RESEARCH ARTICLE

Blackcurrant proanthocyanidins augment IFN-γ-induced suppression of IL-4 stimulated CCL26 secretion in alveolar epithelial cells

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Received: June 24, 2009
Revised: September 30, 2009
Accepted: October 22, 2009

Epidemiological studies reveal that fruit consumption reduces the prevalence of airway inflammation and childhood asthma. In particular, blackcurrant polyphenolic extracts have been shown to alleviate lung inflammation. Since IL-4-stimulated eotaxin-3 (CCL26) secretion is a major factor in the continuous eosinophil recruitment observed in atopic asthma, our focus was to evaluate the effectiveness of blackcurrant polyphenolic compounds on CCL26 secretion in human alveolar epithelial cells. Our results indicate that a proanthocyanin-enriched blackcurrant extract (BC-P), but not anthocyanin-enriched blackcurrant extract suppressed both IL-4- and IL-13-stimulated CCL26 secretion in a dose-dependent manner. Furthermore pre-incubation of cells with BC-P caused a time-dependent suppression of IL-4-stimulated CCL26 secretion. Moreover, epigallocatechin (EGC), and to a lesser extent epicatechin, metabolites identified in the proanthocyanidin extract, suppressed IL-4-stimulated CCL26 secretion. EGC was also effective at reducing the cellular phosphorylated STAT-6/STAT-6 ratio. Furthermore, both BC-P and purified EGC potentiated the ability of IFN-γ to suppress IL-4-stimulated CCL26 secretion. The progression of an allergic immune response is complex, identifying plant compounds that target specific cellular events and complement the body's own immune actions is important for the development of functional foods. Our findings support the potential for blackcurrant polyphenolic compounds to reduce eosinophil recruitment and alleviate eosinophilic-driven airway inflammation.

Keywords:
Eotaxin-3 / IFN-γ / IL-4 / Lung inflammation / Proanthocyanidins

1 Introduction

The etiology of asthma involves a combination of genetic and environmental factors that are epitomized by a dysregulated allergen-induced immune response to otherwise inert substances [1, 2]. A common feature in allergy-induced asthma is the activation of $CD4^+$ T-helper type 2 lymphocytes (Th2 cells) that promote the classic symptoms of asthma, including chronic eosinophilic-mediated lung inflammation [3]. Amongst the Th2-cell-derived cytokines identified, IL-4 and IL-13 have been associated with eosinophil recruitment into the airways *via* the induction of the eosinophil chemoattractant, eotaxin [4]. Currently, there are three major eotaxin isoforms associated with allergen-induced eosinophilia; eotaxin-1 (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26). The synthesis and secretion of the various eotaxin isoforms are differentially expressed and dependent upon the type and temporal expression profile of respective agonists during the progression of the allergen-induced immune reaction [5, 6]. Furthermore, although reports identify the expression of all three eotaxin isoforms in airway epithelium, only CCL26 has been found to remain

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Abbreviations: ANOVA, analysis of variance; **BC-A,** anthocyanin-enriched blackcurrant extract; **BC-P,** proanthocyanin-enriched blackcurrant extract; **BC-T,** blackcurrant total polyphenolic extract; **CCL11,** eotaxin-1; **CCL26,** eotaxin-3; **EC,** epicatechin; **EGC,** epigallocatechin; **FBS,** fetal bovine serum; **NF-κB,** nuclear factor-kappa B; **STAT,** signal transducer and activators of transcription; **TBS,** tris-buffered saline; **Th2 cells,** $CD4^+$ T-helper type 2 lymphocytes

elevated 24 h after allergen challenge in individuals with atopic asthma [7]. These finding suggests that Th2 cell-derived cytokine-induced CCL26 may be a primary cause in the on-going eosinophil recruitment to the asthmatic airway and that CCL26 plays an important role in the perpetuation of the disease.

A putative therapeutic intervention strategy employed in atopic diseases is to target cellular events that regulate chronic inflammation. Modulation of transcription factor activation has been targeted in number of diseases including asthma [8, 9]. In general, Th2 cells mediators, IL-4 and IL-13, induce an accumulation of cytoplasmic Janus kinases that result in the phosphorylation of the transcription factor, signal transducer and activators of transcription (STAT)-6, in the cell [8], which mediates the action or enhancement of effector responses such as the induction of CCL26 [9]. The inhibition of STAT-6 phosphorylation may therefore prevent IL-4 stimulated CCL26 expression and alleviate eosinophil recruitment into the lung. Another potential cellular target in the amelioration of asthmatic symptoms is the modulation of $CD4^+$ T-helper type 1 lymphocytes (Th1 cells)-stimulated events. In particular, the Th1 cell-derived cytokine, IFN-γ, has been shown to alleviate allergen-induced cellular processes [10] *via* the suppression of STAT-6 phosphorylation [11]. However, in an allergic tissue, Th2 cells are dominant, and so the expression of Th-1 cell cytokines, like IFN-γ may not be generated [12]. Therefore, therapies that modulate specific immune processes that lead to the suppression of CCL26 expression may be beneficial in regulating allergen-induced eosinophilic-driven inflammation.

Foods that may alleviate allergen-induced airway eosinophil inflammation and promote health benefits on lung function are a real possibility. Recent epidemiological studies show that an increase in fresh fruit and vegetables in the daily diet correlates with a lower prevalence of respiratory symptoms and a lower incidence of non-specific lung diseases [13, 14]. An Italian respiratory study of 18 737 children demonstrated that citrus and kiwifruit intake (1–2 times *per* week) gave significant protective effects against wheezing and nocturnal/chronic cough and that this protective effect was stronger in children with a history of asthma [15]. These human studies suggest that certain fruits may contain phytochemicals that reduce the symptoms and prevalence of allergen-induced asthma. Recent animal studies indicate that berryfruits are capable of suppressing airway inflammation [16–18]. In particular, fruit-derived anthocyanins [16, 17] and proanthocyanidins [18] have been shown to attenuate lung inflammation. However, their mode of action is currently unknown and may involve similar (*e.g.* nuclear factor-kappa B (NF-κB) [19–21], haemoxygenase-1 (HO-1) [22]) or currently unknown cellular mechanisms accumulating in the temporal suppression of different eotaxin isoforms that synergistically serve to ameliorate allergy-induced eosinophilic inflammation.

In this study, we specifically identify and evaluate the effectiveness of blackcurrant polyphenolic compounds, anthocyanin and proanthocyanidin, to suppress CCL26 secretion in alveolar epithelial cell-line. CCL26 is a major eotaxin isoform-induced by activated Th2 cell-derived cytokines (IL-4, IL-13) and so its suppression in lung tissue may serve to ameliorate the chronic eosinophilic inflammation observed in asthma patients. Moreover, identifying the specific cellular action of fruit-derived phytochemicals will allow the development of functional foods that benefit individuals susceptible to allergen-induced asthma.

