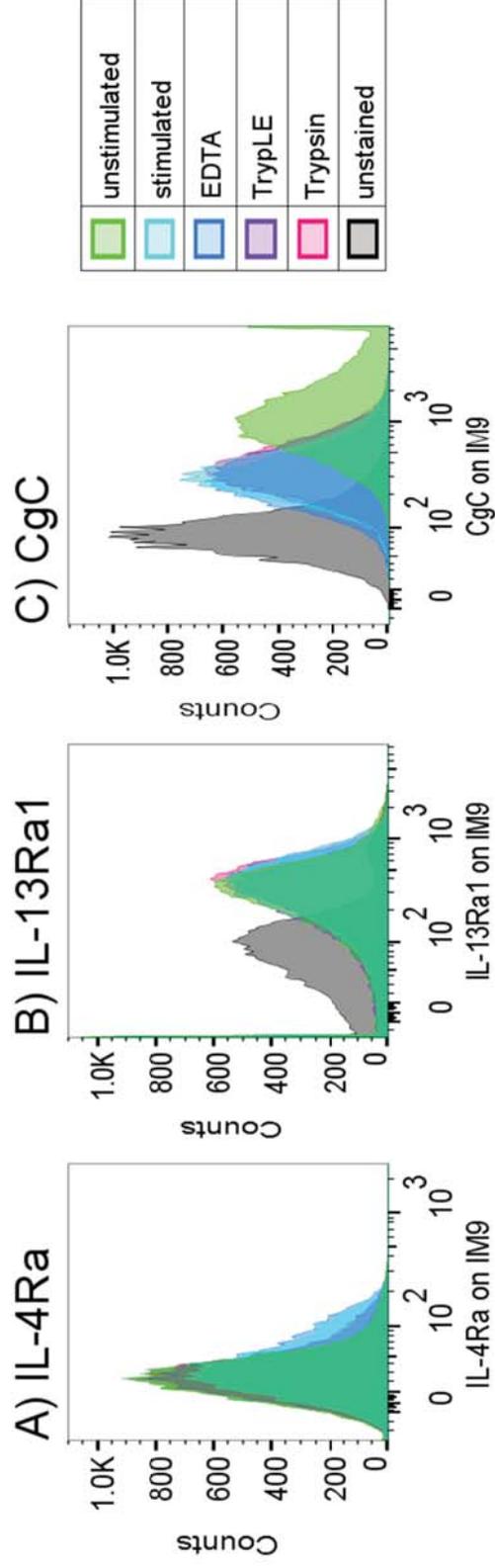


Figure 8-5: Investigation of Trypsin, TrypLE™, and EDTA effect on cytokine receptor expression on IM9 cells

IM9 cells were cultured in suspension and stimulated with 20 ng/mL IL-4 and 20 ng/mL TNF α or left unstimulated (green) for 48 h. A portion of the stimulated (light blue) cells were spun down and resuspended in one of three solutions containing equivalent EDTA (blue), TrypLE™ (purple), or Trypsin (pink) concentrations used for detachment of adherent cells. Cells were counted, aliquoted into a 96-well plate at 5×10^5 in 200 μ L, and stained (1:50) with single antibodies for IL-4R α (A), IL-13R α 1 (B), or CgC (C) as described in the materials and methods section or left unstained (black). Cells were analysed by flow cytometry using a BD FACSVerser™. Results are one sample per condition from one experiment.

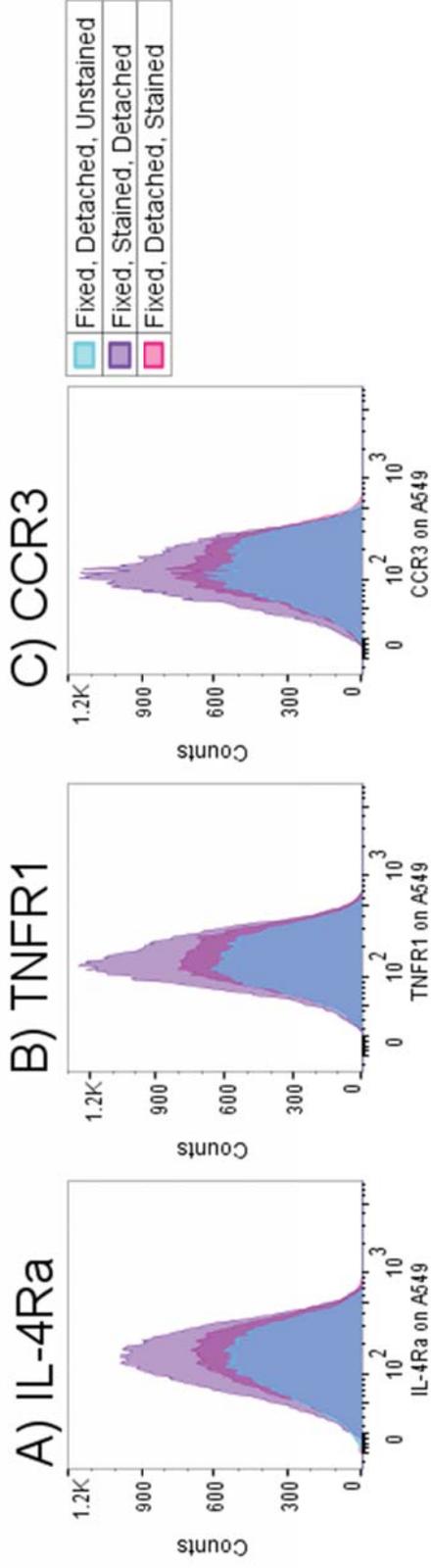


Figure 8-6: Protocol modifications for detecting IL-4Ra, TNF R1, and CCR3 on A549 cells

A549 cells were seeded at 5×10^5 in 12-well plates overnight. Cells were fixed with 0.5% formaldehyde for 5 min using a slow drip technique and then either detached with TrypLE™ followed by antibody staining (1:50) as described in the materials and methods (pink) or antibody stained in well and then detached (purple). Control cells were detached with TrypLE™ and left unstained (light blue). Cells were analysed by flow cytometry using a BD FACSVerse™. Results are one sample per condition from one experiment.

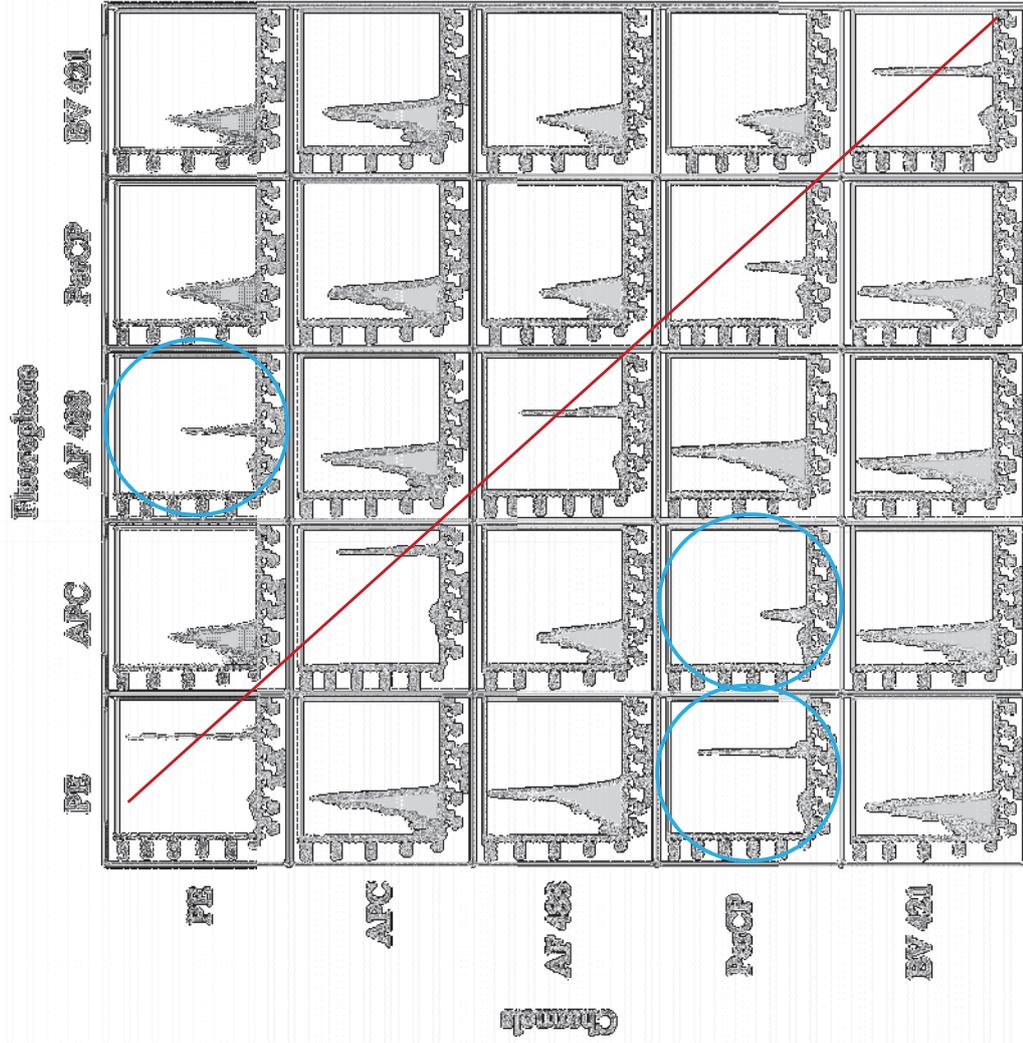


Figure 8-7: Compensation beads used during multi-colour flow cytometry

Two drops of each the positive and negative beads were added to cell staining buffer along with one of the five antibodies of interest (1:50 antibody dilution) then analysed by flow cytometry using a BD FACSVerserTM. Histograms for each fluorophore in its target channel (red line) demonstrate that each antibody is capable of binding the beads because there are two distinct peaks. The ratio between the target channel and spill over (blue circles) into other channels determines a software-generated percentage values which is used to compensate for the spill over when analysing experimental samples. Abbreviates used: AF 488, Alexa Fluor[®] 488; BV 421, Brilliant VioletTM 421. Results are representative of all samples in experiment 4 (Table 8-2).

