This is the published version of:

Liravi, B., et al. (2015) Dynamics of IL-4 and IL-13 expression in the airways of sheep following allergen challenge. BMC Pulmonary Medicine, 15(1), pp. 1-11.

Available online at http://doi.org/10.1186/s12890-015-0097-9

Copyright © 2015 Liravi, B., et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0) (http://creativecommons.org/licenses/by/4.0/). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.
Dynamics of IL-4 and IL-13 expression in the airways of sheep following allergen challenge

Bahar Liravi 1, David Piedrafita 2, Gary Nguyen 1 and Robert J. Bischof 3*

Abstract

Background: IL-4 and IL-13 play a critical yet poorly understood role in orchestrating the recruitment and activation of effector cells of the asthmatic response and driving the pathophysiology of allergic asthma. The house dust mite (HDM) sheep asthma model displays many features of the human condition and is an ideal model to further elucidate the involvement of these critical Th2 cytokines. We hypothesized that airway exposure to HDM allergen would induce or elevate the expression profile of IL-4 and IL-13 during the allergic airway response in this large animal model of asthma.

Methods: Bronchoalveolar lavage (BAL) samples were collected from saline- and house dust mite (HDM)-challenged lung lobes of sensitized sheep from 0 to 48 h post-challenge. BAL cytokines (IL-4, IL-13, IL-6, IL-10, TNF-α) were each measured by ELISA. IL-4 and IL-13 expression was assessed in BAL leukocytes by flow cytometry and in airway tissue sections by immunohistology.

Results: IL-4 and IL-13 were increased in BAL samples following airway allergen challenge. HDM challenge resulted in a significant increase in BAL IL-4 levels at 4 h compared to saline-challenged airways, while BAL IL-13 levels were elevated at all time-points after allergen challenge. IL-6 levels were maintained following HDM challenge but declined after saline challenge, while HDM administration resulted in an acute elevation in IL-10 at 4 h but no change in TNF-α levels over time. Lymphocytes were the main early source of IL-4, with IL-4 release by alveolar macrophages (AMs) prominent from 24 h post-allergen challenge. IL-13 producing AMs were increased at 4 and 24 h following HDM compared to saline challenge, and tissue staining provided evidence of IL-13 expression in airway epithelium as well as immune cells in airway tissue.

Conclusion: In a sheep model of allergic asthma, airway inflammation is accompanied by the temporal release of key cytokines following allergen exposure that primarily reflects the Th2-driven nature of the immune response in asthma. The present study demonstrates for the first time the involvement of IL-4 and IL-13 in a relevant large animal model of allergic airways disease.

Background

Asthma is a chronic inflammatory disease of the lungs characterized by inflammation, airway hyperresponsiveness (AHR) and airway wall remodelling. Atopic asthmatics display high levels of allergen-specific immunoglobulin E (IgE) antibodies, and this is associated with the development of a type 2 immune response with evidence of elevated expression levels of T-helper type 2 (Th2) cytokines [1]. The Th2 cytokines, including interleukin (IL)-4, IL-5, IL-9, IL-13 and IL-25, together promote key pathophysiological features of asthma including allergen-specific IgE, airway inflammation (characterized by activated lymphocytes, eosinophils, mast cells and macrophages), damage to the airway epithelium, mucus gland hyperplasia and structural remodelling of the airway wall [2–4]. Other cytokines have been implicated in the pathogenesis of asthma. For example, IL-6 is a marker of inflammation and serves as an important regulator of effector CD4+ T cell fate by promoting IL-4 production during Th2 differentiation while inhibiting Th1 differentiation [5]. An
immunoregulatory role for IL-6 in asthma and other pulmonary diseases where the lung epithelium is damaged has been highlighted [6]. There is strong evidence that IL-4 and IL-13 play a crucial role in orchestrating the recruitment and activation of the effector cells of the asthmatic response. IL-4 is an essential trigger for Th2 lymphocyte differentiation, and both IL-4 and IL-13 can induce IgE class switching in B lymphocytes. Additionally, IL-4 and IL-13 act on bronchial epithelial, endothelial and airway smooth muscle cells, collectively leading to many of the pathophysiological features of asthma [3, 7].

Clinical observations of IL-4 in allergic asthma include increased IL-4 in the serum and bronchoalveolar lavage (BAL) of allergic individuals [8, 9], while nebulized IL-4 given to patients with mild asthma results in a significant increase in AHR associated with the elevation of sputum eosinophil numbers [10]. Studies using IL-4 deficient mice indicate that the main role of IL-4 in allergic airway inflammation is during the initial priming of Th2 effector cells [3, 11].