2 Material and methods

2.1 Materials

All cell culture media, fetal bovine serum (FBS) and supplements were purchased from Invitrogen NZ (Auckland, NZ). The alveolar epithelial (A549) cell-line was purchased from American Tissue Cell Collection, (ATCCTM ♯CCL-185 (c/o Cryosite, Lane Cove NSW, Australia)). Human recombinant IL-4 and INFγ, anti-human STAT-6 and phosphorylated STAT-6 antibodies, plus CCL26 DuoSet ELISA kit were purchased from R&D systems (Pharmaco, Auckland, NZ). Anti-human phosphorylated STAT-1 and STAT-1 monoclonal antibodies were purchased from BD Biosciences (Auckland, NZ), whereas an anti-actin monoclonal antibody (clone AC-40) was purchased from Sigma-Aldrich, (Auckland, NZ). WST-1 reagent was purchased from Roche (Auckland, NZ). Purified epigallocatechin (EGC) and epicatechin (EC) compounds were purchased from Sigma-Aldrich. Kaleidoscope protein markers, Bradford protein assay kit, goat anti-mouse IgG conjugated to horse radish peroxidase and immobilon P membrane were purchased from Bio-Rad Laboratories (Auckland, NZ) Chemiluminence kit and Kodak BioMax XAR film were purchased from Pierce (Global Science, Auckland, NZ) and Radiographic supplies (Christchurch, NZ), respectively. Unless otherwise stated, all other reagents were purchased from BioLab (Auckland, NZ).

2.2 Blackcurrant polyphenolic extracts

Polyphenolic compounds were extracted from New Zealand blackcurrants (*Ribes nigrum cf.,* Ben Ard) fractionated and assessed for anthocyanin and proanthocyanidin composition. Briefly, freshly picked blackcurrants were immediately frozen and stored at −80°C until required. Polyphenolic compounds were extracted using a methodology previously described [23]. This process provided six fractions, which were initially characterized by HPLC. In this study we selected, in addition to a total polyphenolic blackcurrant extract, two sub-extracts that were enriched (>90%) with either anthocyanin- (BC-A) or proanthocyanidin-(BC-P) polyphenolics compounds. The proanthocyanidin enriched blackcurrant extract was analyzed by LC-ESI-MS combined

with photodiode array detection, as described previously by this group [24].

2.3 Alveolar epithelial cell culture

Cells were grown under standard tissue culture conditions *i.e.* 37°C in a 95% humidified atmosphere at 5% CO_2 in DMEM Ham's F12K medium containing 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 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. All experiments were performed in the absence of FBS; removal of FBS did not affect the viability of the cells.

2.4 Blackcurrant polyphenolic extract modulation of CCL26 secretion

2.4.1 Characterization experiments

Preliminary experiments were carried out to optimize the time (24 or 48 h)-and concentration (0–100 ng/mL)-dependent effect of IL-4 or IL-13 on CCL26 generation in A549 cells. Supernatants were collected and frozen at −80°C until CCL26 measurement by ELISA.

2.4.2 Dose-response

A549 cells were co-incubated with control (containing DMSO equivalent to that contained within the diluted extract) media or 0.5–10 μg/mL blackcurrant total polyphenolic extract (BC-T), BC-A or BC-P (diluted from 100 mg/mL DMSO stocks) in the absence or presence of IL-4 (10 ng/mL) or IL-13 (10 ng/mL) for 24 h. Supernatants were processed as described above.

2.4.3 Time-dependent effect

A549 cells were pre-incubated with control (diluted DMSO) medium or BC-P (5 μg/mL) for 1–24 h. Cells were washed twice (media removed, replacing with fresh media and gently agitated for 5 min) and finally placed in fresh media before being stimulated with IL-4 (10 ng/mL) for a further 24 h. Supernatants were processed as described above.

2.4.4 Recovery period

A549 cells were pre-incubated with control (diluted DMSO) medium or BC-P extract (5 μg/mL) for 6 h, washed twice (as stated above) and either (i) stimulated with IL-4 (10 ng/mL) for 24 h, or (ii) incubated with medium alone for 1–24 h, then stimulated with IL-4 for a further 24 h.

2.4.5 Proanthocyanidin compounds

Cells were pre-incubated with control (diluted DMSO) medium or 0.1–10 μg/mL purified EGC or EC (diluted from 100 mg/mL DMSO stocks) for 6 h, washed twice (as stated above) and stimulated with IL-4 (10 ng/mL) for a further 24 h. Supernatants were processed as described above.

2.5 Blackcurrant polyphenolic extract effect on cell viability

Cell viability was assessed using a method described by Tan *et al.* [25]. Briefly, A549 cells were seeded into sterile 96 well plates and grown until confluent. Polyphenolic extracts, BC-T, BC-A or BC-P (0.5–10 μg/mL), control (diluted DMSO) media or positive (hydrogen peroxide (5 mM)) control were incubated for 24 h. Cells were washed twice with fresh media (as described above) and then incubated with WST-1 reagent (10 μL) for 4 h, followed by absorbance measurement at 450 nm, with reference absorbance at 620 nm. Cell viability was calculated as a percentage of change in absorbance after subtraction of control.

2.6 IFN-γ and proanthocyanidins modulation of CCL26 secretion

2.6.1 IFN-γ alone

A549 cells were concomitantly incubated with IFN-γ (0.01–100 ng/mL) and IL-4 (10 ng/mL) for 24 h. Supernatants were collected and measured for CCL26 as described above.

2.6.2 IFN-γ plus BC-P extract, EGC or EC

A549 cells were pre-incubated with control (diluted DMSO) medium, BC-P extract (0.5 μg/mL) EC (10 μg/mL) or EGC (0.5 μg/mL) for 6 h, washed twice (as described above) and then incubated with IFN-γ (0.05–5 μg/mL) in the presence of IL-4 (10 ng/mL) for a further 24 h. Supernatants were collected and processed as described above.

2.7 Assessment of cellular events

2.7.1 STAT-6 activation

A549 cells were pre-incubated with control (diluted DMSO) medium or (i) the BCP extract (5 μg/mL) or EGC (5 μg/mL)

for 6 h, washed twice (as described above) and then stimulated with IL-4 (10 ng/mL) for 0–120 min or (ii) control (diluted DMSO) media, EC (10 μg/mL) or EGC (0.5 μg/mL) for 6 h, washed twice (as described above) and then incubated with either media or IFN-γ (1 ng/mL) for a further 24 h, followed by IL-4 (10 ng/mL) stimulation for 60 min. At the end of time-points, cells were immediately placed on ice and whole cell extracts were prepared as described below.

2.7.2 STAT-1 activation

A549 cells were pre-incubated with control (diluted DMSO) media or EGC (5 μg/mL) for 6 h, washed twice (as described above) and then stimulated with IFN-γ (10 ng/mL) for 0–90 min. At the end of time-points, cells were immediately placed on ice and whole cell extracts were prepared as described below.

2.8 Cell extract analysis

2.8.1 Cell lysate preparation

Preparation of cell lysate and assessment of phosphorylated STAT levels was previously described Moynihan *et al.* [26]. Briefly, cells were placed on ice, washed with 1 mL ice-cold phosphate-buffered saline, pH 7.4 (PBS) and then re-suspended in 200 μL ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, proteinase inhibitor cocktail (Roche), 1 mM activated Na_3VO_4, 1 mM NaF), and incubated on ice for 5 min. The cell lysate was then frozen at −80°C.