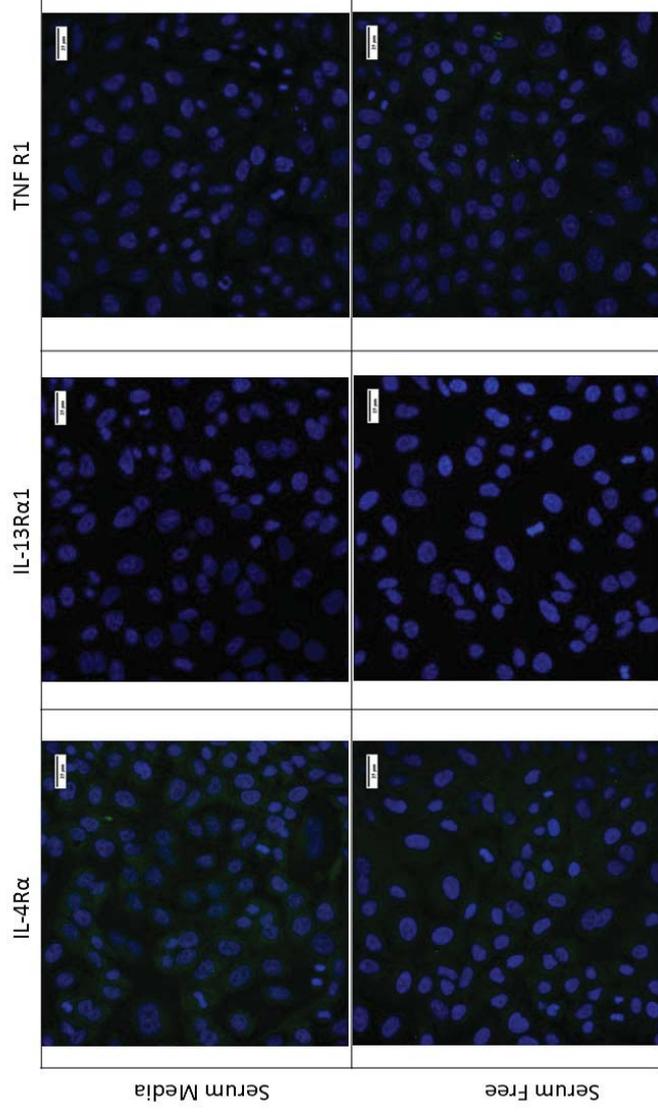


Figure 8-8: Fluorescent micrographs of A549 cells stained for cytokine receptors

A549 cells seeded at 3×10^5 per well in 6-well plates with a 18 x 18 mm ½ oz cover slip in the bottom of the well. Cells were grown to form a monolayer (80%) and then the media was replaced with either fresh serum media or serum-free media for an additional 24 h. Cells were stained with antibodies as described in the materials and methods. The antibody dilution for IL-4Rα, IL-13Rα1, and TNF R1 were 1:20 (1.25 µg/mL), 1:200 (1 µg/mL), and 1:100 (1 µg/mL) respectively. Coverslips were mounted on slides using VectorShield hard set mounting media. Slides were then imaged on a Leica SP5 DM6000B scanning confocal microscope with the assistance of Manawatu Microscopy & Imaging Centre. Results are one sample per condition from one experiment.

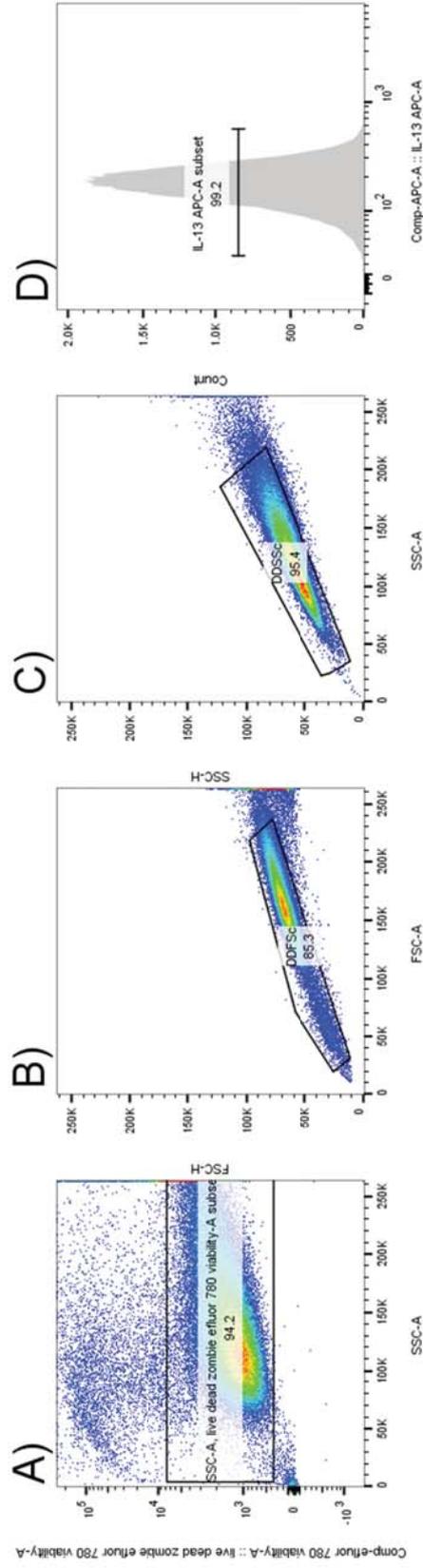


Figure 8-9: Gating strategy for Zombie NIR™ viability co-stained with IL-13R α 1 on A549 cells

A549 cells were seeded at 5×10^5 per well in 12-well plates, serum starved for 24 h, and then exposed to DMSO or procyanidin A2 (5 μ M) for 2 h. Cells were detached with TrypLE™ and stained (1:50) with a co-stain of the Zombie NIR™ viability kit and IL-13R α 1 as described in the materials and methods. Cells were analysed by flow cytometry using a BD FACSVerser™. Samples were gated inclusively for living cells using the fluorescence of the Zombie NIR™ and side scatter (A), living cells were gated for exclusion of doublets with forward scatter (B) and side scatter (C) and then gated for inclusion of IL-13R α 1 fluorescence (D). Results are representative of samples in experiment 5 & 6 (Table 8-2).

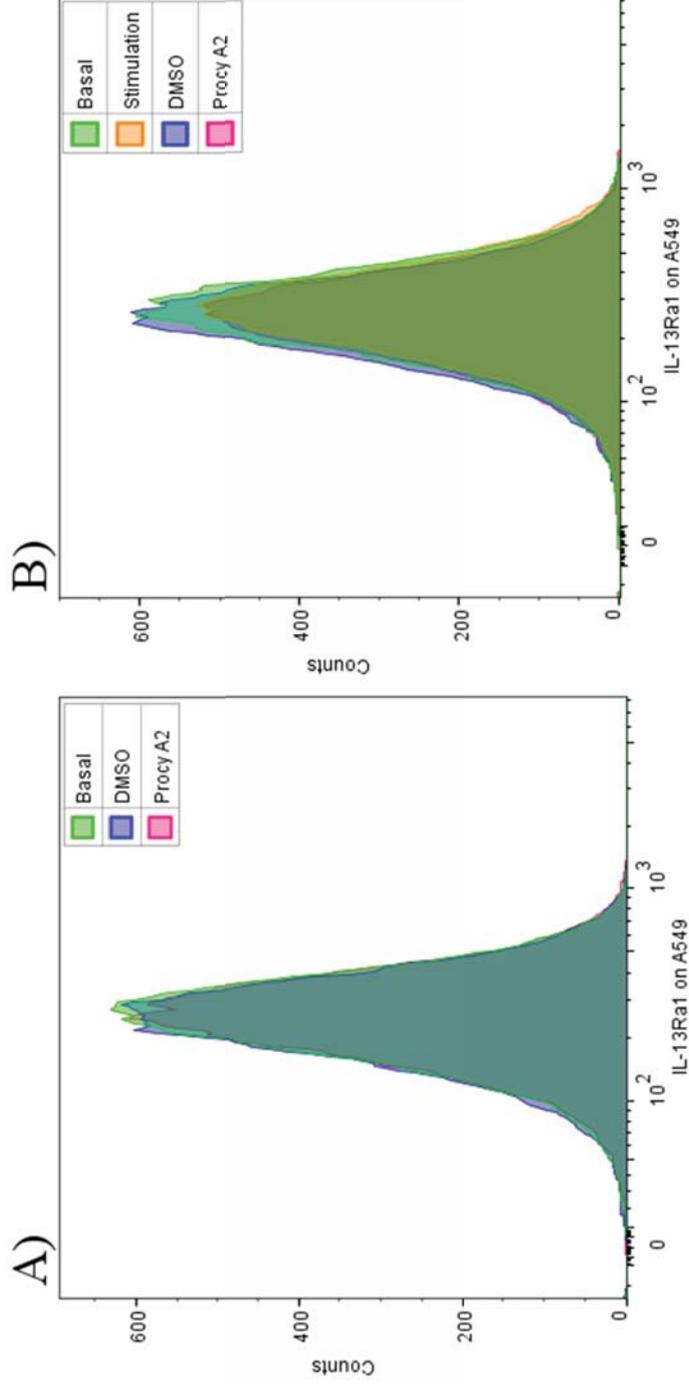


Figure 8-10: Procyanidin A2 effect on the expression of IL-13R α 1 on A549 cells

A549 cells were seeded at 5×10^5 per well in 12-well plates, serum starved for 24 h, exposed to procyanidin A2 (procy A2; 5 μ M) or DMSO control for 2 h, and then stimulated with 5 ng/mL IL-4 or left unstimulated. Cells were detached with TrypLE™ and stained (1:50) with a co-stain of the Zombie NIR™ viability kit and IL-13R α 1 as described in the materials and methods. Cells were analysed by flow cytometry using a BD FACSVerser™. A) A representative histogram of basal (green), DMSO exposed (blue), or procy A2 exposed (pink) IL-13R α 1 expression on unstimulated A549 cells. B) A representative histogram of basal (green), stimulated (orange), DMSO exposed (blue), or procy A2 exposed (pink) IL-13R α 1 expression on unstimulated A549 cells. Histograms are representative of average median fluorescence intensity values from 2 separate experiments and 6 individual measurements.