In murine and human studies, IL-13 has been shown to be directly responsible for eosinophil survival and proliferation within lung tissue, the release of mediators responsible for bronchoconstriction and the induction of mucus hypersecretion (reviewed in [12]). IL-13 mRNA expression has been reported in bronchial biopsies from both allergic and non-allergic asthmatic subjects [13], and IL-13 in BAL following allergen provocation of asthmatic subjects is strongly correlated with an increase in eosinophil numbers [14, 15]. In mice, human IL-13 promotes many of the inflammatory changes associated with asthma, such as inflammatory cell infiltration in the lungs and AHR and goblet cell metaplasia [16, 17]; treatment with an anti-IL-13 monoclonal antibody (mAb) has been shown to be effective in mice [18], although poorer outcomes have been realised to date in human clinical trials [19, 20].

A sheep model of asthma based on the relevant allergen house dust mite (HDM) displays many key features of the human asthmatic condition [21, 22]. Sheep sensitized to HDM develop allergen-specific IgE responses, inflammation of the airways following airway allergen challenge that includes profound eosinophilia, airway epithelial mucus hypersecretion, airway wall remodelling and early and late-phase asthmatic responses and AHR following airway allergen challenge [22–24]. Unfortunately, our current understanding of the role of Th2 cytokines in larger animal models of asthma, where experimental therapeutic and in real-time manipulation is viable, is limited to a description or phenotypic analysis of inflammatory cells involved in the allergic response (reviewed in [25, 26]), and a single study in non-human primates reporting elevated mRNA expression of IL-4 and IL-13 in HDM-challenged airways [27].

The current study aimed to investigate the expression of IL-4 and IL-13, as well as IL-6, IL-10 and TNF-α, in the sheep model of asthma following allergen challenge of the airways. We hypothesized that airway allergen challenge would induce an elevated expression profile of two critical Th2 cytokines, IL-4 and IL-13, in the HDM sheep model of allergic asthma.

**Methods**

**Animals and HDM sensitization and challenge protocols**

Merino-cross ewes (8–12 months old), treated orally with anthelmintic to eliminate any worm parasites prior to the experiment, were housed in indoor pens and fed ad libitum. All experimental work was approved by the Monash University Animal Ethics Committee.

Sheep were sensitized by subcutaneous immunizations with a solubilized preparation of house dust mite extract (HDM; CSL Ltd, Parkville, Australia) as detailed elsewhere [23, 28]. Sensitized animals (HDM-specific IgE responders) were rested for 3 weeks, then given segmental airway challenges with HDM (500 μg in 5 ml) at weekly intervals over 3 weeks (ie. three challenges over 3 weeks) using a flexible fibre-optic endoscope (Model FG-16×, Pentax, NJ, USA). The airway challenges for each animal involved a segmental bolus infusion of 5 ml HDM solution into each of 3 discrete lung lobes (HDM administered only to the left caudal, right caudal and right middle lobes; see Fig. 1 and [22]) and a 5 ml infusion of sterile saline into each of three control lung lobes (saline administered...
only to the left cranial, right cranial and accessory lobes; Fig. 1 and [22]). Thus, over the entire period of airway challenges, each separate lung lobe was exposed to only saline or HDM; further, in each animal and on each challenge occasion, HDM and saline segmental challenges were given at the same time-point. At the time of the third and final airway challenge with saline/HDM, BAL collections were performed on individual lobes for subsequent cellular and cytokine analyses (as detailed below).

Bronchoalveolar lavage (BAL) sampling

On the occasion of the final (3rd) airway challenge, BAL collections with sterile saline were performed on separate lung lobes at 0, 4, 24 and 48 h following airway saline/HDM challenge using a fibre-optic endoscope [23, 28] and samples placed on ice before being centrifuged at 400 g to separate BAL cells from BAL fluid. BAL fluid samples were concentrated by centrifugation filtration using 3 kDa nominal molecular weight limit devices (Amicon Ultra-15, Millipore, Bedford, MA, USA) following the manufacturer instructions and stored at −80 °C prior to cytokine quantitation (see below). Pelleted BAL cells were resuspended in 5 ml of sterile PBS and used for flow cytometry (see below). Total cell counts were determined using a haemocytometer and presented as cells/ml BAL fluid. Differential cell counts (total of 200 cells) were performed on cytospot preparations stained with Kwik Diff™ solution (Thermo Fisher Scientific, MA, USA) to enumerate proportions of macrophages, lymphocytes, eosinophils and neutrophils.

