

Blackcurrant phenolic extract induces hemoxygenase-1 expression in lung epithelium; involvement in the suppression of Th2 cytokine-induced CCL26 expression

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Introduction

Eosinophilic induced inflammation enhances the airway hyper-responsiveness and obstruction observed in asthma (1). Inflammation cytokines liberated from the T-lymphocyte subset (Th2) during the course of asthma are instrumental in exacerbating its symptoms by promoting further eosinophilic induced inflammation (Figure 1).

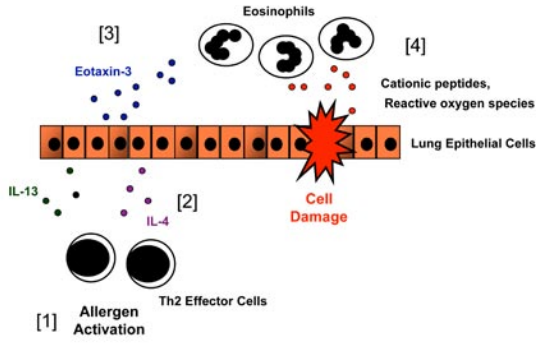


Figure 1: Stages of allergic lung inflammation. Allergen activation [1] stimulates a T cell expansion that leads to the generation of Th2 cytokines [2]. IL-13 and IL-4 induce eotaxin-3 generation from lung epithelial cells [3] which activate eosinophils to liberate damaging cationic peptides and reactive oxygen species [4].

Studies suggest a correlation with healthy lung function and fruit consumption (2, 3). Fruits are high in various polyphenolic compounds and there is evidence developing that such compounds may have immune modulatory actions which are additional to the antioxidant properties. One such mechanism is the induction of heat shock proteins (HSP) by fruit polyphenolic compounds, which may lead to time protection. Fruit polyphenolics may have potential in alleviating allergy-induced inflammation.

Aim

In this study we evaluated the ability of a blackcurrant phenolic extract to induce small HSP in lung epithelium and explore its putative role in attenuating eosinophilic inflammation by specifically investigating its modulation of Th2 cytokine (IL-4/IL-13) induced CCL26 [a major eosinophilic chemottractant in allergy-induced inflammation] expression.

Methodology

A phenolic extract from blackcurrant [*Ribes nigrum* L cv Ben Ard] harvested from New Zealand sources was used in this study. Two lung epithelial cell-lines [alveoli [A549] and bronchial [BEAS2B] cells respectively] were preincubated with phenolic extract of blackcurrant and then analyzed initially for small heat shock protein expression [HSP] followed by exploring their putative involvement in the pathology of Th2-induced eosinophilic airway inflammation.

Preparation of polyphenolic extracts

- Fruits were harvested and snap frozen. Polyphenolics were isolated from the aqueous extract by absorption onto XAD-7 followed by elution with methanol.
- The methanol was evaporated to dryness leaving a red powder, which was dissolved in DMSO for use in study [stock concentration 100mg/ml].

Analysis of small HSP expression

- Cells [1×10^6 cells] were grown in T75 flasks until confluency and then incubated with blackcurrant extract [5ug/ml] for specified time [0-6hrs].
- Protein [20ug], extracted from the cells, was separated by SDS-electrophoresis and analyzed by Western blotting using monoclonal antibodies directed against specific small HSP's; HSP32/HO-1, HSP27, HSP20, α B-crystallin.

Evaluation of Th2-induced CCL26 expression

- Lung epithelial cells [3×10^5 cell/well] were grown to confluency in 12 well plates then preincubated with the blackcurrant phenolic extract [0.5-10ug/ml] or hemin chloride [0.05-10ug/ml] and then challenged with Th2 cytokines; IL-4 [10ng/ml] or IL-13 [10ng/ml].
- Accumulation of CCL26 was determined over 24hrs using a specific CCL26 DuoSet ELISA [R&D systems].
- Involvement of HSP32/HO-1 was explored by the addition of Zinc [II] protoporphyrin IX [ZnPPiX] [1-5uM], which selectively blocks HSP32/HO-1 activity, during both the preincubation with blackcurrant extract and stimulation with Th2 cytokines.

References

- Wills-Karp, M [1999] Immunologic basis of antigen-induced airway hyper-responsiveness. *Annual Rev. Immunol* 17 255-28.
- Butland B.K. et al, [2000]. Diet lung function and lung function decline in a cohort of 2512 middle aged men. *Thorax* 55 102-108
- Forastiere F. et al, [2000]. Consumption of fresh fruit rich in vitamin C and wheezing symptoms in children. *Thorax* 55 283-288.