8.6 Discussion

With this study, I investigated the hypothesis that procyanidin A2 inhibited cytokine-induced CCL11 and CCL26 production in A549 cells through modulation of cytokine receptor expression at the cell surface using flow cytometry. The data generated provided did not support this hypothesis. I sought to measure expression of IL-4R α , TNF R1, IL-13R α 1, and CCR3 on the surface of A549 cells. The IL-13R α 1 subunit was the sole detected receptor on A549 cells and no change in basal or cytokine-induced expression was measured following a 2 h exposure to procyanidin A2 (5 μ M) compared with DMSO controls (Figure 8-10). Variations in the cell preparation protocol such as cell stimulation (Figure 8-3), cell differentiation stage (Figure 8-4), or use of fixation (Figure 8-6) did not allow for the detection of IL-4R α , TNF R1, or CCR3. Positive detection of IL-13R α 1 on A549 cells, as well as CgC and IL-13R α 1 on the IM9 cell line confirmed that the antibody staining protocol was appropriate, and was unlikely to be the source of technical difficulties in detecting IL-4R α , TNF R1, and CCR3 on A549 cells.

It was reported in the literature that A549 cells express the IL-4R α and IL-13R α 1 subunits, and do not express CgC or IL-13R α 2 through the use of flow cytometry [253]. Comparatively, A549 cells have more IL-13R α 1 molecules per cell than IL-4R α molecules, with the IL-4R α fluorescence peak barely detectable on the flow cytometry histogram [253]. Binding experiments have demonstrated IL-4R α and IL-13R α 1 expression levels can vary greatly depending on the cell type. Renal cell cancer lines were measured to express as much as 150,000 IL-13 binding sites per cell; whereas, multiple T-cell lymphoma cell-lines and the B-cell lymphoma cell-line Raji had undetectable levels of IL-13 binding sites [254]. Other epithelial cell lines, Calu-6 (lung) and HeLa (cervix) express approximately 400-500 molecules/cell of IL-4R α ,

while Raji has reported expression of 2,200 molecules per cell of IL-4R α [255]. The combination of a lower concentration of IL-4R α receptors to detect on A549 cells and the IL-4R α antibody being labelled with the least bright fluorophore (PerCP) in the panel could have contributed to the trouble detecting it; whereas, the IL-13R α 1 subunit was greater in concentration and labelled with APC one of the brightest fluorophores. Furthermore, both type I and II IL-4 receptors were demonstrated to have ineffective and short-lived dimerization in the plasma membrane [256]. A constitutive internalization mechanism was proposed as compensation for the weak affinities of the type II receptor subunits [256,257]. The IL-4R α subunit can accumulate in small vesicles stably positioned close to the plasma membrane which would make detection from extracellular antibody staining difficult. It is possible that the inclusion of a permeabilization step for flow cytometry samples would have allowed for the detection of the IL-4R α subunit, but this would have no longer distinguished between intra- and extracellular localization of the receptor. Furthermore, permeabilization was included during confocal fluorescence microscopy and did not allow for visual confirmation of antibody staining (Figure 8-8). IL-4R α expression on primary epithelial cells was demonstrated to increase with long-term air-liquid interface culturing [258]. A variation in culture conditions to a 14-day culture (potentially more differentiation) in submersion here did not allow for detectable levels of IL-4R α (Figure 8-4). Additionally, I could have investigated IL-4R α expression by attempting to use IL-13 to induce expression, however, remaining consistent with previous work in this project (stimulation with IL-4) was preferred.

The data presented here investigating IL-13R α 1 in both basal and cytokine-induced conditions did not support the hypothesis that procyanidin A2 affects receptor expression on A549 cells. Despite this, the possibility remains that procyanidin A2 could affect

inhibition of cytokine-induced CCL11 and CCL26 through indirect effects on cytokine receptors, as cytokine signalling is suggested to operate on a spectrum, and not as distinctly on or off [259]. The structure of the dimeric receptor complex [260] as well as the interplay between antigen and receptor can affect the potency of downstream signalling [261].

With respect to TNF α signalling, two distinct TNF receptors have been identified, a 55 kDa TNF R1 and a 75 kDa TNF RII. Both are single transmembrane glycoproteins that through ligand binding homotrimerize in the plasma membrane. They differ by the presence of an intracellular death domain on TNF R1, which is able to drive either apoptosis or inflammation [262]. A549 cells are reported in the literature to express the TNF R1 receptor but not TNF RII [263]. Expression of TNF R1 ranges from 4,000 – 130,000 TNF α binding sites [212,263]. Following binding of TNF α to TNF R1, the receptor portion that extends into the extracellular space, the ectodomain, is shed and signalling pathways progress internally [262]. Constitutive proteolytic cleavage of the ectodomain that generates soluble TNF R1 (sTNF R1) could be responsible for the struggles here in identifying TNF R1 at the cell surface with flow cytometry. Indeed, sTNF R1 has been measured in the culture media of A549 cells [264].

The CCR3 receptor is expressed on eosinophils, basophils, mast cells, dendritic cells, T helper cells, and airway epithelial cells [249]. Specifically, it is reported to be present on the A549 cell-line [249,265,266]. It responds to CCL11 and CCL26 as well as to other chemokines such as monocyte-chemotactic protein 3 (CCL7), monocyte-chemotactic protein 4 (CCL13), and RANTES (CCL5) [267]. Bronchial expression of CCR3 is associated with asthma disease severity in atopic asthmatics [250,268]. Furthermore, plasma concentrations of CCL5 and Th2 cell expression of CCR3 are

elevated in asthmatic patients [251]. The dynamic of CCL26 with CCR3 in airway epithelial cells is particularly intriguing. Both ligand and receptor are produced on the same cell and allow for CCL26 auto-regulation of its own receptor CCR3 on A549 cells [266]. Interestingly, eosinophils exposed to supernatant of A549 cells which were subject to short-interfering RNAs (siRNA) for either CCL26 or CCR3 had reduced reactive oxygen species release [249,265]. This relationship between CCL26 and CCR3 could have potentially lead to additional experiments involving epithelial cells co-cultured with immune cells, which was the original intent for investigating CCR3 on A549 cells. It is unfortunate detection of CCR3 was not successful, it may have been improved by using a higher concentration of IL-4 (i.e. 100 ng/mL) to stimulate the cell as this was reported in the literature [249,265].

The use of flow cytometry further in this project could have assisted the progression into complementary areas of immunology. Polyphenolic-induced modulation of cell plasma membrane proteins has been reported in the literature, specifically with dendritic cells (DC) and this could have been an area of investigation. Quercetin, curcumin, ellagic acid, and silibinin (a flavonoid found in milk thistle—*silybum marianum*), were all demonstrated to suppress co-stimulatory molecules CD80 and CD86 expression on DCs [269-272]. CD80 and CD86 are necessary for T cell activation and survival. The reduction of these co-stimulatory molecules subsequently reduced DC maturation and possibility T cell activation. It has been suggested that polyphenol exposure can drive DCs toward an anti-inflammatory profile [273]. There was previous experience in the Food and Wellness group with using blood samples to isolate monocytes and a protocol for inducing them to differentiate into DCs. Unfortunately, due to limited access to the FACSVerse™ flow cytometer, the opportunity of undertaking research using monocyte-derived DCs wasn't possible and I

progressed this thesis work by investigating possible cellular mechanisms by which procyanidin A2 inhibits cytokine-induced CCL11 and CCL26.

Chapter Nine

Evaluating procyanidin–induced
alterations in membrane fluidity

9.1 Evaluating procyanidin–induced alterations in membrane fluidity

Contribution Declaration

This thesis chapter is not published elsewhere.

The content of this chapter was written by Sara Coleman (100%).

9.2 Abstract

BACKGROUND: Procyanidin A2 has newly demonstrated biological activity as an inhibitor of cytokine-induced eotaxin production in A549 cells. The molecular mechanism responsible for this inhibition is unclear. Flavonoids and A-type proanthocyanidins are indicated as eliciting biological activity through interactions with the cytoplasmic membrane.

OBJECTIVE: Here, I evaluated alterations in membrane fluidity as one potential mechanism by which procyanidin A2 inhibits cytokine-induced CCL11 and CCL26 in human lung epithelial cells.

METHODS: I used A549 cells seeded into 12-well plates and optimized a membrane fluidity bioassay using the fluorescent, amphiphilic, molecular probe 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO). Cells were exposed to procyanidins for 1 h prior to incubation with 20 μ M DiO at 37°C for 1 h. Following washing with PBS, fluorescence intensity (ex 485 nm/ em 520 nm) was measured in a BMG POLARStar Omega plate reader.

RESULTS: A549 cells exposed to procyanidins had no detectable difference in fluorescence intensity compared to DMSO. Procyanidin A2 (10 μ M) trended toward reduced fluorescence intensity (a more rigid membrane). Unfortunately robust methods for the evaluation of membrane fluidity were not achieved in this study. As a consequence good experimental control data were lacking and data on effects of procyanidins were weak and not able to be substantiated. Despite this the data are shown here for completeness and in the context of a body of work for a PhD thesis.

CONCLUSIONS: These data provide no evidence to support the hypothesis that alterations in membrane fluidity could be one possible mechanism by which procyanidin A2 is capable of inhibiting cytokine-induced CCL11 and CCL26 production in A549 cells. More validation of the DiO assay and/or a complimentary method for measuring membrane fluidity would be required in order to draw stronger conclusions which time did not allow.

9.3 Introduction

Biological membranes are organized, pliable layers of lipids interspaced with proteins that form a boundary in an organism. They act as selective barriers which allow some substances to pass and restrict others. The outer boundary of a cell is the plasma membrane [274]. The fluid-mosaic model of membrane structure is the most prominent description for understanding the properties of cellular membranes [275]. This model's specifics include biological membranes as a dynamic two-dimensional liquid where nonpolar fatty acid chains of phospholipids are sequestered together away from water and polar heads are in contact with the exterior surface [276]. There have been revisions to this model. The plasma membrane bilayer is understood as asymmetric and proteins are no longer thought of as having ubiquitous lateral movement, as some proteins can be confined to specific regions of the plasma membrane [277]. Additionally, the actin filament membrane, concentrated raft domains, and extracellular dynamic protein complexes are all suggested to have important roles in the function of the plasma membrane [278].