Cytokine detection in BAL samples

Protein levels of IL-4, IL-6, IL-10, IL-13 and TNF-α in BAL samples were determined using ovine-specific ELISAs. Antibodies and standards used are detailed in Table 1, with IL-4, IL-6, IL-10 and TNF-α ELISA protocols as previously reported [29–31], and the IL-13 ELISA protocol developed and optimized in-house.

BAL cell characterization by flow cytometry

For intracellular cytokine staining, BAL cells were resuspended in cell culture medium (DMEM containing 10 %

| ELISA | Antibody (Ab)/cytokine details | Clone/type | Dilution | Source |
|-------|------------------------------|------------|----------|--------|
| IL-4  | rov IL-4 (cytokine standard) | -          | -        | Moredun a |
|       | Anti-bovine IL-4 mAb (coating) | CC313 | 1:400 | AbD Serotec b |
|       | Anti-bovine IL-4/biotinylated mAb (detecting) | CC314 | 1:1600 | AbD Serotec |
|       | Streptavidin/HRP | - | 1:1000 | Dako c |
| IL-6  | rov IL-6 (cytokine standard) | -          | -        | CAB d |
|       | Anti-ovine IL-6 mAb (coating) | 486 | 1:400 | Epitope e |
|       | Anti-ovine IL-6, pAb (detecting) | rabbit pAb | 1:5000 | CAB |
|       | Anti-rabbit Ig/HRP | - | 1:1000 | Dako |
| IL-10 | rbov IL-10 (cytokine standard) | -          | -        | Moredun a |
|       | Anti-bovine IL-10 mAb (coating) | CC318 | 1:200 | AbD Serotec |
|       | Anti-bovine IL-10/biotinylated mAb (detecting) | CC320 | 1:1000 | AbD Serotec |
|       | Streptavidin/HRP | - | 1:1000 | Dako |
| IL-13 | rbov IL-13 (cytokine standard) | -          | -        | Kingfisher f |
|       | Anti-bovine IL-13, pAb (coating) | rabbit pAb | 1:800 | Kingfisher |
|       | Anti-bovine IL-13/biotinylated pAb (detecting) | rabbit pAb | 1:2000 | Kingfisher |
|       | Streptavidin/HRP | - | 1:1000 | Dako |
| TNFα | rov TNFα (cytokine standard) | -          | -        | CAB |
|       | Anti-ovine TNFα mAb ascites (coating) | 6.09 | 1:200 | CAB |
|       | Anti-ovine TNFα pAb (detecting) | rabbit pAb | 1:500 | CAB |
|       | Anti-rabbit Ig/HRP | - | 1:1000 | Dako |

rov Recombinant ovine, rbov Recombinant bovine, mAb Monoclonal antibody, pAb polyclonal antibody

aMoredun: Dr Gary Entrican, Moredun Research Institute, Edinburgh, Scotland
bAbD Serotec: AbD Serotec, UK
cDako: Agilent Technologies Inc., CA, USA
dCAB: Centre for Animal Biotechnology, School of Veterinary Science, Melbourne, Australia
eEpitope: Epitope Technologies Pty Ltd, VIC, Australia
fKingfisher: Kingfisher Biotech, Inc, MN, USA
fetal bovine serum (FBS) and 0.1 % gentamycin; Life Technologies, CA, USA) at 10^6 cells/ml and incubated in the presence of Brefeldin A (20 μg/ml; Cell Signaling Technology, MA, USA) for 2 h, then washed and resuspended in intracellular blocking buffer (PBS, 0.1 % saponin, 5 % normal horse serum, 5 % normal sheep serum (NSS), 5 % FBS, 0.01 % sodium azide) to reduce non-specific binding. Cells were then incubated for 30 min at 4 °C with antibodies (Abs) to IL-4 (FITC-conjugated anti-bovine IL-4 mAb, 1:100; AbD Serotec, UK), or IL-13 (rabbit anti-bovine IL-13 polyclonal antibody (pAb), 1:100; Kingfisher Biotech Inc., MN, USA) with matching secondary Ab (Alexa Fluor™ 488 goat anti-rabbit IgG, 1:200; Jackson ImmunoResearch Laboratories Inc., PA, USA). Cells were washed and fixed in 2 % paraformaldehyde (PFA; Fluka, St. Gallen, Switzerland) in PBS and stored at 4 °C prior to analysis by flow cytometry. Leukocyte cell populations were gated on the basis of forward scatter (FSC) and side scatter (SSC) characteristics [23] and 10,000 events were acquired using a BD LSR II flow cytometer (Becton Dickinson Biosciences, CA, USA), with data analyzed using FlowJo™ software (TreeStar Inc, OR, USA).