2.8.2 SDS-PAGE and immunoblotting

Approximately 60 μg protein was separated by SDS-PAGE under denaturing conditions (8% resolution gels), transferred to immobilon P membrane, washed in Tris-buffered saline (TBS, 10mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl), and then blocked for 1 h at room temperature in 5% non-fat skimmed milk powder in TBS. Membranes were probed with the different primary monoclonal antibodies diluted in 4% skimmed milk/TBS; (i) anti-human phosphorylated STAT-6 (0.5 μg/mL) polyclonal antibody or (ii) anti-human phosphorylated STAT-1 (1:1000 dilution) monoclonal antibody overnight at 4°C. Blots were then washed three times (10 min) with TBS containing 0.5% v/v Tween-20, and then incubated for 1 h with either goat anti-rabbit IgG (1:1000 dilution) or goat anti-mouse IgG (1:3000 dilution) conjugated to horse radish peroxidase, before being washed a further five times (10 min) with TBS/Tween-20. The membrane bound antibody was then detected by chemiluminesence on Kodak BioMax XAR film. To verify even protein loading simultaneous blots for STAT-6 (0.5 μg/mL), STAT-1 (1:1000 dilution) and actin (1:2000 dilution) were carried out using the same protocol described above. Semi-quantitative densitometry analysis of immune-reactivity was measured using ImageJ 1.410 software (National Institute of Health, USA). Results are expressed as an abituary optical value for cellular phosphorylated STAT-6 or STAT-1 and expressed as a ratio of respective STAT-6 or STAT-1 levels measured in the same whole cell extract. In addition, changes in STAT-6 and STAT-1 levels were evaluated by cellular actin levels.

2.9 Statistical analysis

Results were analyzed using paired or unpaired Student *t*-test or one-way analysis of variance (ANOVA). Where appropriate the original data were expressed as a ratio of control. Statistical significance for all indices was set at $p < 0.05$ with a confidence level of 95%.

3 Results

3.1 Blackcurrant polyphenolic extracts exerted a differential action on IL-4-stimulated CCL26 secretion

Preliminary experiments demonstrated that IL-4 and IL-13 induced a concentration- and time-dependent secretion of CCL26 from A549 cells, which was optimal after 24 h using 10 ng/mL of either IL-4 (1.7 ± 0.3 ng/mL) or IL-13 (2.1 ± 0.2 ng/mL). Pre-incubation of A549 cells with BC-T, BC-A or BC-P extracts (0.5–10 μg/mL) for 24 h had no adverse effect on cell viability (Fig. 1A) or baseline CCL26 secretion (data not shown). Concomitant incubation of A549 cells with individual polyphenolic blackcurrant extracts together with IL-4 (10 ng/mL) or IL-13 (10 ng/mL) caused a dose-dependent inhibition of IL-4- and IL-13-stimulated CCL26 secretion (Fig. 1B), whereas no change was observed when the BC-A extract was used.

3.2 Blackcurrant proanthocyanidins modulated IL-4-stimulated CCL26 secretion

We next focused on characterizing the inhibitory action of the BC-P.

3.2.1 Time course

Pre-incubation of cells with BC-P prior to IL-4 stimulation caused a time-dependent suppression of stimulated CCL26 secretion (Fig. 2A). A 6 h pre-incubation of BC-P with cells induced a 36% ($p < 0.05$) suppression of IL-4-stimulated CCL26 secretion (1.4 ± 0.15 *versus* 0.9 ± 0.7 ng/mL, control *versus* BC-P), which was still present after a 24 h pre-incubation of cells with the BC-P extract.

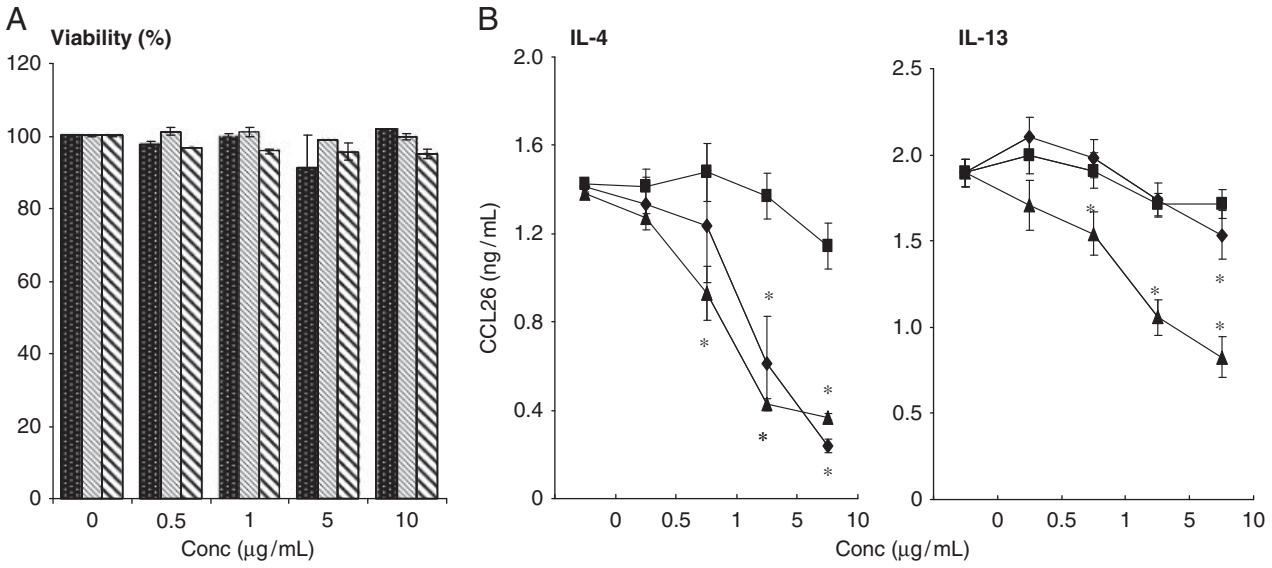

Figure 1. Blackcurrant polyphenolic extract modulation of IL-4-stimulated CCL26 secretion. Solvent extracts of the three blackcurrant polyphenolic extracts; BC-T, anthocyanin-enriched blackcurrant extract (BC-A) and proanthocyanidin-enriched blackcurrant extract (BC-P) were incubated with alveolar epithelial (A549) cell-line and assessed for (A) cell viability and (B) modulation of CCL26 secretion. (A) Blackcurrant polyphenolic extracts (0.5–10 μg/mL); BC-T (speckled bars), BC-A (fine stripped bar) or BC-P (coarse stripped bar) or control (diluted DMSO) were incubated with cells for 24 h, washed twice and WST-1 reagent was used to assess cell proliferation. Cell viability was calculated as percentage of control; cells incubated with diluted DMSO/media only. Results are expressed as mean ± SEM, $n = 3$ separate experiments. No statistical difference (ANOVA) was observed. (B) A549 cells were concomitantly incubated with blackcurrant polyphenolic extracts (0.5–10 μg/mL); BC-T (diamonds), BC-A (squares) or BC-P (triangles) in the presence of IL-4 (10 ng/mL) or IL-13 (10 ng/mL) for 24 h. Collected supernatants were measured for CCL26 secretion using appropriate ELISA. Data were calculated as IL-4- or IL-13-stimulated CCL26 secretion (ng/mL). Results are expressed as mean ± SEM, $n = 4$ separate experiments. $^*p < 0.05$ represents statistical difference (ANOVA) from time zero.