Flavonoids are a class of polyphenolic secondary plant metabolites that are accepted as biologically active in the context of human health. Mechanisms of action for flavonoid biological activity include capacity as an antioxidant, controlling signal transduction pathways, and interaction with biological membranes [279]. Specifically, flavonoid interactions with lipid bilayers of the cell membrane are primarily dictated by polyphenol polarity and structure, which has been shown to impact biological activity [236,280]. Flavonoids at concentrations ranging from 1- 10 μM have been demonstrated to decrease the membrane fluidity of synthetic membranes [238].

Previous research investigating the mechanism of action for a subclass of flavonoids, A-type procyanidins, has focused on their anti-bacterial properties in relation to urinary tract infections [281]. Data presented within this thesis have demonstrated A-type procyanidins, specifically procyanidin A2, are capable of inhibiting *in vitro* production of two biomarkers, CCL11 and CCL26, relevant to the inappropriate inflammation present in allergic asthma, though the mechanism of action is still unclear [165,166]. The ability of A-type procyanidins to modulate membrane fluidity has been shown in 3T3-L1 adipocytes; however, no published study has utilized measurements of fluorescence intensity to investigate procyanidins in lung epithelial cells in relation to allergic asthma. In this study, I investigated dimeric procyanidin compounds procyanidin A2 and procyanidin B2 for *in vitro* efficacy of modulating membrane fluidity in A549 human alveolar epithelial cells.

For this work, I used the amphiphilic, fluorescent, molecular probe 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO). Many fluorescent molecular probes are commercially available and differ based on how they localize in biological membranes and the wavelengths of excitation and emission. DiO was suitable here because it is comprised of a charged fluorophore that localizes the probe at the cell membrane's surface as the lipophilic tails anchor the probe to the membrane (Figure 9 -1) and it has an emission wavelength that is compatible with our laboratory equipment. Thus, I measured fluorescence intensity from the DIO probe as a means to investigate membrane fluidity modulation following procyanidin exposure *in vitro*.

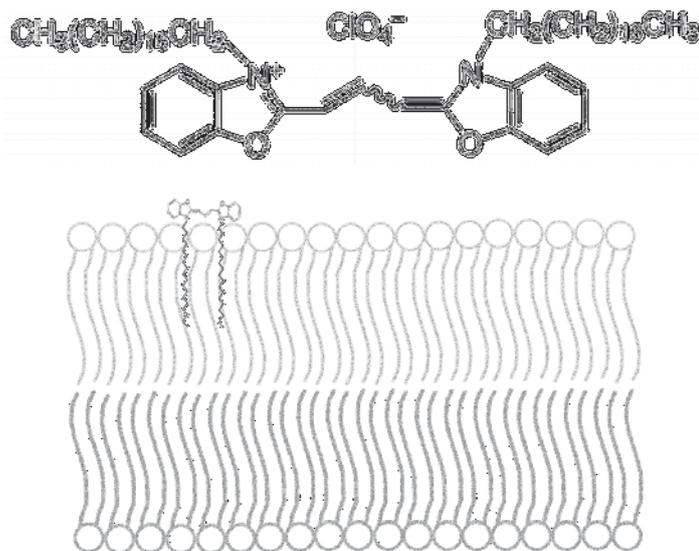


Figure 9-1: Schematic of DiO insertion into a phospholipid bilayer

9.4 Materials and methods

9.4.1 Materials

The human alveolar epithelial (A549) cell-line was purchased from the American Type Culture Collection (ATCC® CCL-185™ (c/o Cryosite, New South Wales, Australia)). Cell culture media, phosphate buffered saline (PBS), penicillin-streptomycin-neomycin antibiotic mixture, 100x L-glutamine, 2.5% trypsin, and the 3,3'-di(1-dodecyl-4-dimethylaminopropyl)indocarbocyanine perchlorate (DiO) molecular probe were purchased from Life Technologies/ Thermo Fisher Scientific (Auckland, New Zealand). Fetal bovine serum (FBS) was purchased from Moregate Biotech (Hamilton, New Zealand). Procyanidin A2 (HPLC $\geq 99\%$, epicatechin-(4 β -8, 2 β -O-7)-epicatechin) was purchased from Extrasynthese (Genay Cedex, France). Dimethyl sulfoxide (DMSO) and all other chemicals not specifically listed were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

9.4.2 Cell culture conditions

Cells were grown under standard tissue culture conditions of 37°C in a 95% humidified atmosphere at 5% CO₂ in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 2 mM L-glutamine, 50 μ g/mL penicillin, 50 μ g/mL streptomycin, 100 μ g/mL neomycin, and 10% FBS. The cell cultures were grown to form a monolayer (80% confluence) and then growth arrested for 24 h in the

absence of FBS before conducting experiments. Experiments used A549 cells of passage 92-100.

9.4.3 Developing conditions for using DiO molecular probe

The manufacturer's instructions state the DiO probe excites optimally at 484 nm and emits at 501 nm (Figure 9-2) [282]. Due to the narrow Stokes shift, preliminary experiments measured the fluorescence intensity of 1, 5, and 10 μM DiO using four excitation/emission wavelength combinations, 480/500, 480/520, 485/500, and 485/520 nm. Experiments used A549 cells seeded at 3×10^5 per well in 12-well plates with culture and growth arrest as described in 9.4.2. The DiO molecular probe was dissolved in DMSO to 1 mg/mL as directed by the manufacture and stored in aliquots shielded from light at room temperature until use. Prior to use, the stock solution was sonicated in a water bath for 30 min and diluted to desired concentration in PBS containing MgCl_2 and CaCl_2 . Cells were incubated with DiO for 1 h at 37°C , washed two times with PBS containing MgCl_2 and CaCl_2 , and fluorescence intensity measured using 15 point orbital averaging with a BMG POLARstar Omega plate reader. Instrumental gain was constant at 1500 logarithmic decibel units (dB). DiO probe saturation was investigated for a concentration range of 1 μM to 70 μM using an identical cell set up. Additionally, an incubation time course for 15 to 60 min was investigated using an identical cell set up.

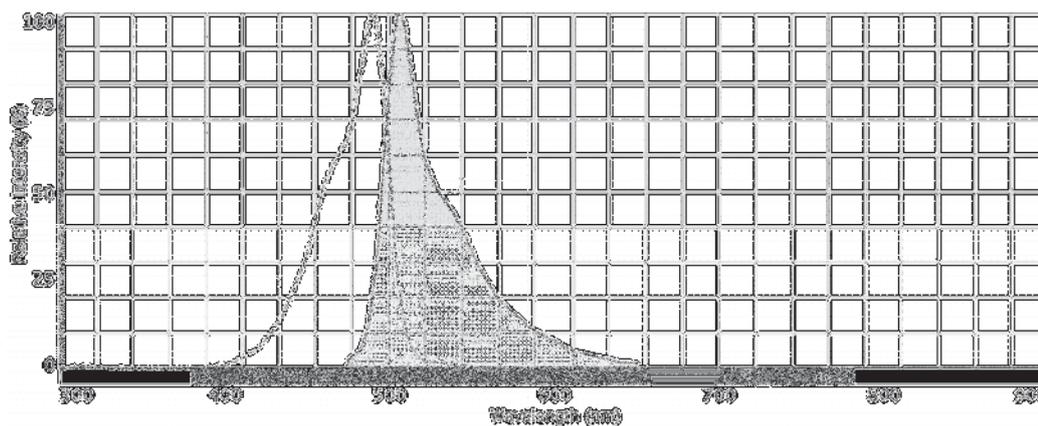


Figure 9-2: Fluorescence spectra for DiO probe

The dotted line represents the excitation spectra. The filled line represents the emissions spectra. Spectra image from:

<https://www.thermofisher.com/order/catalog/product/D275>

9.4.4 Membrane fluidity bioassay

The assay investigating modulation of membrane fluidity by procyanidins used A549 cells seeded at 2×10^4 per well in 96-well plates with culture and growth arrest as described in section 9.4.2. Cells were exposed to 1, 5, or 10 μM procyanidin A2 and B2 for 1 h in serum free media, washed 2x with PBS containing MgCl_2 and CaCl_2 , and then stained with 20 μM DiO for 1 h at 37°C. Preparation of procyanidins has been previously described in section 7.4.4. Experiments included polyphenols apigenin and phloretin (1 μM and 10 μM of each; Figure 9-3) as well as the unsaturated fatty acid eicosapentaenoic acid (EPA; 1 and 10 μM) as potential positive controls. Following DiO incubation, cells were washed two times and left in PBS containing MgCl_2 and CaCl_2 . Fluorescence intensity was measured with 485/ 520 nm excitation/ emission wavelengths in a BM POLARstar Omega Plate Reader. Instrumental gain was constant at 1500 logarithmic decibel units.

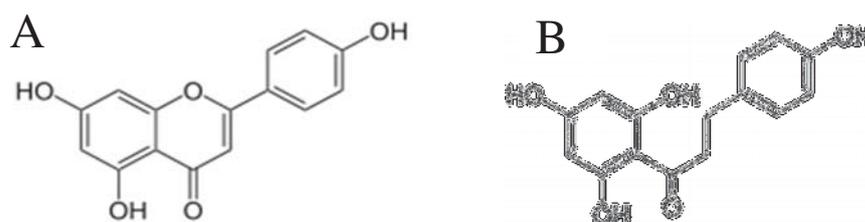


Figure 9-3: Apigenin (A) and phloretin (B) chemical structures

9.4.5 Statistics

Statistical analysis of data was performed using SigmaPlot 12.5 (Systat Software Inc., London, UK). The mean fluorescence intensity values of DiO incubated A549 cells following procyanidin exposure were analysed with a one-way ANOVA. Data that did not reach a $p < 0.05$ threshold were listed as not significant (NS).