Results

The Blackcurrant phenol extract [5ug/ml] caused a prominent increase in HSP32/HO-1 [hemoxygenase-1] expression [optimum after 6hrs] in both lung epithelial cell-lines. The blackcurrant phenolic extract had no effect on the expression of other small HSP; HSP27, HSP20 and α B-crystallin [Figure 2].

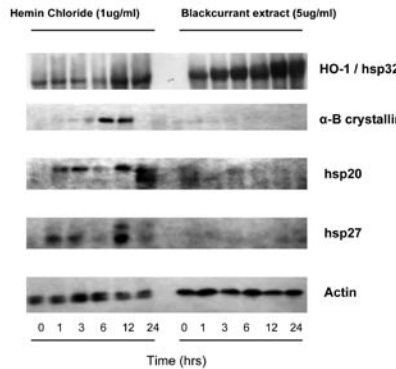


Figure 2: Small HSP expression induced by the 5ug/ml blackcurrant phenolic extract

Simultaneous incubation of the blackcurrant phenolic extract [0-10ng/ml] with Th2 cytokines; IL-4 & IL-13 for 24hrs mediated a concentration-dependent suppression of both IL-4- and IL-13-induced CCL26 expression on both lung cell lines [Figure 3]. The blackcurrant phenolic extract alone had no effect on CCL26 expression or viability of both lung epithelial cell lines [data not shown].

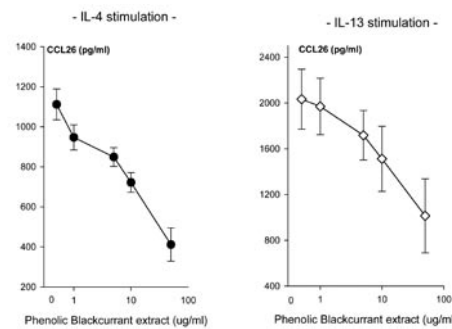


Figure 3: Dose effect of phenolic extracts of blackcurrant and boysenberry on IL-4- and IL-13-induced CCL26 generation

Preincubation [3-6hrs] of the blackcurrant phenolic extract [10ug/ml] with lung epithelial cells followed by Th2 cytokine challenge displayed the optimal suppression of both IL-4- and IL-13-induced CCL26 expression [Figure 4]. In addition, suppression of IL-4- and IL-13-induced CCL26 expression by blackcurrant phenolic extract was maintained for 3 hrs after its removal [Figure 5].

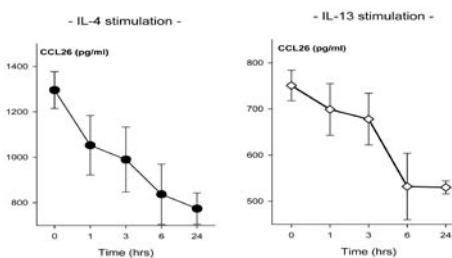


Figure 4: Time profile of suppression of IL-4 and IL-13 stimulated CCL26 generation by the blackcurrant phenolic extract (10ug/ml)

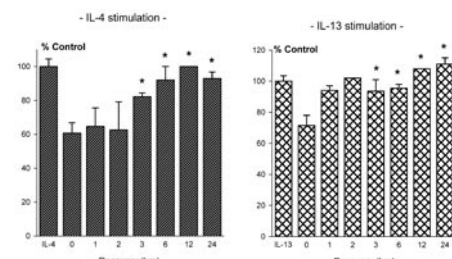


Figure 5: Recovery time course inhibition of blackcurrant phenolic extract (10ug/ml) on IL-4- and IL-13-induced CCL26 generation

Exploration into the involvement of HSP32/HO-1 showed that the presence of ZnPPiX during the preincubation of blackcurrant phenolic extract [5ug/ml] with lung epithelial cells (during the stimulation with either IL-4 or IL-13) dose-dependently estimated the suppressive effect of blackcurrant phenolic extract on Th2 cytokine-induced CCL26 expression [Figure 6].