3.2.2 Recovery

We also explored the time required for cells to recover from exposure to the BC-P extract (Fig. 2B). A549 cells were pre-incubated with either control (diluted DMSO) media or BC-P for 6 h, washed twice (see Section 2 for details) with fresh media, and the recovery of IL-4-stimulated CCL26 secretion was monitored over 24 h. Pre-incubation of cells with the BC-P extract caused a 39% (1.75 ± 0.25 *versus* 1.06 ± 0.12 ng/mL, control *versus* BC-P, $p < 0.01$) suppression, which was time-dependently removed when the cells were washed and incubated with media for 1–24 h prior to IL-4 stimulation. The inhibitory effect of BC-P extract was no longer evident after a 24 h wash-out period, IL-4 (0.75 ± 0.25 *versus* 0.74 ± 0.25 ng/mL, control *versus* BC-P, $p < 0.05$).

3.2.3 Proanthocyanidin metabolites

Fullscan (200–500 *m/z*) LC-ES-MS analysis of the BC-P identified four catechin compounds. The chromatogram shown in Fig. 3A, revealed that gallocatechin (GC), catechin (C) and epicatechin (EC) were in relative low abundance compared with EGC in our BC-P extract. Since EGC is a major component of blackcurrant proanthocyanidins (prodelphindins), we next investigated its effect on IL-4-stimulated CLL26 secretion. We also examined the effect of EC, which although was present only in low abundance in our particular extract is another major blackcurrant proanthocyanidin (procyanidins). A 6 h pre-incubation of A549 cells with either purified EGC or EC had no effect on baseline CCL26 secretion, however, a differential inhibitory effect was observed on IL-4-stimulated CCL26 secretion. Pre-incubation of cells with EGC prior to IL-4 stimulation caused a concentration-dependent inhibition of CCL26 secretion, which became significant ($p < 0.05$) using 1 μg/mL (1651 ± 48 *versus* 1133 ± 78 pg/mL, control *versus* EGC). In contrast, IL-4-stimulated CCL26 secretion was only slightly inhibited when cells were pre-treated with 10 μg/mL EC; 30% inhibition (1651 ± 48 *versus* 1210 ± 251 pg/mL, control *versus* 10 μg/mL EC, $p < 0.05$).

3.2.4 Cellular events

Using semi-quantitative reverse transcription-PCR we confirmed that pre-incubation of A549 epithelial cells with the either the BC-P extract or purified EGC for 6 h prior to IL-4 stimulation for 4 h suppressed CCL26 mRNA expression; 50 and 55%, respectively, when expressed as a ratio of

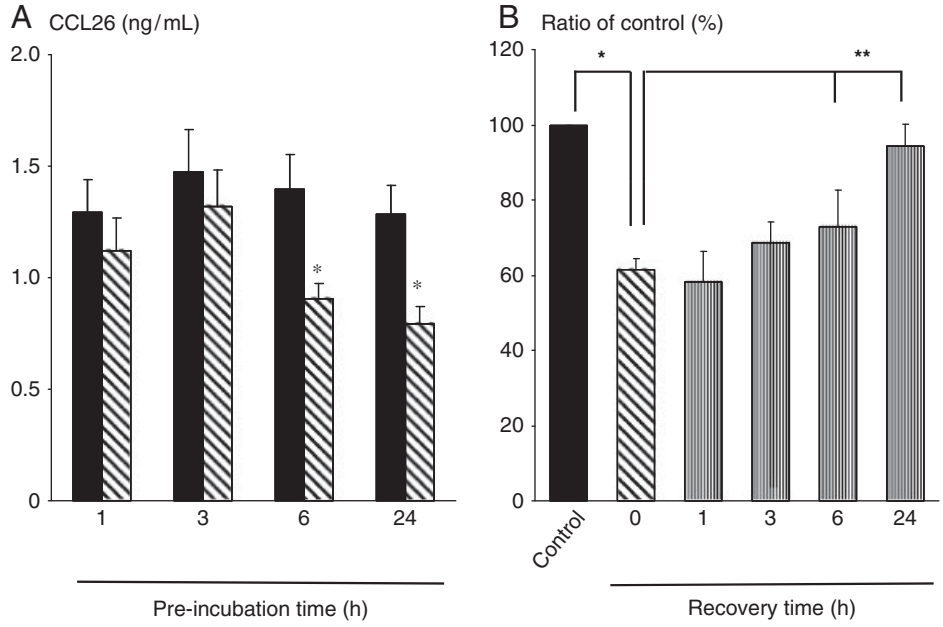

Figure 2. Characterization of proanthocyanidin enriched blackcurrant extract-induced suppression of IL-4-stimulated CCL26 generation. (A) Alveolar epithelial (A549) cells were incubated with either control (diluted DMSO) media (filled bars) or 5 μg/mL blackcurrant proanthocyanidin extract (BC-P) (open bars) for 1–24 h, washed twice and incubated for a further 24 h in the presence of IL-4 (10 ng/mL). Supernatants were collected and measured for CCL26 secretion. Data were calculated as IL-4-stimulated CCL26 secretion (ng/mL). Results are expressed as mean ± SEM, $n = 5$ separate experiments. $^{*}p < 0.05$ represents statistical (paired Student *t*-test) difference form corresponding control at a specific time point. (B) A549 epithelial cells were pre-incubated for 6 h with control media or BC-P (5 μg/mL), washed twice and either (i) stimulated with IL-4 for 24 h or (ii) further incubated with media alone for 1–24 h prior to IL-4 stimulation. At the end of each time point the collected supernatant was measured for CCL26 secretion. Data was calculated as IL-4-stimulated CCL26 secretion (ratio of control). Results are expressed as mean ± SEM, $n = 5$ separate experiments. $^{*}p < 0.01$ represents statistical (paired Student *t*-test) difference from cell pre-incubated with media only, $^{**}p < 0.05$ represents statistical (paired Student *t*-test) difference from cell pre-incubated with BC-P.

glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene) transcription (data not shown). Since IL-4 (and IL-13)-stimulated CCL26 expression involves the activation of STAT-6, and not NF-κB [5], we explored the effect of BC-P extract and EGC on the phosphorylation of STAT-6 (indicator of activation) induced by IL-4. Pre-incubation (6 h) of A549 cells with either BC-P (5 μg/mL) or EGC (5 ug/mL) prior to IL-4 stimulation (0–120 min) modulated IL-4-induced STAT-6 phosphorylation (Fig. 4). Analysis of immoblots of SDS-PAGE separated cell lysate revealed that a pre-incubation of either BC-P or purified EGC had no effect on the level of STAT-6; loading verified using cellular actin levels (data not shown). Expression of phosphorylated STAT-6 (pSTAT-6) levels as a ratio of corresponding cellular STAT-6 levels revealed that IL-4-induced a time-dependent increase in the amount of cellular pSTAT6/STAT6 ratio in cells treated with control media. Pre-incubation of cells with BC-P (5 μg/mL) caused a slight decrease in cellular pSTAT-6/STAT-6 ratio compared with control but was not significant. Interestingly, after a 120 min IL-4 stimulation, cells pre-incubated with BC-P showed a greater (non significant) cellular pSTAT-6/STAT-6 ratio than control. In contrast, pre-incubation of cells with EGC (5 μg/mL) significantly ($p < 0.05$) reduced the cellular pSTAT-6/STAT-6 ratio, which was initially observed after 60 min IL-4 stimulation.