9.5 Results

9.5.1 Optimal conditions for use of DiO molecular probe

The fluorescence intensity values for A549 cells stained with 1, 5, and 10 μM DiO then measured with four sets of wavelength combinations are displayed in Figure 9-4. Fluorescence values are highest with the 485-500 nm filter combination (baseline:

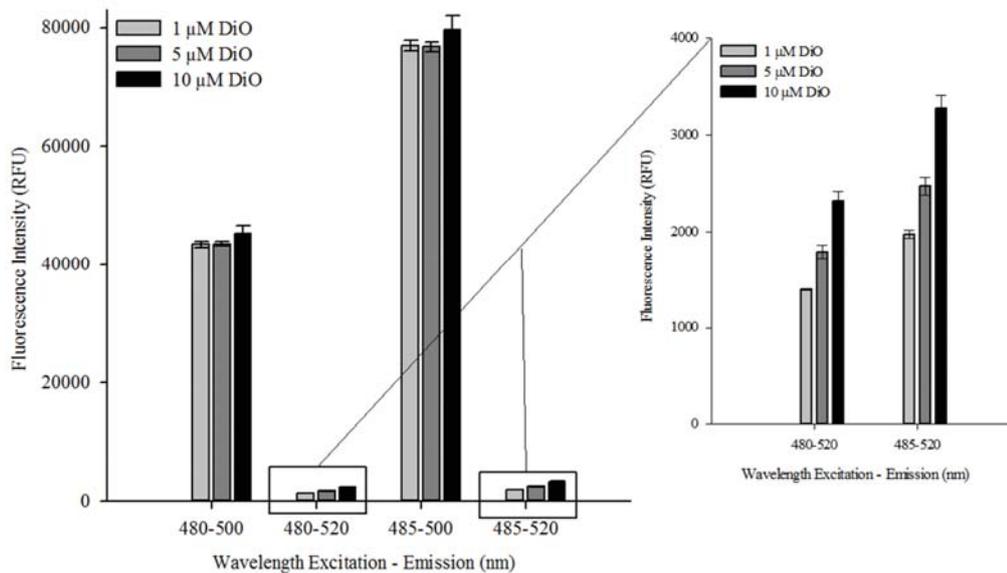


Figure 9-4: Excitation and emission wavelength optimization for DiO probe

A549 cells were seeded at 3×10^5 in 12-well plates, serum starved for 24 h, then stained with 1, 5, or 10 μM DiO for 1 h at 37°C. Fluorescence intensity was then measured at ex/em wavelengths of 480/500, 480/520, 485/500, and 485/520 nm using 15 point orbital averaging with a BMG POLARstar plate reader. The results are expressed as mean \pm SEM, which were from 1 experiment and 3 individual measurements.

78,856 \pm 2,408; 1 μM : 77,005 \pm 890; 5 μM : 76,803 \pm 815, 10 μM : 79,675 \pm 2428 RFU), which corresponds with spectrum peaks for the DiO probe given by the manufacturer (484 -501 nm). Measurements using the 480-500 nm filters are roughly half that of the 485-500 nm values (Baseline: 44,688 \pm 1,311; 1 μM : 43,483 \pm 507; 5 μM 43,618 \pm 377; 10 μM 45,271 \pm 1,341 RFU). Both combinations using the 500 nm emission filter display spectral spill-over as a result of the narrow Stokes shift and do not accurately reflect the DiO concentration gradient. The 480-520 nm and the 485-520 nm filter sets both produced much lower fluorescence intensity readings (480-520 nm baseline: 1,338 \pm 21; 1 μM : 1,397 \pm 11; 5 μM : 1,792 \pm 67, 10 μM : 2319 \pm 99 RFU; 485-520 nm baseline: 1,926 \pm 22; 1 μM : 1,975 \pm 42; 5 μM : 2,477 \pm 91, 10 μM : 3,273 \pm 142 RFU). The 520 nm emissions filter is a suboptimal wavelength in the fluorescence spectrum; however, it is far enough away from the excitation filters of 480 nm and 485 nm to no longer have spectral spill-over. The DiO probe concentration differences can be

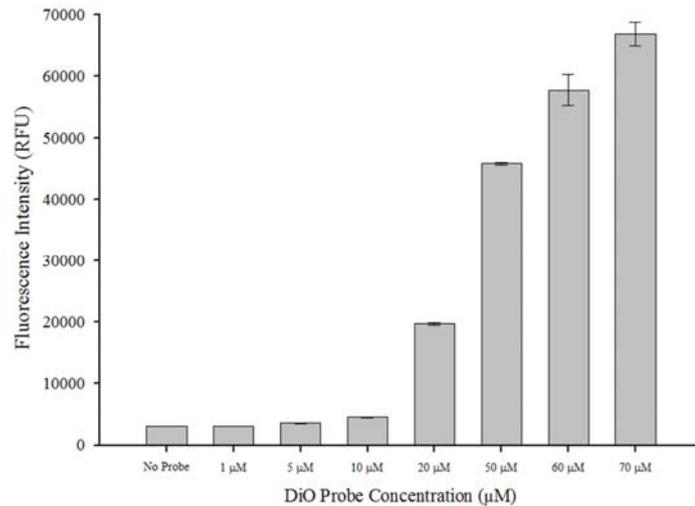


Figure 9-5: DiO molecular probe incubation concentration gradient

A549 cells were seeded at 3×10^5 in 12-well plates, serum starved for 24 h, and then stained with 1- 70 µM DiO for 1 h at 37°C. Fluorescence intensity was then measured at ex 485/em 520 nm using 15 point orbital averaging with a BMG POLARstar plate reader. The results are expressed as mean ± SEM, which were from 1 experiment and 3 individual measurements.

detected with the 520 nm emission filter. Thus, the 485-520 nm filter set was chosen as the preferred bioassay wavelengths.

The incubation concentration gradient using 1 – 70 µM DiO probe on A549 cells is displayed in Figure 9-5. A549 cells alone with no staining had fluorescence intensity at $3,054 \pm 31$ RFU. Staining using 1, 5, and 10 µM increased fluorescence intensity to $3,113 \pm 11$, $3,565 \pm 81$, $3,565 \pm 81$ and $4,564 \pm 69$ RFU respectively. A noticeable jump in fluorescence intensity was measured with 20 µM DiO ($19,758 \pm 199$ RFU). Fluorescence intensity then increased steadily for 50 µM, 60 µM, up to the final measured concentration of 70 µM (50 µM: $45,898 \pm 236$; 60 µM: $57,751 \pm 2558$; 70 µM: $66,821 \pm 1905$ RFU). From these data, 20 µM DiO was determined as an adequate concentration for use in the bioassay.

Optimal time was investigated for DiO incubation on A549 cells. A time course for 20 µM DiO can be seen in Figure 9-6. A blank well containing PBS was measured at 4,278 RFU, which represented the background fluorescence of PBS and the plastic (Figure 9-6). A well containing the 20 µM DiO solution but no cells had a fluorescence intensity of 19,170 RFU (Figure 9-6). This well was a cause for concern as the DiO probe should not fluoresce unless inserted into a cell membrane. Baseline fluorescence

of unexposed A549 cells was $3,058 \pm 25$ RFU. Fluorescence intensity for A549 cells exposed for 15, 30 or 60 min with $20 \mu\text{M}$ DiO were $6,924 \pm 172$, $10,627 \pm 119$, and $18,684 \pm 675$ RFU respectively. A 60 min incubation time showed 98% saturation compared with the $20 \mu\text{M}$ no cells well. From these data, 60 min was chosen as the optimal incubation time.

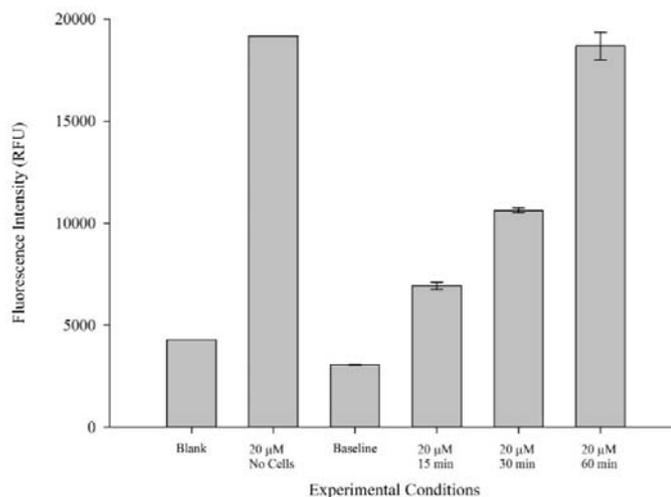


Figure 9-6: DiO molecular probe incubation time course

A549 cells were seeded at 3×10^5 in 12-well plates, and then stained with $20 \mu\text{M}$ DiO for 15-60 min at 37°C . Fluorescence intensity was then measured at ex 485/em 520 nm using 15 point orbital averaging with a BMG POLARstar plate reader. The results are expressed as mean \pm SEM, which were from 1 experiment and 3 individual measurements.

9.5.2 Membrane Fluidity

I used the DiO probe optimization data to create a bioassay to investigate membrane fluidity. As mentioned, the DiO probe is amphiphilic and is comprised of a charged fluorophore that localizes the probe at the cell membrane's surface as the lipophilic tails anchors the probe to the membrane. In theory, the more fluid the plasma membrane is, the greater ability for the probe to insert itself into the plasma membrane and thus great fluorescence intensity in the bioassay. Conversely, reduced fluorescence would equate with a more rigid membrane and less ability of the probe to insert into the membrane. I investigated changes in fluorescence intensity following a 1 h incubation with procyanidins. The 1 h time point was chosen due to previous data collected investigating time course of inhibition for procyanidin A2 on cytokine-induced CCL26 production (section 7.5.4). Experiments included polyphenols apigenin and phloretin as

positive controls that have been documented in the literature as modulating liposomal fluidity [283]. Furthermore, unsaturated fatty acid EPA was included as a positive control as incorporation of fatty acids into the plasma membrane is suggested to promote membrane fluidity [284]. The raw values for fluorescence intensity following procyanidin exposure can be seen in Figure 9-7. The membrane fluidity bioassay did not detect any difference in fluorescence intensity when comparing 1 and 10 μM EPA, apigenin, phloretin to DMSO, nor was there any difference detected in fluorescence intensity when comparing 1, 5, and 10 μM procyanidin A2, and procyanidin B2 to DMSO (bars left to right in Figure 9-6: 1 μM : EPA 18,313 \pm 6,986; apigenin 20,701 \pm 7,617; phloretin 19,792 \pm 7,388, DMSO 19,149 \pm 7,580; procy A2 21,833 \pm 7,807; procy B2 21,497 \pm 7,698; 5 μM : DMSO 19,947 \pm 7,659; procy A2 20,410 \pm 6,924; procy B2 20,492 \pm 7,419; 10 μM EPA 15,635 \pm 6,363; apigenin 18,839 \pm 7,097;