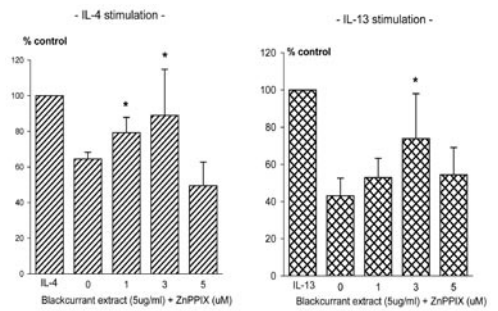


Figure 6: Attenuation by ZnPPiX (inhibitor of hemoxygenase activity) on the blackcurrant phenolic extract's (5ug/ml) ability to suppress IL-4- and IL-13- stimulated CCL26 generation

Further support of this process was demonstrated when preincubation [6hrs] with hemin chloride [known generator of HSP32/HO-1] prior to stimulation of lung cells with Th2 cytokines displayed a dose-dependent suppression of both IL-4- and IL-13-induced CCL26 expression [Figure 7].

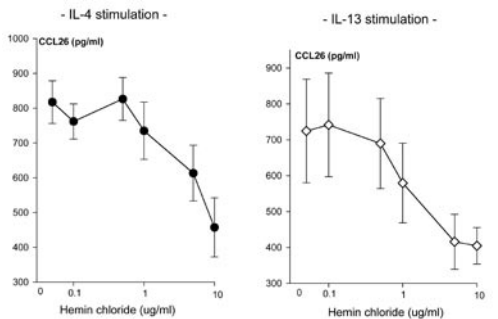


Figure 7: Dose effect of heme chloride on IL-4- and IL-13-induced CCL26 generation

Conclusions

Our results suggest that the blackcurrant phenolic extract upregulates HSP32/HO-1 in lung epithelial cells, which may serve to modulate Th2 cytokines [IL-4/IL-13]-induced eosinophilic inflammation. Fruits or derived functional foods from New Zealand blackcurrants may be beneficial in alleviating airway inflammation.



Introduction

Allergy-induced expression of Th2 cytokines (IL-4 and IL-13) in the lung promotes eosinophilic inflammation. Inflammation cytokines liberated from the T-lymphocyte subset (Th2) during the course of asthma are instrumental in exacerbating its symptoms by promoting further eosinophilic induced inflammation 91, Figure 10.

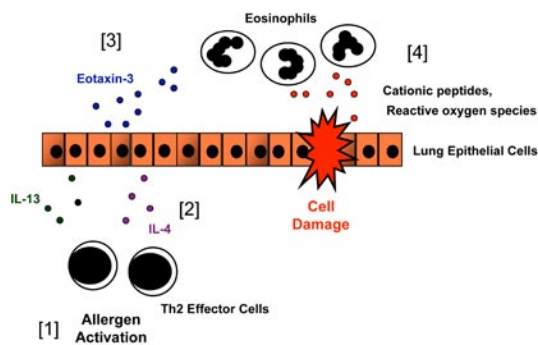


Figure 1: Stages of allergic lung inflammation. Allergen activation [1] stimulates a T cell expansion that leads to the generation of Th2 cytokines [2]. IL-13 and IL-4 induce eosinophilic generation from lung epithelial cells [3] which activate eosinophils to liberate damaging cationic peptides and reactive oxygen species [4].

Studies suggest a correlation with healthy lung function and fruit consumption [2, 3]. Fruits are high in various polyphenolic compounds and there is evidence developing that such compounds may have immune modulatory actions which are additional to antioxidant properties. Our recent findings suggest that some polyphenolic compounds may suppress the induction of eosinophilic chemotactants such as eotaxins (e.g. CCL26), in lung epithelium (poster presentation this meeting by Hurst SM). Furthermore, the Th1 cytokine, IFN γ has also been shown to modulate both Th2 cytokine expression and eosinophilic inflammation.

Aim

In this study we explore the effect of phenolic extracts of blackcurrant and boysenberry fruits on Th2-cytokine [IL-4 and IL-13]-induced CCL26 [eotaxin-3] expression in human lung epithelial cells and explore the putative interaction between the blackcurrant phenolic extract and IFN γ .

Methodology

A phenolic extract from blackcurrant and boysenberry harvested from New Zealand sources was used in this study. In our experiments we used 2 lung epithelial cell-lines (alveoli [A549] and bronchial [BEAS2B] cells respectively) which we preincubated with the phenolic extracts or the Th1 cytokine IFN γ . Cells were then analyzed for involvement in the pathology of Th2- induced eosinophilic airway inflammation.