**Immunostaining of lung tissue**
Lung tissues were collected at post-mortem, 48 h post-allergen challenge, and embedded in Optimal Cutting Temperature (OCT) medium (Tissue Tek, Miles Inc., PA, USA) and stored at −80 °C prior to immunostaining. Frozen tissue sections (5 μM) were cut onto glass microscope slides, then air-dried and fixed in 2 % PFA. Slides were washed in PBS/0.1 % Tween 20 and incubated with anti- IL-4 (anti-bovine IL-4 mAb, 1:100, clone CC313; AbD Serotec) or a combination of anti-IL-13 (as detailed above) and anti-CD45 (common leukocyte marker; [32]) antibodies. Secondary Abs included Alexa Fluor™ 594 anti-mouse Ig (1:1000; Life Technologies) for IL-4 and CD45, and Alexa Fluor™ 488 anti-rabbit IgG (1:200; Jackson ImmunoResearch) for IL-13 detection. Slides were then washed and mounted with Mowiol mounting medium (Calbiochem, CA, USA) containing 4’, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1:5000; Life Technologies).

**Statistical analysis**
To assess the effect of saline or HDM challenge as well as time following challenge on BAL cell numbers, cell percentages, cytokine levels and cytokine expressing cells, a two-way ANOVA test was performed followed by a Holm-Sidak test to correct for multiple comparisons. Differences were considered significant for p < 0.05. All data are reported as the mean ± standard deviation (SD).

**Results**
**Analysis of BAL cells following airway allergen challenge**
BAL leukocyte numbers from HDM- and saline-challenged lungs before and at 4, 24 and 48 h post-challenge are shown in Fig. 2. In saline-challenged lobes there was little change in leukocyte numbers across the different time points. Conversely, in HDM-challenged lung lobes there was initially a decline in total BAL leukocyte number at 4 h, followed by a significant increase by 24 h post-challenge (Fig. 2).

While macrophages were the major cell component in BAL, there was an initial decline in numbers at 4 h after saline or HDM challenge, followed by a return to similar baseline levels within 48 h post-challenge (Fig. 3a). Macrophages represented ~90 % of BAL at baseline but this was reduced at all time points post-challenge (Fig. 3e). Lymphocyte numbers showed little change over time (Fig. 3b), although the percentage of lymphocytes in BAL increased at 4 h in saline- and HDM-challenged lobes compared to pre-challenge (Fig. 3f). There was a significant increase in neutrophils in BAL over the first 4–24 h after saline or HDM challenge (Fig. 3c, g). In contrast, eosinophils that were absent in BAL prior to airway challenge were recruited into BAL following HDM challenge, reaching their maximum at 24–48 h (Fig. 3d, h).

**BAL cytokine profiles**
IL-4, IL-13, IL-6, IL-10 and TNF-α levels were assessed in BAL samples collected after saline or HDM challenge (Fig. 4). BAL IL-4 levels were greater in HDM-challenged lobes compared to saline at 4 h but decreased between 4 and 48 h after HDM challenge (Fig. 4a). IL-13 levels in HDM-challenged lobes were significantly higher than saline-challenged lobes at each of the time points examined (Fig. 4b). IL-6 levels at 4 h post-challenge showed no significant difference between HDM and saline treated lobes, while greater levels of IL-6 were detected in HDM-challenged lobes compared to saline.
Fig. 3 Kinetics of leukocyte sub-population traffic into BAL fluid over time following saline and HDM bronchial challenges, showing changes in (a-d) cell number and (e-h) cell percentage over time. Data presented as mean counts ± SD for n = 6 sheep (*denotes significant difference between HDM and saline-challenged lobes at the corresponding time point: *p < 0.05, **p < 0.01; * denotes significant difference between time points: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

Fig. 4 Cytokine protein levels over time (0–48 h) showing (a) IL-4, (b) IL-13, (c) IL-6, (d) IL-10 and (E) TNF-α in BAL fluid following saline and HDM bronchial challenges. Data presented as means ± SD for n = 10 sheep (*denotes significant difference between HDM and saline-challenged lobes at the corresponding time point: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; * denotes significant difference between time points: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)
at 24 and 48 h (Fig. 4c). Further, there was a significant decline in BAL IL-6 levels from the 0 h/4 h time-point to 24 and 48 h after saline challenge. IL-10 levels were increased at 4 h and decreased at 48 h in HDM-compared to saline-challenged lobes (Fig. 4d). TNF-α levels in HDM-challenged lobes were similar to saline-challenged lobes at the examined time points and there was no change in BAL over time (Fig. 4e).