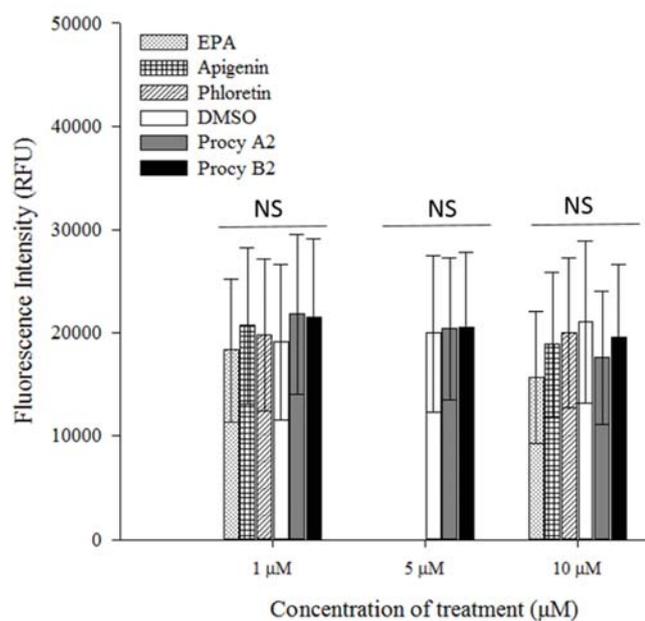


Figure 9-7: Membrane fluidity bioassay

A549 cells were seeded at 2×10^4 in 96-well plates, and then exposed to 1, 5, or 10 μM procyanidin (procy) A2 and B2, positive controls 1 and 10 μM EPA, apigenin, or phloretin, or DMSO for 1 h in serum free media. Fluorescence intensity was measured at ex 485/em 520 nm using 15 point orbital averaging with a BMG POLARstar plate reader. The results are expressed as mean \pm SEM, which were from 3 experiment and 9 individual measurements. Data that did not reach a $p < 0.05$ threshold were listed as not significant (NS).

phloretin $20,010 \pm 7,317$; DMSO $21,054 \pm 7,954$; procy A2 $17,629 \pm 6,490$; procy B2 $19,548 \pm 7,087$)

9.6 Discussion

Structurally-defined dimeric procyanidins were investigated within this doctoral project for their ability to modulate airway inflammation in relation to one specific endotype of eosinophilic allergic asthma. Procyanidin A2 was demonstrated to inhibit cytokine-induced eotaxin production in A549 cells. Here, I sought to investigate modulation of membrane fluidity as one mechanism by which procyanidin A2 could be effecting inhibition. Membrane fluidity is a characteristic of cellular structure that can be affected by exogenous compounds. The cell cytoplasmic membrane is composed of a lipid bilayer and associated embedded proteins; it is an intrinsic component for extracellular signals to be converted into intracellular cell signalling events [237]. The fluid mosaic model is the most widely accepted model for understanding biological membranes [275].

To investigate membrane fluidity as a possible mechanism, I developed a bioassay using the fluorescent amphiphilic molecular probe DiO, which can incorporate into the cytoplasmic membrane and was thought to correlate with the fluidity of the plasma membrane. The bioassay measured fluorescence intensity following exposure to various polyphenolic compounds to ascertain if polyphenolic exposure would affect DiO incorporation into the plasma membrane and thus affect membrane fluidity. The bioassay developed was lacking in robust methodological and experimental controls. The data collected did not reveal any difference in fluorescence intensity of procyanidin exposed A549 cells compared to DMSO exposed cells.

The DiO probe has been previously used by Zhu et al. to demonstrate that A-type proanthocyanidin compounds can inhibit differentiation in 3T3-L1 mouse adipocyte cell line through modulation of cytoplasmic membrane fluidity, hydrophobicity, and permeability [241]. The DiO probe was used in conjunction with fluorescent microscopy and here I attempted to expand DiO utility by measuring fluorescence with a BMG POLARstar Omega plate reader. However, there was an inconsistency during the preliminary development of the assay. The DiO molecular probe was described by the manufacturer as 'being weakly fluorescent in water but

highly fluorescent and quite photo-stable when incorporated into a membrane' [285]. Figure 9-6 shows that in the absence of A549 cells that 20 μ M DiO diluted in PBS containing $MgCl_2$ and $CaCl_2$ is fluorescent. The legitimacy of the assay was based on the assumption that the probe is incorporated into the cellular membrane. Attempts to contact the manufacture to resolve how the DiO probe transitioned from weakly fluorescent to highly fluorescent upon incorporation into a membrane did not clarify this anomaly as they could not provide details of the shift. To compensate, a repeated wash step was included to reduce non-specific binding of a perpetually fluorescent molecular probe.

The bioassay I developed included the use of potential positive controls EPA and two other plant-origin polyphenols, apigenin and phloretin. These compounds have demonstrated effects in modulating membrane fluidity [283,284,286]. The bioassay developed for this work was not able to detect any modulation in membrane fluidity by these positive controls (Figure 9-7). They were used successfully in equivalent concentrations to my work here but the method of measuring membrane fluidity was fluorescence polarization. The methodology for measuring membrane fluidity in the experiments here could have potentially been improved by using this similar but more widely used fluorescence technique.

Fluorescence polarization, also referred to as fluorescence anisotropy, uses a membrane bound fluorophore excited with polarized light, and measures the emitted light in two planes, parallel and perpendicular to the polarization plane of excited light [287]. The definition of anisotropy, the property of being directionally dependent implying different properties in different directions, gives a clue to the theoretical principle behind fluorescence polarization. Fluorescence intensity measurements reveal localization but not organization. Fluorescence polarization allows exploration of order and disorder [288]. Carbocyanine dye orientation in a phospholipid bilayer is such that the DiO conjugated bridge lies in a plane parallel to the surface of the epithelial cell [289] (Figure 9-1) and because of this it is suitable for fluorescence intensity measurements. Fluorescence polarization would require a probe in which the fluorophore enters the membrane, such as 1,6-diphenyl-1,3,5-hexatriene (DPH) (Figure 9-8).

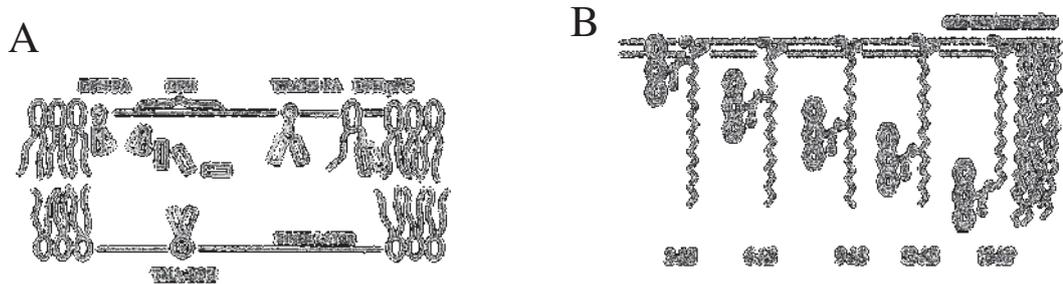


Figure 9-8: Schematic diagram of locations of fluorescent probes

DPH (A) and n-(9-anthroyloxy) steric acids (B) in a membrane. Modified from Szollosi [291]

I undertook fluorescence polarization experiments for this project but returned no useable data due to technical limitations. I used the BMG POLARstar Omega plate reader which is capable of reading fluorescence polarization, however there were technical issues in properly calibrating the two emissions photomultiplier to the DPH fluorophore which I could not overcome despite technical support. This calibration with the intended experimental fluorophore is a critical step in instrumental set-up as polarization is a calculated value. If the two photomultiplier tubes are not calibrated to account for the inescapable error of differing length light paths, the calculated values can be negative. Within the Plant and Food Research, Palmerston North, facility there is another instrument which is capable of fluorescence polarization measurements with a capable and experienced scientist who was willing to assist in the experiments; however, their Tecan Safire Plate reader was not capable of being used with fluorophores that excite at less than 400 nm. The DPH fluorophore excites at 355 nm and emits at 460 nm. Thus, use of another instrument was not an option to improve methodology if I wanted to maintain consistency with previous literature.

Biological membrane order can be measured with fluorescence polarization assays as well as with flow cytometry. There is precedence for using fluorophores including pyrenes, coumarines, fluoresceins, Bodipy, and cyanine dyes [290]. Experiments by Tsuchiya et al. used n-anthroyloxy stearic acid (n-AS) to show an increase in fluorescence polarization (decreased membrane fluidity or a more rigid membrane) by flavonoids in liposomes [283]. The n-AS probes create a gradient in the membrane to allow for measurement of fluidity at different positions in the membrane [291] (Figure 9-8). Similarly, fluorescence polarization assays have used the DPH molecular probe with A549 cells [292,293]. Most recently, Zhu et al. demonstrated a reduction in

membrane fluidity by A-type proanthocyanidins in 3T3-L1 mouse adipocytes, though the lowest concentration included was 10 $\mu\text{g/mL}$ (17 μM) which is double the human physiological threshold [240]. Conversely, epicatechin, catechin, and B-type coco hexamers did not affect membrane fluidity of biomimetic membranes when measured by a DPH fluorescence polarization assay [227].

In conclusion, modulation of membrane fluidity remains one possible mechanism by which procyanidin A2 could inhibit cytokine-induced eotaxin production in A549 cells. The membrane fluidity bioassay developed in this work was not sufficiently robust in design to allow for measuring differences in fluorescence intensity of positive controls or experimental procyanidin treatments. Improvements to methodology such as shifting to measuring fluorescent polarization were unable to provide additional data due to technical limitations. More work would have been needed in order to investigate if procyanidin A2 can modulate membrane fluidity in A549 cells.