Preparation of polyphenolic extracts

- Fruits were harvested and snap frozen.
- Extracts were extracted in acetone, evaporated to dryness, and reconstituted in water.
- Polyphenolics were isolated from the aqueous extract by absorption onto XAD-7 followed by elution with methanol.
- The methanol was evaporated to dryness leaving a red powder, which was dissolved in DMSO for use in study (stock concentration 100mg/ml).

Evaluation of Th2-induced CCL26 expression

- Lung epithelial cells (3×10^5 cells/well) were grown to confluency in 12 well plates then simultaneously incubated with the blackcurrant or boysenberry phenolic extract (0.5-10ug/ml) or recombinant IFN γ (0.01-100ng/ml) or combinations in the presence of Th2 cytokines; IL-4 (10ng/ml) for 24hrs.
- Accumulation of CCL26 was determined using a specific CCL26 DuoSet ELISA (R&D systems).

Assessment of CCL26 transcript

- CCL26 transcript stability:** Lung epithelial cells (5×10^5 cells/flask) were grown to confluency and stimulated with IL-4 (10ng/ml) for 4hrs. The cells were then washed and incubated with actinomycin D (5ug/ml) +/- blackcurrant phenolic extract (5ug/ml) for specified times (0.5-6hrs).
- CCL26 mRNA expression:** Lung epithelial cells (5×10^5 cells/flask) were grown to confluency and incubated +/- blackcurrant phenolic extract (5ug/ml) for specified times (1-6hrs). The cells were then washed and incubated +/- IL-4 (10ng/ml) for 4hrs.
- Total RNA was extracted and RT-PCR using superscript III/Taq reagents (Invitrogen) and specific nucleotide primers designed from human CCL26 cDNA. GAPDH was used as control for RNA loading.

Determination of STAT-6 phosphorylation

- Lung epithelial cells (1×10^6 cells/flask) were grown to confluency and were incubated with blackcurrant, boysenberry or IFN γ or specified combination for 24hrs.
- Cells were then washed and stimulated with IL-4 (5ng/ml) for 15mins, placed immediately on ice and nuclear proteins extracted. 10ug protein was separated by SDS-electrophoresis and analyzed by Western blot using specific monoclonal antibodies directed against either STAT-6 (R&D systems) or phosphorylated STAT-6 (Sapphire BioScience).

Results

Simultaneous incubation of blackcurrant phenolic extract (0-10ng/ml) or boysenberry (0-10ug/ml) with the Th2 cytokine, IL-4 for 24hrs mediated a concentration-dependent suppression of IL-4-induced CCL26 expression on both lung cell lines. (Figure 2). In addition, neither the blackcurrant or boysenberry phenolic extracts alone had an effect on CCL26 expression or viability of both lung epithelial cell-lines (data not shown).

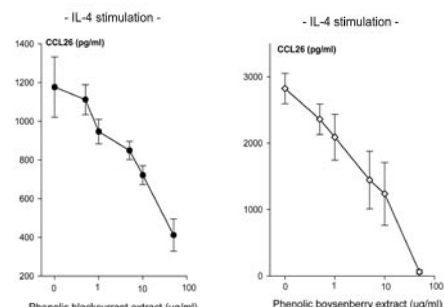


Figure 2: Dose effect of a blackcurrant and boysenberry extract on IL-4-induced CCL26 expression

Simultaneous incubation of the lung cells with blackcurrant phenolic (0.1-5ug/ml) extract in the presence of a sub-threshold concentration of boysenberry phenolic extract (0.1ug/ml) mediated potentiation of the blackcurrant phenolic extract's ability to suppress IL-4-induced CCL26 expression (Figure 3). Similar results were obtained with IL-13 (data not shown).

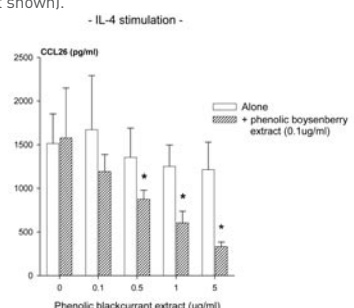


Figure 3: Boysenberry phenolic extract (0.1ug/ml) potentiation of blackcurrant phenolic extract (0.1-5ug/ml) suppression of IL-4-stimulated CCL26 generation

The blackcurrant phenolic extract had no effect on the stability of IL-4-induced CCL26 mRNA in lung epithelial cells (Figure 4) plus no adverse affect was identified on the bioavailability of IL-4 (data not shown) in experiments.