**Intracellular expression of IL-4 and IL-13 in BAL cells**

BAL cell subpopulations were identified in the pre- (0 h) and post-challenge (4 h, 24 h, 48 h) BAL samples, with gating based on forward- and side- light scatter (FSC, SSC) properties as reported elsewhere [23]. BAL cell subpopulations were then examined for intracellular IL-4 and IL-13 expression and analyzed by flow cytometry.

There was a marked but transient increase in the proportion and total number of IL-4⁺ lymphocytes from 0 to 4 h in HDM-challenged lungs, followed by a return to baseline levels from 24 h post-challenge; the increase in IL-4 expression at 4 h was significant compared to saline-challenged lobes at that time-point (Fig. 5a & d). There was a significant increase in the percentage and total number of IL-4⁺ macrophages at 24 h post-HDM challenge compared to earlier time-points and saline challenge (at 24 h), with a subsequent decline to baseline levels at 48 h post-challenge (Fig. 5b & e). Of the granulocytes recruited into the lungs at 24 h post-HDM challenge, the proportion and the total number of these cells expressing IL-4 was greater in HDM- compared to saline-challenged lobes, although no difference was observed at 48 h (Fig. 5c & f).

A significant decline in the proportion of lymphocytes expressing IL-13 was seen in both saline- and HDM-challenged lobes from 4 to 24 h post challenge followed by an increase in saline-challenged lungs at 48 h (Fig. 6a); a similar pattern of change in cell number was seen following saline challenge (Fig. 6d). The percentage of IL-13⁺ macrophages in HDM-challenged lobes was higher than in saline-challenged lobes at 4 and 24 h post-challenge (Fig. 6b). In HDM-challenged lungs, there was a decrease in the proportion of macrophages positive for IL-13 from 4 to 24 h, followed by an increase in the proportion (but not total cell number) of both macrophages and granulocytes at 48 h (Fig. 6b, c, e & f). The total number of IL-13 expressing macrophages was not

![Fig. 5](image-url)
significantly altered in HDM-challenged lobes but the number of these cells in saline-challenged lobes increased at 48 h compared to 4 and 24 h (Fig. 6e).

**IL-4 and IL-13 expression in lung tissue**

Post-mortem lung tissues were collected 48 h after a HDM allergen challenge and frozen tissue sections cut for immunostaining to examine IL-4 and IL-13 expression (Fig. 7). Intracellular expression of IL-4 and IL-13 was observed in cells within the lung tissues, and this was localized particularly within and below the epithelial layer (Fig. 7b and d). Dual staining of the lung tissue sections for IL-13 and CD45 (leukocyte common antigen) clearly showed that IL-13 was expressed in both immune (IL-13+CD45+) and non-immune (IL-13−CD45−) cells within the HDM-challenged airway tissues (Fig. 7d).

**Discussion**

The pathophysiological features of allergic asthma are characterized by airway inflammation and structural and functional changes in the lung. These responses are directed by the actions of a number of key cytokines. IL-4 and IL-13 are two such cytokines that have been shown to play a central role in directing the pathophysiological changes in allergic asthma [3]. The role of Th2 cytokines has been extensively studied in small animal models of asthmatic disease and much of our current understanding arises from basic mechanistic, knockout and transgenic studies, and therapeutic interventions [26]. There is clear potential and much interest in the development of targeted anti-Th2 cytokine therapies for asthma [33]. However, mixed success with regard to anti-IL-4 and anti-IL-13 interventions in clinical trials to date [19, 20] confirms the need for a better understanding of the mechanisms of pathogenesis underlying the different Th2 ‘endotypes’ seen in asthma [34–37]. The ability to perform detailed kinetic studies and real-time measurements as well as dose—response efficacy studies and therapeutic interventions, requires the development and validation of large animal models that provide clarity of disease onset and progression. Such investigations will help to improve our understanding of the role for IL-4 and IL-13 in human asthmatic disease and identify effective, targeted therapeutic strategies [22].