Chapter Ten

Thesis Discussion

10.1 Summary of thesis conclusions

This doctoral project investigated dimeric fruit procyanidins for their ability to modulate biomarkers of airway epithelium inflammation *in vitro*. I successfully optimized cytokine-induced airway inflammation model systems for CCL11 and CCL26 production, while TSLP production in A549 cells was undetectable. Cytokine-induced CCL11 production was inhibited when A549 cells were exposed to 1, 5, or 10 μM procyanidin A, but not procyanidin B1 or B2, prior (6 h) to an inflammatory insult. Co-incubation of A549 cells with procyanidin A2 and procyanidin B2 demonstrated no evidence of a synergistic relationship for inhibiting cytokine-induced CCL11 production in my model system. Similarly, A549 cells exposed to procyanidin A2 (5 μM or 10 μM), and to a lesser extent procyanidin B2, had reduced production of cytokine-induced CCL26 production. An inhibition time course demonstrated procyanidin A2 had greatest inhibition efficacy on cytokine-induced CCL26 production when incubated for 2 – 6 h prior to an inflammatory insult. Comparison of procyanidin A2 inhibition to the known CCL26 inhibitor, IFN γ , demonstrated that procyanidin A2 and IFN γ did not share the same temporal inhibition patterns. Furthermore, experiments investigating concomitant incubation of procyanidin A2 and IFN γ demonstrated that procyanidin A2 could interfere with IFN γ -mediated CCL26 inhibition. Experiments measuring CCL26 suggested that procyanidin A2 inhibition was likely through a mechanism different than IFN γ . This project then investigated two possible mechanisms responsible for the procyanidin A-mediated inhibition of cytokine-induced CCL11 and CCL26: the modulation of cytokine receptor expression, and modulation of plasma membrane fluidity. However, there was no evidence to support either of these modes of action. To the best of my knowledge, the data presented in this thesis collectively demonstrate for the first time the ability of procyanidin A2 to inhibit cytokine-induced eotaxin

production from the lung epithelium *in vitro* and support further investigation of procyanidin A2 as a modulator of airway inflammation.

The procyanidins investigated in this thesis project were chosen based on their abundance in fruit foods [294] (also see chapter two) as well as commercial availability of structurally-defined isolated compounds. Additionally, limited procyanidin bioavailability reported in the literature gave support to evaluating procyanidin dimers [295-299]. It is unclear at this point if procyanidin A2, itself, is eliciting the inhibitory effects or if the results are due to a metabolite. Investigation of the degradation kinetics of procyanidin A2 and procyanidin B2 demonstrate that dimer stability in water is temperature and pH dependent; at pH 7.4, roughly 95% of procyanidin A2 and 75% of procyanidin B2 remain after 4 h in an aqueous environment [300]. As discussed in section 3.5.2, an *in vitro* fermentation model using human faecal microbiota demonstrated procyanidin B2 and procyanidin A2 can both be metabolized by human faecal bacteria [159]; though, comparatively, procyanidin A2 was more resistant to microbial catabolism than procyanidin B2, with at least 50% original procyanidin A2 remaining after 24 h of fermentation [159]. Additionally, *in vitro* stability after gastric and intestinal simulation of digestion showed that procyanidin B2 was isomerized or degraded after 1 h in gastric conditions, and completely degraded after 2 – 8 h in intestinal conditions [301]. These data collectively suggest that procyanidin A2 is a more stable molecule in physiological contexts than procyanidin B2, which could be a reason for the differing inhibition efficacy on cytokine-induced CCL11 and CCL26 demonstrated in this thesis. Interestingly, apple extracts containing dimeric and oligomeric procyanidins (B-type) have been demonstrated to inhibit cytokine-induced CCL11, suggesting stability of B-type procyanidins may increase when in a complex mixture of polyphenols or that increased degrees of polymerization support B-type

procyanidins to inhibit eotaxin production in A549 cells [302]. The possibility remains that two different metabolite profiles are generated from procyanidin A2 and procyanidin B2 degradation and a uniquely procyanidin A2-originating product is the biologically active molecule. However, together the literature and my data suggest procyanidin A2 itself as being a biologically active compound.

The A549 alveolar epithelial cell culture model system used for evaluating procyanidins utilized cells grown in submersion culture. The A549 cell-line has been demonstrated to polarize [180,303], which would create distinct apical and basal surfaces. Tight junctions, among other functions, regulate the paracellular transport of ions and small molecules and inhibit the mixing of proteins in the apical and basal membranes. Fruit procyanidin oral consumption would imply lung epithelium exposure to procyanidins would be a result of systemic circulation and diffusion through the basement membrane. In the human body, the monolayer of lung epithelial cells are supported by a basement membrane/ extra cellular matrix and this could affect the demonstrated inhibition of cytokine-induced CCL11 and CCL26. The extracellular matrix protein profile is altered in asthmatics and contributes to airway remodelling of asthma [304]. Small molecules from the blood must diffuse through the basement membrane, consisting of laminin, type IV collagen, and fibronectin, to reach the basal side of the epithelia [305,306]. The modulation of CCL11 and CCL26 production demonstrated here in apical procyanidin application may differ from basal application of procyanidins. Apical application of procyanidins, as mentioned in section 7.6, could be modelled to closer resemble physiological conditions by culturing at an air-liquid interface. Air-liquid interface culturing is common with normal human bronchial cells, which results in mixed populations of ciliated, mucus-secreting goblet, and basal cells [307]. As mentioned in section 8.6, A549 cells can develop differently when cultured at

air-liquid interface. Similarly, choice of culture media and culture time have been demonstrated to affect the development of multilamellar bodies in A549 cells [308]. Long-term (25 day) culture of A549 cells in Ham's F12 media resulted in the development of multilamellar bodies, whereas, 11 day culture in DMEM did not [308]. Multilamellar bodies are membrane-bound cellular organelles that store lipids. In type II alveolar cells, they function as secretory granules for depositing surfactant on the surface of the alveolae [309]. Surfactant reduces the surface tension at the air-cell junction to prevent the lung from collapsing during respiration. The presence of surfactant *in vivo* could affect the anti-inflammatory effects which I demonstrated here *in vitro*. For example, it has been reported surfactant lipids regulate induced IL-8 production in A549 cells [310]. For this project, I chose to remain consistent with the substantial literature which used A549 cultured in submersion, while acknowledging its limitations, and cultured in DMEM/F12 media, a 1:1 mixture of DMEM and Ham's F12 media.

10.2 Discussion of investigated biomarkers

The inappropriate inflammation at the airway epithelium was used to investigate the biological activity of procyanidins because it was suggested as a promising site for affecting asthma [311] and the large global burden of asthma [312,313] prompted a need for preventative and/or management strategies. The three biomarkers of airway inflammation investigated, TSLP, CCL11, and CCL26, were chosen because of their prominent roles in perpetuating two of the defining features of allergic asthma: skewing of T cell maturation toward a Th2 profile (TSLP), and the recruitment of eosinophils into the lung tissue (CCL11 and CCL26). Additionally, CCL11 and CCL26 can also stimulate eosinophils to generate reactive oxygen species [314,315]. Interestingly, CCL26 was shown to be a more potent recruiter of human eosinophils because it has

two temporal phases of attracting eosinophils: one in conjunction with CCL11 at 0–6 h, and a second phase on its own at 12–18 h [316]. Thus, modulation of these specific biomarkers by procyanidins stood to have a reasonable chance of translating *in vitro* results into clinical efficacy.

The biomarkers used here for investigating modulation of airway inflammation at the epithelium are not exclusive to lung inflammation. Th2 cytokine, IL-4, can also stimulate production of CCL26 in keratinocytes [317,318] and a human monoclonal antibody, dupilumab, against the IL-4 receptor subunit, IL-4R α , has demonstrated clinical efficacy in atopic dermatitis patients [319]. Furthermore, TSLP is produced from induced keratinocytes, which has been shown to then aggravate experimental allergic asthma in mice [320]. Interestingly, A-type proanthocyanidins are able to reduce pro-inflammatory cytokine (IL-6 and TNF α) production in LPS-stimulated human myeloma cells, and could reduce melanogenesis [321]. IL-4 can induce production of CCL11 in human airway smooth muscle cells [322,323]. Additionally, CCL26 is produced by active lesions of ulcerative colitis and IL-4 can enhance CCL26 expression in human colonic myofibroblasts [324]. Collectively, these studies support further investigation of procyanidin A2 modulation of inflammation at other barrier locations outside the lung.

In contrast to investigation of TSLP, CCL11, and CCL26 biomarkers in different barrier locations, it would also be interesting to investigate procyanidin A2 modulation of biomarkers which pertain to other endotypes of asthma, namely neutrophilic (severe) asthma [325]. For example, IL-8 is produced by the airway epithelium and recruits neutrophils into lung tissue as well as stimulates neutrophils to express cell surface proteins which allow adherence to activated endothelial cells [326]. Proanthocyanidins

from blackcurrant have been demonstrated to reduce endothelial surface markers, and inhibit leukocyte infiltration [326,327]. Similarly, granulocyte-macrophage colony-stimulating factor (GM-CSF) is also produced by epithelial cells and promotes stem cells to produce granulocytes [8]. Modulation of IL-8 or GM-CSF by procyanidin A2 could support lung health in neutrophilic asthma.