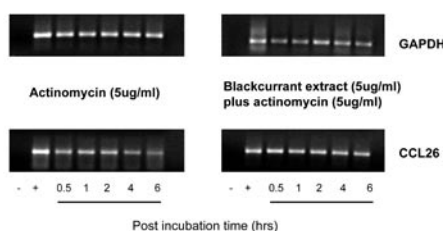


Figure 4: The effect of blackcurrant phenolic extract (5ug/ml) on the stability of IL-4-induced CCL26 transcript

Conclusions

These data suggest that blackcurrant and boysenberry phenolic extracts act synergistically to suppress IL-4-induced CCL26 in lung epithelial cells, which involves the prevention of STAT-6 phosphorylation and subsequent CCL26 transcription. IFN γ enhances the ability of blackcurrant phenolic extract to suppress IL-4-induced CCL26 expression, which in part, involves the prevention of STAT-6 phosphorylation. These data give mechanistic insights into the potential value of fruits or derived-functional foods that might be beneficial in alleviating airway inflammation.

Preincubation (6-24hrs) of blackcurrant phenolic extract (5ug/ml) with lung epithelial cells demonstrated an attenuation of IL-4-induced phosphorylation of STAT-6 transcription factor (Figure 4). Further experiments showed that the blackcurrant phenolic (5ug/ml) extracted caused a suppression of IL-4-induced CCL26 mRNA expression, which was evident by 4hrs. Furthermore, preincubation of blackcurrant phenolic extract (5ug/ml) with the lung cells alone did not induced CCL26mRNA expression (Figure 6).

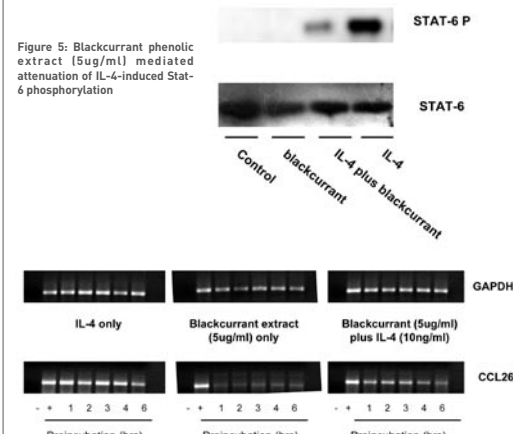


Figure 6: Effect of blackcurrant phenolic extract (5ug/ml) on IL-4-induced CCL26 expression

Simultaneous incubation of IFN γ (0.01-100ng/ml) with lung epithelial cells for 24hrs demonstrated a concentration-dependent suppression of IL-4-induced CCL26 expression. Co-incubation of the lung cells with the blackcurrant phenolic extract (0.1-5ug/ml) and a sub-threshold concentration of IFN γ (1ng/ml) caused an additive effect on the suppression of IL-4-induced CCL26 expression (Figure 7). Similar results were observed with IL-13 (data not shown).

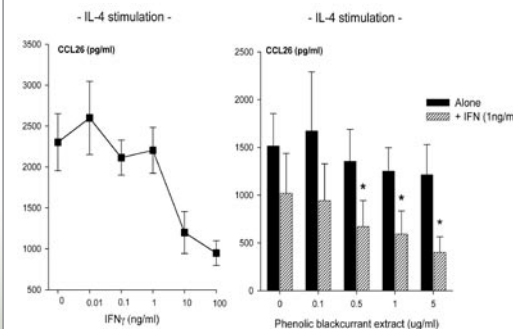


Figure 7: Dose effect of IFN γ +/- blackcurrant phenolic extract on IL-4-stimulated CCL26 generation

Co-incubation of lung cells with the blackcurrant (0.5ug/ml) and boysenberry (0.5ug/ml) phenolic extract mediated an enhanced suppression of STAT-6 phosphorylation when incubated alone (Figure 8). Furthermore, IFN γ suppressed IL-4-induced STAT-6 phosphorylation in lung epithelial cells.

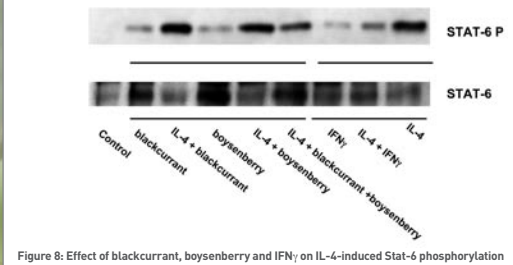


Figure 8: Effect of blackcurrant, boysenberry and IFN γ on IL-4-induced Stat-6 phosphorylation