There is comparatively little known about the role for Th2 cytokines in allergic airways disease in larger animal...
models such as dog, sheep, horse and non-human pri-
mates, although it appears Type 2 or Th\textsubscript{2} immune path-
ways are involved, establishing some parallels between
these larger animal models and human allergic asthma
[26, 38–40]. In the present study we used a sheep model
of allergic asthma to investigate the kinetics of expres-
sion of IL-4 and IL-13 in allergen-challenged airways,
with the goal to extend our understanding of Th\textsubscript{2}-driven
mechanisms in this large animal model system.

IL-4 and IL-13 are required for the IgE class switching
in B lymphocytes [41], and increased BAL levels of IL-4
and IL-13 are consistent with elevated allergen-specific
IgE in serum [23, 28] and BAL fluid [28] in the sheep
asthma model. The early elevation of these Th\textsubscript{2} cytokines
followed by the recruitment of neutrophils, lymphocytes
and macrophages in this model, precedes the late phase
allergic response associated with the appearance of eosin-
ophils from 24 to 48 h after allergen exposure. The fact
that eosinophils were detected in saline-treated control
lung lobes might suggest some degree of a systemic (ie
lung-wide) response to segmental lung allergen exposure
in this model. Indeed, altered BAL cellularity in control
lobes has also been observed by others that have used re-
peated segmental airway challenge to study inflammation/
infection in sheep [42]. In our own experience and that of
others [43], it has been shown in sheep that repeated BAL
sampling has no effect itself on BAL cellularity over time,
and sampling from different lung lobes is comparable.

We found elevated IL-4 levels in BAL across all time-
points examined following allergen challenge. Lympho-
cytes, most likely CD\textsuperscript{4}+ T cells [23], were a major source
of IL-4 in the immediate hours (4 h) after allergen chal-
lenge, while IL-4 expressing alveolar macrophages (AMs)
were predominant in BAL at the later (24 and 48 h) time-
points. IL-13 was elevated in BAL at 4 h post challenge,
with lymphocytes (4 h) and AMs (4–48 h) found to be the
major sources of IL-13 post allergen challenge. The
immunostaining of airway tissue showed that the bron-
chial epithelium also serves as a cellular source for IL-13
in sheep airway tissues following allergen challenge, simi-
lar to that reported elsewhere [44]. Eosinophils and mast
cells may also be a source of IL-4 and IL-13 [3, 4], and
in this study and earlier investigations these cells have
been shown to be a key feature of the inflammation
seen in the sheep model of allergic asthma [22]. Indeed,
the greater levels of intracellular IL-13 expression in BAL
granulocytes from 24 to 48 h post-allergen challenge is
associated with an increased eosinophil recruitment into
BAL at this time. While not shown in the present study, a significant correlation between IL-13 expression and eosinophils in BAL has been reported elsewhere [14, 15].

The elevated IL-4 and IL-13 levels seen in the present study reinforces the idea that Th2 lymphocytes are key cellular players in directing the early immune response to allergen re-exposure in the sheep asthma model. Moreover, our observations with respect to the cellular source of these cytokines also implicate significant changes in the airway macrophage population and their involvement in the pathophysiology of allergic airway responses in sheep.

AMs are the principal cellular sentinels of the respiratory tract that are continually exposed to potentially inflammatory stimuli, and there is good evidence to suggest they become differentially activated during asthma responses [45]. Both IL-4 and IL-13 are involved in the alternative activation, or differentiation of the 'M2 class' of macrophages [46, 47] and recent studies have identified important roles for airway macrophages in allergic inflammation, including promotion of Th2 reactivity and airway tissue remodelling [48, 49]. M2 macrophages have been shown to be more abundant in the BAL and airway tissues of asthmatics compared with healthy subjects [50]. Further, enhanced levels of IL-13 producing macrophages have been found in the BAL from subjects with severe asthma, suggesting that M2 macrophages may contribute to reduced lung function in asthma patients [51].

In the present study, there was an initial decline observed in AM numbers following allergen challenge but also a clear shift in their functional phenotype, with an increase in the proportion of IL-4 (24 h) and IL-13 (4 and 24 h) producing AMs, suggesting the emergence of alternatively activated macrophages [52, 53] and a likely contribution to the key pathophysiological features of the sheep allergic asthma model. We have also found in preliminary studies that AMs collected from sheep asthmatic airways are altered in their ability to release cytokines in response to various stimuli, and display a diminished capacity for phagocytosis ex vivo (Liravi et al., unpublished data), typical of the changes seen in alveolar macrophages in asthma [54]. While it was shown that IL-13 expression in AMs was elevated at 4 and 24 h in allergen challenged airways, we found a similar proportion of IL-13 expressing AMs in saline and allergen-challenged airways at 48 h. Constitutive expression of IL-13 by AMs in normal subjects and elevated expression in subjects with airway disease may reflect the broad role for IL-13 in homeostasis and disease [49, 55].