10.3 Procyanidins and the balance between Th1/ Th2 immunity

In health, Th2 immunity is antagonized by Th1 immunity and vice versa in order to maintain homeostasis [328]. It has been suggested that the prevalence of Th2-dominated allergy in modern times, as humans have migrated to temperate climates, is a consequence of the advantages our ancestors gained from strong Th2 responses for protection against helminth infections in tropical climates [329,330]. One strategy for reducing the burden of Th2 dominated allergic asthma has been the use of Th1 cytokine, IFN γ . For example, inhaled IFN γ can reduce airway inflammation and avoids unwanted side effects of systemic administration [218,221]. Additionally, Th1 cytokines, IFN γ and IL-1 β , can induce the production of macrophage inflammatory protein 1-alpha (CCL3) from A549 cells and CCL3 can inhibit IL-4 induced CCL26 production [331]. In the work presented here, procyanidin A2 interfered with IFN γ - mediated CCL26 inhibition (Figure 7-7). I suggested the interrupted IFN γ signalling was through procyanidin A2 interactions with the cellular plasma membrane (section 7.6). Biological activity at the cell surface that influences cell-cell interactions was also suggested as the mechanism for highly oligomeric procyanidins (≥ 5 DP) suppressing Th1 immunity related to autoimmune inflammation [332]. At first consideration, procyanidins impeding Th1 immunity seems counter to regaining a healthful balance of Th1 and Th2 immunity in allergic asthma, because Th2-mediated disease is reduced by the presence of Th1 immunity. Indeed, deficiencies of type I interferons allow deregulation of ILC2

cells which instigate type 2 immunopathology in viral respiratory-induced asthma [333]. However, the reduction in Th1 immunity by oligomeric procyanidins was mediated through reduced antigen-presentation capabilities of macrophages and by inhibiting T cells [332]. Similarly, as mentioned in 8.6, quercetin, curcumin, ellagic acid, and silibinin, were all demonstrated to suppress co-stimulatory molecules CD80 and CD86 expression on DCs [269-272]. The reduction of these co-stimulatory molecules subsequently reduced DC maturation. Thus, procyanidins through interactions with the cell surface may control the maturation of antigen presenting cells in both Th1-mediated autoimmune disease and Th2-mediated allergic disease to reduce the progression of pathology and promote immune homeostasis. To evaluate this hypothesis, experiments specifically investigating A-type procyanidins effects on DC maturation in the context of allergic asthma are needed.

10.4 Avenues for future research

Looking forward, research will need to consider the inter-individual metabolic differences when evaluating proanthocyanidin interventions. Individual differences, such a microbiome profile, will be particularly important if consumption of a food is the intended route of administration. Clusters (enterotypes) of microbial compositions have been demonstrated in the gut [334] and long-term dietary patterns have been associated with specific gut microbial enterotypes [335]. Additionally, it was demonstrated that the dynamics between microbial communities are universal and the inter-individual variability originates from the colonizing species [336]. Dietary fibre metabolism into short-chain fatty acids (SCFA) is understood to inhibit inflammatory pathways of macrophages and DCs as well as maintain intestinal epithelial integrity and health [337]. Gut microbiota metabolism of dietary fibre increases circulating SCFAs which impair Th2 effector functions in the lung, thus demonstrating the ability of host-

microbiome crosstalk to influence peripheral tissues [338]. Strategies for preventing the development of allergic asthma do include appropriate development of both the gut and lung microbiomes [339]. Interestingly, bacteria from the oral cavity are the dominant factor in shaping the airway microbiota [340]. Collectively, these studies suggest that the microbiome enterotype should be considered when investigating procyanidin A2 in a whole body system. Furthermore, because of the fibre content of fruits, consumption of the whole fruit, not just the bioactive polyphenols, may provide additional support of the airways.

Following prudent evaluation of procyanidin A2 on immune cells such as eosinophils and DCs *in vitro*, this project's findings could progress by investigating a procyanidin A2 rich fruit, such as cranberries, in an animal model of allergic asthma. There are limitations to mouse models of human allergic asthma, such as prolonged mouse exposure to OVA resulting in tolerance or the weak induction of IgE in the house dust mite model (HDM) [341-343]. Additionally, CCL26 production is exclusive to humans and would not be applicable for study in mice [344]. There would still be much to gain from investigating A-type procyanidins rich foods in a whole body system, such as: evaluation of airway inflammation *in vivo*, effects on immune cell migration into the lung tissue, maturation of immune cells *in vivo*, airway hyper-responsiveness, and airway tissue remodelling. In this project, I was mindful of physiological relevance when selecting the investigated concentrations of procyanidins. If an animal study evaluating procyanidin A2 were conducted and found to reduce inflammation, an equivalent dose for a human trial could be chosen through the body surface area normalization method, which calculates a ratio from oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function [345].

Understanding the biological activity and mechanisms of action by which A-type procyanidins could control inappropriate airway inflammation associated with allergic asthma would allow for their focused use as a non-pharmaceutical means to prevent inflammatory illness and assist with improving human health.

Appendix I

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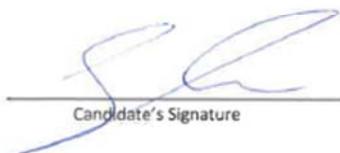
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Chapter 3

FRUIT PROCYANIDINS: MODULATING INFLAMMATION TO PROMOTE HEALTH

Sara L. Coleman^{1,2,*}, Roger D. Hurst¹, Gregory M. Sawyer¹, and Marlena C. Kruger²

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ABSTRACT

Procyanidins are polyphenolic compounds found in relatively high concentrations in fruits that have come to be known as biologically active in the context of promoting human health. Epidemiological evidence suggests that people who consume diets high in fruits and vegetables are less susceptible to inflammation-related diseases and diseases of multifactorial pathogenesis such as metabolic syndrome, atherosclerosis, asthma, and cancer. Procyanidins are one of three types of proanthocyanidins and differ from the others based on the hydroxylation pattern of their B ring. They differ amongst themselves according to the type of linkage that connects the flavan-3-ol monomeric units. Despite progress, there is still a lack of measurement technologies to delineate the structure of higher polymers accurately, although specific dimer designations have been identified. Research is working toward understanding the health-promoting properties of procyanidins, specifically moving beyond their antioxidant capabilities. Bioavailability of designated dimers *in vivo* is not well understood, though there are data to suggest that *in vitro* experiments could be relevant in the human system. Population studies have shown an inverse relationship between procyanidin consumption and ill health, especially inflammatory conditions. Here we present a focused review of the properties of individual procyanidins and suspected mechanisms by which they could potentially regulate inflammation. Modulation of cytokine secretion, mechanisms involving signal transduction pathways - NF- κ B, STAT, and MAPK, and procyanidin influence on the chemotaxis of inflammatory cells into the tissues, will all be discussed.

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The *in vitro* evaluation of isolated procyanidins as modulators of cytokine-induced eotaxin production in human alveolar epithelial cells

Sara L. Coleman^{a,b,*}, Roger D. Hurst^a, Gregory M. Sawyer^a and Marlena C. Kruger^b

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Abstract.

BACKGROUND: Populations that consume procyanidin-rich diets are less susceptible to inflammatory disease. Allergic asthma is an inflammatory lung disease perpetuated by a hyperreactive airway epithelium and eosinophil infiltration into the lung. Eotaxin-1 (CCL11) mediates eosinophil migration into tissues and its modulation could represent a means to assist the management of airway inflammation.

OBJECTIVE: Here we evaluated procyanidins as a means of modulating CCL11 production *in vitro*.

METHODS: We used human lung epithelial cells (A549) and optimized the conditions to induce CCL11 production *in vitro*. Cells were exposed to procyanidins for 6 h prior to an inflammatory insult of 5 ng/mL IL-4 with 5 ng/mL TNF α for 48 h. An enzyme-linked immunosorbent assay was used to measure CCL11 production.

RESULTS: Cells exposed to 5 μ M procyanidin A2 prior to the inflammatory challenge showed significantly inhibited (36%) CCL11 production. Under the same conditions, procyanidins B1 and B2 elicited no effect. Furthermore, combinations of procyanidins A2 and B2 (5 μ M total) demonstrated no evidence of a synergistic interaction.

CONCLUSIONS: These data demonstrate that the regulation of CCL11 by lung epithelial cells is not ubiquitous among the three investigated procyanidins. We demonstrate a differential inhibition of CCL11 by A-type and B-type procyanidins. This evidence supports further studies into procyanidins, specifically A-type, for managing inappropriate airway inflammation.

Keywords: A549, eotaxin-1 (CCL11), IL-4, lung inflammation, procyanidins

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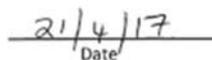
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Procyanidin A2 Modulates IL-4-Induced CCL26 Production in Human Alveolar Epithelial Cells

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Abstract: Allergic asthma is an inflammatory lung disease that is partly sustained by the chemokine eotaxin-3 (CCL26), which extends eosinophil migration into tissues long after allergen exposure. Modulation of CCL26 could represent a means to mitigate airway inflammation. Here we evaluated procyanidin A2 as a means of modulating CCL26 production and investigated interactions with the known inflammation modulator, Interferon γ (IFN γ). We used the human lung epithelial cell line A549 and optimized the conditions for inducing CCL26. Cells were exposed to a range of procyanidin A2 or IFN γ concentrations for varied lengths of time prior to an inflammatory insult of interleukin-4 (IL-4) for 24 h. An enzyme-linked immunosorbent assay was used to measure CCL26 production. Exposing cells to 5 μ M procyanidin A2 (prior to IL-4) reduced CCL26 production by 35% compared with control. Greatest inhibition by procyanidin A2 was seen with a 2 h exposure prior to IL-4, whereas IFN γ inhibition was greatest at 24 h. Concomitant incubation of procyanidin A2 and IFN γ did not extend the inhibitory efficacy of procyanidin A2. These data provide evidence that procyanidin A2 can modulate IL-4-induced CCL26 production by A549 lung epithelial cells and that it does so in a manner that is different from IFN γ .

Keywords: airway inflammation; eotaxin-3 (CCL26); IFN γ ; IL-4; procyanidin A2

1. Introduction

Procyanidins are polyphenolic secondary plant metabolites that are recognized as biologically active in the context of human health. They are the product of polymerization of monomeric flavan-3-ol units by a currently undefined mechanism in plants [1]. The monomeric units can be linked together in various patterns, which can then dictate their ability to influence specific biological pathways. Polyphenolic compounds are thought to have evolved within plants to assist in coping with physiological stresses such as drought, excess UV exposure and/or pathogenic invasion [2]. Furthermore, there is accumulating evidence that plant polyphenols may have a role in managing stress in the human body [3,4]. Unfortunately, the average consumption of fruit and vegetables per person in the US is half of the USDA recommended daily intake of 1.5–2 cups and 2–3 cups, respectively [5]. Thus, concentrated extracts or isolated bioactive plant compounds could supplement the typical western diet for improved health and wellbeing.

Inflammatory diseases, such as allergic asthma, are less prevalent in populations that consume procyanidin-rich diets [6,7]. The lung epithelium is responsible for regulating pulmonary inflammation and secretes a range of cytokines, which coordinate physiological responses that restrict airflow. Eosinophil migration into the lung tissue is a defining feature of the inflammation that perpetuates

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References Cited

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