3.3 Blackcurrant proanthocyanidins enhanced IFN-γ ability to inhibit IL-4-stimulated CCL26 secretion

3.3.1 CCL26 secretion

Concomitant incubation of A549 cells with IFN-γ (0.01–100 ng/mL) and IL-4 (10 ng/mL) for 24 h caused a dose-dependent suppression of stimulated CCL26 secretion (Fig. 5A), which was maximal using 10 ng/mL IFN-γ (2080 ± 130 *versus* 1251 ± 158 pg/mL, control *versus* IFN-γ, $p < 0.05$). Since both IFN-γ and BC-P independently inhibited IL-4-stimulated CCL26 secretion, we explored the possibility that blackcurrant proanthocyanidins and IFN-γ may complement each others inhibitory action. We found that pre-incubation of cells with either control (diluted DMSO) media alone or a sub-threshold concentrations of the BC-P (0.5 μg/mL) extract prior to the concomitant incubation of IFN-γ and IL-4 enhanced the suppressive effect of IFN-γ in a dose-dependent manner (Fig. 5B). Moreover, concomitant incubation of either

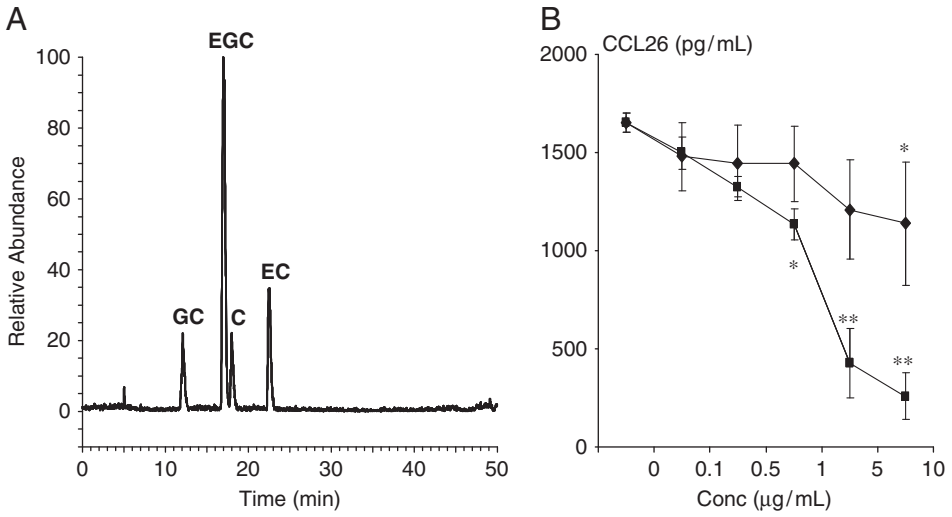

Figure 3. Proanthocyanidin subunits exerted a differential inhibitory action on IL-4-stimulated CCL26 secretion. (A) Solvent extracts of the proanthocyanidin enriched blackcurrant extract (BC-P) were subjected to fullscan spectrum analysis of mass/charge (*m/z*) ratios from 200–500 nm using LC-ESI-MS. The base peak plot chromatogram (200-500 m/z) shows the relative abundance of four catechin compounds; gallocatechin (GC), catechin (C), epicatechin (EC) and epigallocatechin (EGC) (B) Alveolar epithelial (A549) cells were pre-incubated with control (diluted DMSO) media or 0.1–10 μg/mL purified EGC (squares) or EC (diamonds) for 6 h, washed twice and then stimulated with IL-4 (10 ng/mL) for 24 h. Supernatants were collected and measured for CCL26 secretion using ELISA. Data were calculated as IL-4-stimulated CCL26 pg/mL. Results are expressed as mean ± SEM, $n = 5$ separate experiments. *$p < 0.05$ and **$p < 0.01$ represent statistical (ANOVA) difference from cells pre-incubated with DMSO/media only.

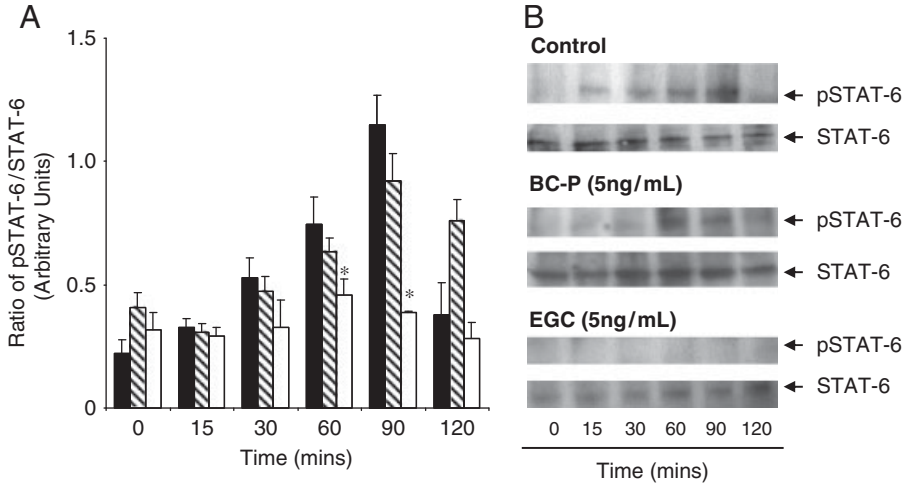

Figure 4. BC-P and EGC modulated IL-4-stimulated STAT-6 phosphorylation. Alveolar epithelial (A549) cells were pre-incubated with control (diluted DMSO) media (filled bars) or 5 μg/mL BC-P (coarse striped bars) or 5 μg/mL EGC (open bars) for 6 h, prior to 0–120 min stimulation with IL-4 (10 ng/mL). 60 μg/mL whole cell extracts (described in Section 2) were separated by SDS-PAGE, transferred to immobilon-P membrane, and screened using either a polyclonal antibody directed against phosphorylated STAT-6 or a monoclonal anti-STAT-6 antibody. (A) Data were calculated as a ratio of phosphorylated STAT-6 to STAT-6 within the same cell extract using densitometry analysis (ImageJ 1.410, National Institute of Health, USA). Results are expressed as mean ± SEM, $n = 3$ independent experiments. *$p < 0.05$ represents statistical (paired Student *t*-test) difference from cells pre-incubated with control media only and those with either BC-P or EGC. (B) Depicts representative immunoblots from one experiment.