IL-4 is known to direct the differentiation of AMs and their release of pro-inflammatory cytokines such as IL-6 and TNFα [56]. In the present study TNFα levels in BAL showed minimal change following allergen or saline exposure, however BAL IL-6 levels were elevated at all time-points in response to allergen, with Th2 lymphocytes, AMs and/or airway epithelial cells [31] the most likely source. Indeed, the inflammatory pathways underlying mucus hyperplasia and AHR seen in the sheep asthma model may be partly dependent on IL-6 signalling, although the contribution of classical or IL-6 trans-signalling, as recently investigated in mice and human subjects [57], is yet to be elucidated in the sheep model. IL-10 levels in BAL were highest at 4 h but lower at 48 h following allergen challenge compared to saline challenge. In the present study we did not investigate the cellular source of IL-10. However, sheep AMs are able to produce IL-10 in response to IL-13 ex vivo (Liravi et al., unpublished data) and macrophages from asthmatics are known to secrete elevated IL-10 levels [58]. On the other hand, Tregs (regulatory T lymphocytes) could also be a source of IL-10, although these cells are less functional and their numbers are reduced in asthma [59].

IL-4 and IL-13 overwhelmingly have an impact on airway inflammation, directing the recruitment and activation of immune cells including airway dendritic cells, alveolar macrophages, eosinophils, mast cells and neutrophils, mucin production by airway epithelial cells and airway wall remodelling in response to allergen exposure [4, 12]. These features have all previously been reported in the sheep asthma model [24, 28], and together with findings from our present study on cytokine data not previously documented for any other large animal model of asthma, strongly implicates a role for Th2 cytokines in the inflammatory and disease processes in this model system.

Future studies in the sheep asthma model may investigate the changes in expression and subsequent therapeutic targeting of other Th2-associated mediators such as IL-17, IL-22, IL-25 and IL-33 [60–63], or miRNAs thought to play a role in directing the pathways of IL-4 and/or IL-13 production in allergic airways, including miR-21, miR-145 and miR-155 [64–67].

**Conclusion**

The results of this study provide further insight into the kinetics of cytokine expression in allergen-challenged airways, and for the first time in a large animal model demonstrate a Th2 polarized cytokine profile featuring IL-4 and IL-13 associated with allergen-induced airway inflammation.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

BL, RB and DP contributed to the design and conduct of the experiments and GN assisted with bronchoscopies. RB supervised the work and RB and DP assisted BL with data analysis and the final draft of the manuscript. All authors read and approved the final manuscript.

**Acknowledgments**

BL was supported by an Australian Postgraduate Award (APA).
Author details
1 Biotechnology Research Laboratories, Department of Physiology, Monash University, Clayton 3800 VIC, Australia. 2 School of Applied and Biomedical Sciences, Federation University, Churchill 3842 VIC, Australia. 3 The Ritchie Centre, Hudson Institute of Medical Research, Clayton 3168 VIC, Australia.