1 ng/mL (1320 ± 291 *versus* 966 ± 191 pg/mL, IFN-γ *versus* IFN-γ plus BC-P extract) or 5 ng/mL (1293 ± 274 *versus* 703 ± 90 pg/mL, IFN-γ *versus* IFN-γ plus BC-P, $p < 0.05$) IFN-γ and IL-4 with cells pre-incubated with BC-P extract revealed an inhibition of CCL26 secretion that was greater than the sum of the inhibitory actions observed by the BC-P extract or IFN-γ alone. Pre-incubation of cells with EGC (0.5 μg/mL) followed by concominant incubation with IFN-γ (0.5-5 ng/mL) and IL-4 (10 ng/mL) caused an inhibition that was greater ($p < 0.05$) than the

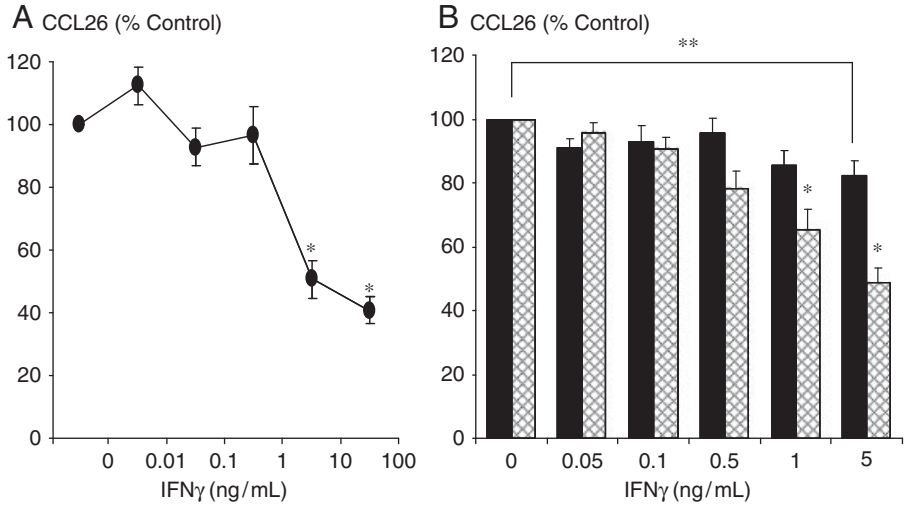

Figure 5. BC-P enhanced IFN-γ ability to suppress IL-4-stimulated CCL26 secretion. (A) Alveolar epithelial (A549) cells were concomitantly incubated with media or IFN-γ (0.01–100 ng/mL) and IL-4 (10 ng/mL) for 24 h. Collected supernatant was measured for CCL26 secretion by ELISA. Data were calculated as IL-4-stimulated CCL26 secretion (% control). Results are expressed as mean ± SEM, $n = 5$. $^{*}p < 0.01$ represents statistical difference (ANOVA) from time zero. (B) A549 cells were pre-incubated with control (diluted DMSO) media (filled bars) or 0.5 μg/mL blackcurrant proanthocyanidin extract (BC-P, crossed bars) for 6 h, washed twice and then concomitantly incubated with media or IFN-γ (0.05–5 ng/mL) and IL-4 (10 ng/mL) for 24 h. Supernatant was collected and measured for CCL26 secretion by ELISA. Data were calculated as IL-4-stimulated CCL26 secretion (% control). Results are expressed as mean ± SEM, $n = 5$. $^{*}p < 0.01$ represents statistical (paired Student *t*-test) difference between cells pre-incubated with media or BC-P cells in the presence of IFN-γ. $^{**}p < 0.05$ represents statistical (paired Student *t*-test) difference between cells pre-incubated with IFN-γ alone.

sum of EGC or IFN-γ alone (Fig. 6). In contrast, pre-incubation of cells with EC (10 μg/mL) had no effect on the inhibitory action of IFN-γ (5 ng/mL) on IL-4-stimulated CCL26 secretion.

3.3.2 Cellular events

3.3.2.1 STAT-6 activation

Pre-incubation of cells with either control (diluted DMSO) media, sub-threshold concentration of either EGC (0.5 μg/mL) or IFN-γ (1 ng/mL) or EC (10 μg/mL) alone for 6 h had no effect on the increased cellular pSTAT-6/STAT-6 ratio induced by IL-4 stimulation after 60 min (Fig. 7). However, a subsequent 24 h incubation of cells with IFN-γ (1 ng/mL) after pre-treatment with EGC (0.5 μg/mL) significantly reduced ($p < 0.05$) the cellular pSTAT-6/STAT-6 ratio induced by IL-4, whereas no synergistic effect was observed in cells pre-treated with EC (10 μg/mL). Furthermore, pre-incubation of cells with EC, EGC or IFN-γ had no effect on cellular STAT-6 levels detected by immunoblot; protein loading verified by cellular actin levels detected in parallel blots.

3.3.2.2 STAT-1 activation

IFN-γ-induced inhibitory action on IL-4-stimulated CCL26 expression involves a suppression of STAT-1 activation. We examined whether the inhibitory action of EGC involved a similar pathway. A 6 h pre-incubation of cells with EGC (5 ug/mL) prior to IFN-γ (10 ng/mL) stimulation (0–90 min) revealed a similar time-dependent increase in the cellular phosphorylated STAT-1/STAT-1 ratio as control cells (Fig. 8). Furthermore, assessment of cellular STAT-1 levels revealed no obvious change in the temporal profile over the 90 min IFN-γ stimulation period and were similar in control or EGC-treated cells; protein loading verified by cellular actin cells.

4 Discussion

The search for suitable foods as a natural alternative or as a complement to traditional therapies for the prevention and/or alleviation of inflammatory related diseases has become the focus of recent research. In particular, berryfruit consumption has been shown to alleviate lung inflammation in animal models [16–18]. In this study we provide supportive evidence that blackcurrant-derived polyphenolic compounds, in particular proanthocyanidins, have the potential to modulate cellular events leading to the suppression of IL-4 and IL-13-stimulated CCL26 secretion, a primary eosinophilic chemokine that facilitates chronic lung inflammation in asthma patients.

The composition of berryfruit-derived polyphenolic subunits appear to demonstrate similar biological outcomes [19–22, 27, 28]. Both anthocyanins and proanthocyanidins have been shown to ameliorate lung inflammation by suppressing oxidative stress and inflammation [16–18];

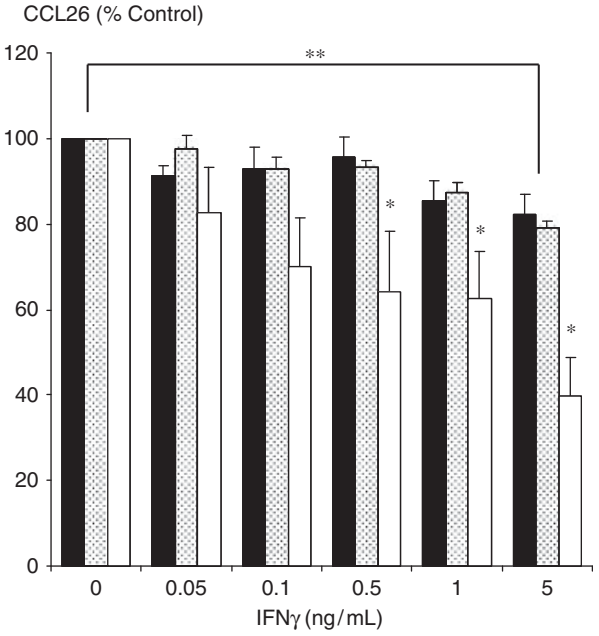

Figure 6. EGC modulation of IFN-γ inhibition of IL-4-stimulated CCL26 secretion. Alveolar epithelial (A549) cells were pre-incubated with control (diluted DMSO) media (filled bars), 10 μg/mL EC (speckled bars) or 0.5 μg/mL EGC (open bars) for 6 h, washed twice and then concomitantly incubated with media or IFN-γ (0.05–5 ng/mL) and IL-4 (10 ng/mL) for a further 24 h. Supernatant was collected and measured for CCL26 secretion using appropriate ELISA. Data were calculated as IL-4-stimulated CCL26 secretion (% control). Results are expressed as mean ± SEM, $n = 5$. $^{*}p < 0.05$ represents statistical (paired Student *t*-test) difference between cells pre-incubated with media or EGC in the presence of IFN-γ. $^{**}p < 0.05$ represents statistical (paired Student *t*-test) difference between cells incubated with IFN-γ alone.

however, the underlying cellular mechanisms that lead to these properties may be diverse and are still relatively unknown. In our study, we found that a blackcurrant proanthocyanidin, but not anthocyanin, extract suppressed IL-4- and IL-13-stimulated CCL26 secretion, suggesting that proanthocyanidin and anthocyanin compounds may modulate distinct (as well as similar) cellular events in the suppression of eosinophilic inflammation. The temporal profile of an allergy-induced immune response involves a variety of coordinated cytokine/chemokine processes [1, 2]. Briefly, after the initial allergen exposure there is an upregulation of pro-inflammatory mediators *e.g.* tumour necrosis factor-α, (TNF-α) which in turn induces CCL11 expression *via* the activation of NF-κB. This is followed by the activation of a number of adaptive immune events including the activation and expression of Th2 cells-derived cytokines (*e.g.* IL-4 and IL-13), which in turn induce CCL26 expression *via* STAT-6. Since both plant-derived anthocyanin and proanthocyanidin extracts have been shown to inhibit NF-κB, it could be suggested that BC-P suppression of eotaxin involves modulation of NF-κB. This may indeed occur for CCL11 expression, where NF-κB and STAT-6 transcription DNA-binding sites on the CCL11 promoter are in close proximity and may contribute to the synergistic action between TNF-α and IL-4 on CCL11 expression [5, 6]; however, may not be the case in the modulation of the CCL26 promoter, where STAT-6 is considered the primary transcription factor [29]. Therefore, the inhibitory action of proanthocyanidin compounds on IL-4-stimulated CCL26 observed in this study may be independent of NF-κB modulation. Moreover, our analysis of blackcurrant proanthocyanidin metabolites, EC and EGC (both shown to suppress NF-κB action [20, 30]), showed a differential effi-

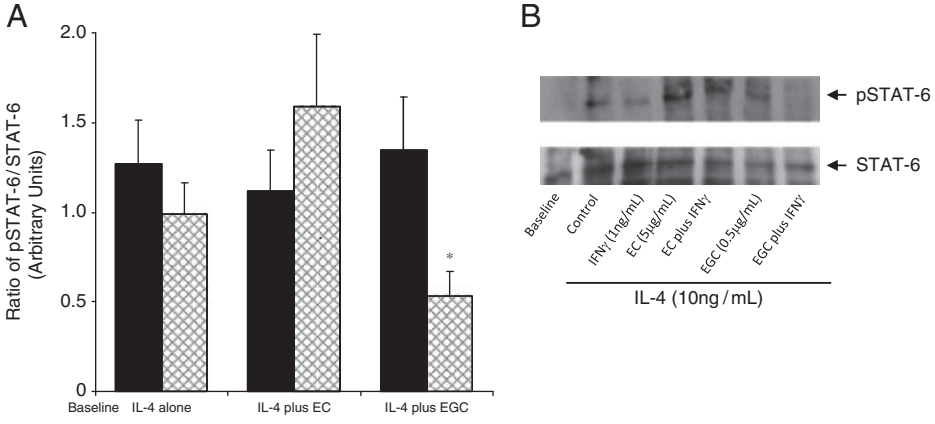

Figure 7. EGC modulation of IFN-γ inhibition of IL-4-activated STAT-6. (A) Alveolar epithelial (A549) cells were either pre-incubated with control (diluted DMSO), EGC (0.5 μg/mL) or EC (10 μg/mL) for 6 h, cells were washed twice, then incubated with either media alone (filled bars) or 1 ng/mL IFN-γ (crossed bars) for a further 24 h before being stimulated with IL-4 (10 ng/mL) for 60 min. 60 μg/mL whole cell extracts (described in Section 2) were separated by SDS-PAGE, transferred to immobilon-P membrane, and screened using either a polyclonal antibody directed against phosphorylated STAT-6 or a monoclonal anti-STAT-6 antibody. (A) Data were calculated as a ratio of phosphorylated STAT-6 to STAT-6 within the same cell extract using densitometry analysis (ImageJ 1.410, National Institute of Health, USA). Results are expressed as mean ± SEM, $n = 3$ independent experiments. $^{*}p < 0.05$ represents statistical (paired Student *t*-test) difference from cells pre-incubated with control media only and those incubated in the presence of IFN-γ. (B) Depicts representative immunoblots from one experiment.

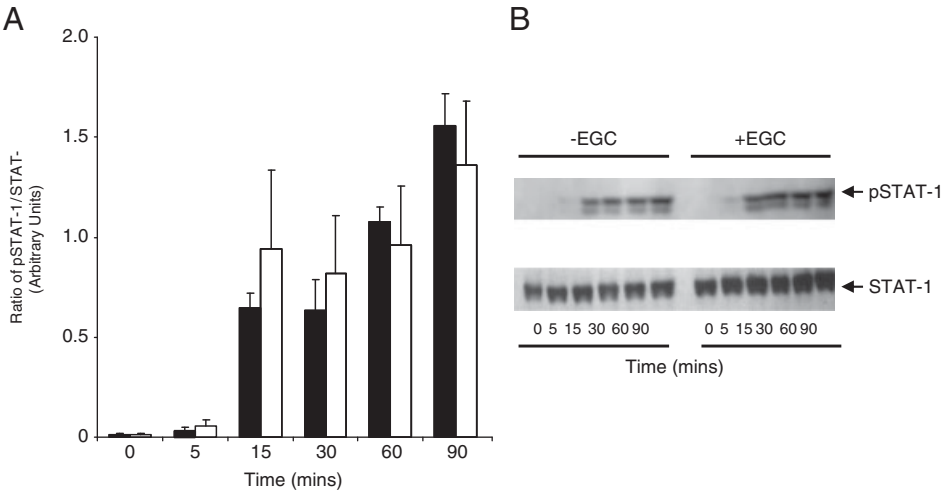

Figure 8. EGC modulation of IFN-γ-induced STAT-1 activation. Alveolar epithelial (A549) cells were pre-incubated with control (diluted DMSO) media (filled bars) or 5 μg/mL EGC (open bars) for 6 h, washed twice and then stimulated with IFN-γ (10 ng/mL) for 0–90 min 60 μg/mL whole cell extracts (described in Section 2) were separated by SDS-PAGE, transferred to immobilon-P membrane, and screened using monoclonal antibodies directed against either phosphorylated STAT-1 or STAT-1. (A) Data were calculated as a ratio of phosphorylated STAT-1 to STAT-1 within the same cell extract using densitometry analysis (ImageJ 1.410, National Institute of Health, USA). Results are expressed as mean ± SEM, $n = 3$ independent experiments. $^{*}p < 0.05$ represents statistical (paired Student *t*-test) difference from cells pre-incubated with control media only and those with EGC. (B) Depicts representative immunoblots from one experiment.

cacy in their ability to suppress IL-4-stimulated CCL26 secretion. EGC not only demonstrated the greater efficacy but also potentiated the inhibitory action of IFN-γ on IL-4-stimulated CCL26 expression. Furthermore, although blackcurrant anthocyanins (and proanthocyanidins) may directly suppress TNFα/IL-4-induced CCL11 expression (this work is on-going in our group), it is also possible that the suppression of eotaxin expression (including CCL26) by anthocyanins observed in an allergy-induced lung inflammation model [16] may be indirectly due to a down-regulation of Th2 cell-derived cytokines, such as IL-13. So although we cannot conclude that STAT-6 and not NF-κB may be involved in BC-P suppression of IL-4 and IL-13-stimulated CCL26 expression, our evidence does support the notion that alternative mechanisms may be involved.

The structure of plant-derived polyphenolic subunits have been shown to dictate its biological function [19–21, 27, 28]. Different polymer lengths identified in proanthocyanidin extract appear to modulate distinct cellular mechanisms leading to the inhibition of oxidative stress and inflammation, for example, large proanthocyanidin polymers interfere with cell–cell interactions [21], mask cell receptor or directly bind to ligand [20], whereas smaller oligomers and individual subunits, (*e.g.* EGC, EC) are intracellular signalling modulations [19, 27, 28]. Therefore, a possible explanation for BC-P ability to inhibit IL-4-stimulated CCL26 secretion may involve proanthocyanidin compounds directly binding to either IL-4/IL-13 or masking of the respective IL-4 or IL-13 receptors. We attempted to limit these possibilities by pre-incubating our cells with the extracts and washing the cells prior to ligand stimulation. However, we concede it is possible that the affinity between these proanthocyanidin compounds and the IL-4 receptor complex may be greater than the washing administered and may contribute to the observed CCL26 suppression. Moreover, although we found that a 6 h wash-out period partially recovery IL-4-stimulated CCL26 secretion, a 24 h wash-out period was required for complete recovery, suggesting that proanthocyanidin compounds may induce receptor internalization. Another possible cell mechanism is BC-P ability to alter the cell's redox status, which may in turn disrupt cellular protein phosphorylation events [31]. EGC has been shown to activate antioxidant responsive element promoter [32] resulting in expression of antioxidant agents such as heme oxygenase-1 which in turn modulate eosinophilic inflammation [22]. Although we observed a temporal suppression of IL-4-stimulated STAT-6 phosphorylation, EGC had no effect on IFN-γ-induced STAT-1 phosphorylation, suggesting that while EGC may not inhibit generic protein phosphorylation, we cannot discount the possibility that changes in the cell's redox status induced by blackcurrant proanthocyanidins may underlie their ability to suppress IL-4-stimulated CCL26 secretion. Furthermore, there is growing number of plant-derived non-cytokine ligands shown to regulate different aspects of cell signalling, for example, retinoic acid inhibition of IL-4-induced eotaxin involves distinctive nuclear receptors that modulate co-stimulators or repressors of STAT-6 rather than the inhibition of STAT-6 phosphorylation translocation to the nucleus [33] and so it is possible, that blackcurrant proanthocyanidins may modulate, currently unknown, regulators of CCL26 expression.

The ability of the BC-P extract to augment IFN-γ-induced suppression of IL-4-stimulated CCL26 secretion suggests

that proanthocyanidin metabolites (especially EGC) may modulate similar cellular events and complement the inhibitory action of IFN-γ on eosinophilic inflammation. IFN-γ has been shown to counteract a number of Th2 cell-mediated cellular events, including eotaxin expression [10–12]. Moreover, IFN-γ negatively regulates STAT6 activation *via* a STAT-dependent pathway [11] and although we found that a sub-threshold concentration of EGC (0.5 μg/mL) acted synergistically with IFN-γ (1 ng/mL) to suppress IL-4-stimulated CCL26 secretion and STAT-6 phosphorylation, EGC had no effect on STAT-1 phosphorylation (data not shown) or IFN-γ-induced STAT-1 phosphorylation. This is supported by other studies [30, 34] that show both EGC and EC had no effect on IFN-γ-mediated STAT-1 activation, whereas, a gallate isoform, EGCG (found primarily in green tea) alleviates IFN-γ-mediated inflammation by preventing STAT-1 phosphorylation.

5 Concluding remarks

Epidemiological studies demonstrate that consumption of selective fruits reduces the incidence and prevalence of asthma especially in children [13, 15]. Moreover proanthocyanidin- and anthocyanin-enriched diets alleviate airway inflammation in animal studies [16, 17, 35]. The bioavailability of plant-derived phytochemicals, although not the focus of this particular study, is an important consideration in the design of a functional food. In particular, blackcurrant-derived proanthocyanidins mainly (>80%) consist of high molecular weight polymers [23], however, recent findings show that these large proanthocyanidins can be broken down by chemical, enzymatic and/or resident microflora in various regions of the digestive tract to release small oligomers and monomers that are easily absorbed, such as EGC and EC [36–39]. Therefore, it is feasible that blackcurrant metabolites, such as EGC, may be able to modulate eotaxin expression in lung tissue. The results in this study, to our knowledge, are the first to demonstrate that blackcurrant proanthocyanidin metabolites suppress IL-4 (and IL-13)-stimulated CCL26 secretion independently, and synergistically with IFN-γ. Understanding which (and how) plant-derived phytochemicals, such as fruit-derived proanthocyanidin and anthocyanin compounds, modulate these cellular events may ultimately lead to the development of functional foods that reduce the risk of allergy-induced asthma and/or allergic conditions in general.

The authors would like to thank Dr. Geoffrey Langford for cultivating and supplying the blackcurrants (Ribes nigrum, cultivar Ben Ard) for this study. Thanks also to Drs Harry Martin and David Stevenson for their constructive advice. This work was funded by The Foundation for Research Science and Technology of New Zealand, grant CO6X0703.

The authors have declared no conflict of interest.

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