Received: 2 April 2015 Accepted: 1 September 2015

Published online: 11 September 2015

References
1. Hall S, Agrawal DK. Key mediators in the immunopathogenesis of allergic asthma. Int Immunopharmacol. 2014;23(1):316–29.
2. Murdoch JR, Lloyd CM. Chronic inflammation and asthma. Mutat Res. 2010;690(1–2):24–39.
3. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol. 2008;8(9):183–92.
4. Holgate ST. Pathogenesis of asthma. Clin Exp Allergy. 2008;38(6):872–97.
5. Kishimoto T. IL-6: from its discovery to clinical applications. Int Immunol. 1995;7(5):251–4.
6. Kroegel C, Julius P, Matthys H, Virchow JcJ, Luttmann W. Endobronchial inflammation by anti-human-interleukin-13 antibody (CAT-354). Clin Exp Immunol. 2010;125(2):312–8.
7. Daher S, Santos LM, Sole D, De Lima MG, Naspitz CK & Musatti CC. IL-13: a mediator of allergic airway inflammation. J Interferon Cytokine Res. 1997;17(3):220–4.
8. Herrick CA, Bottomly K. To respond or not to respond: T cells in allergic asthma. Nat Rev Immunol. 2003;3(5):405–12.
9. Williams-Karp M. Interleukin-13 in asthma pathogenesis. Immunol Rev. 2004;202:175–9.
10. Humbert M, Durham SR, Kimmitt P, Powell N, Assoufi B, Pfister R et al. Requirement for IL-13 independently of IL-4 in experimental asthma. J Immunol. 1999;163(3):899–904.
11. Prieto J, Lensmar C, Roquet A, van der Ploeg I, Gigliotti D, Eklund A & Grunewald J. Increased interleukin-13 mRNA expression in bronchoalveolar lavage cells of atopic patients with mild asthma after repeated low-dose allergen provocations. Respir Med. 2000;94(8):806–14.
12. Grunig G, Warnock M, Wakil AE, Venkaya R, Brombacher F, Rennick DM et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science. 1998;282(5397):2261–6.
13. Bluemle L, Muyumbi J, Khosla B, Neven KY, Karp CL & Donaldson DD. Interleukin-13: central mediator of allergic asthma. Science. 1998;282(5397):2258–61.
14. Blanchard C, Mishra A, Saiato-Akei H, Monik P, Anderson I & Rothenberg ME. Inhibition of human interleukin-13-induced respiratory and oesophageal inflammation by anti-human-interleukin-13 antibody (CAT-354). Clin Exp Allergy. 2005;35(8):1096–103.
15. De Boever EH, Ashman C, Cahm AP, Locantore NW, Overend P, Pouloukis U et al. Efficacy and safety of an anti-IL-13 mAb in patients with severe asthma: a randomized trial. J Allergy Clin Immunol. 2014;133(4):896–9.
16. Piper E, Brightling C, Niven R, Oh C, Faggioni R, Poon K et al. Phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma. Eur Respir J. 2013;41(1):2330–8.
17. Collies DD. Comparative, complementary and relevant: the immunological basis of ovine lung allergic responses. Clin Exp Allergy. 2003;33(3):282–6.
18. Meeuwen EN, Snibson KJ, Hirst SJ, Bischof RJ. Sheep as a model species for the study and treatment of human asthma and other respiratory diseases. Drug Discov Today Dis Models. 2010;6(3):410–6.
macrophages and allergic inflammation during pulmonary infection of mice with Cryptococcus neoformans. J Immunol. 2007;179(8):5367–77.

48. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010;11(10):889–96.

49. Van Dyken SJ, Locksley RM. Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. Annu Rev Immunol. 2013;31:317–43.

50. Melgert BN, ten Hacken NH, Rutgers ED, Timens W, Postma DS & Hyllkema MN. More alternative activation of macrophages in lungs of asthmatic patients. J Allergy Clin Immunol. 2011;127(3):831–3.

51. Yang M, Kumar RK, Hansbro PM & Foster PS. Emerging roles of pulmonary macrophages in driving the development of severe asthma. J Leukoc. Biol. 2012;91(4):557–69.

52. Kang CM, Jang AS, Ahn MH, Shin JA, Kim JH, Choi YS et al. Interleukin-25 and interleukin-13 production by alveolar macrophages in response to particles. Am J Respir Cell Mol Biol. 2005;33(3):290–6.

53. La Flamme AC, Kharkrang M, Stone S, Mirmoeini S, Chuluundorj D & Kyle R. Type II-activated murine macrophages produce IL-4. PLoS One. 2012;7(10), e46989.

54. Balhara J, Gounni AS. The alveolar macrophages in asthma: a double-edged sword. Mucosal Immunol. 2012;5(6):605–9.

55. Hancock A, Armstrong L, Gama R & Millar A. Production of interleukin 13 by alveolar macrophages from normal and fibrotic lung. Am J Respir Cell Mol Biol. 1998;18(1):60–5.

56. Varin A, Mukhopadhyay S, Herbein G & Gordon S. Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion. Blood. 2010;115(2):353–62.

57. Ullah MA, Revez JA, Loh Z, Simpson J, Zhang V, Bain L et al. Allergen-induced IL-6 trans-signaling activates gammadelta T cells to promote type 2 and type 17 airway inflammation. J Allergy Clin Immunol. 2015. doi:10.1016/j.jaci.2015.02.032.

58. Colavita AM, Hastie AT, Musani AI, Pascual RM, Reinach AJ, Lustine HT et al. Kinetics of IL-10 production after segmental antigen challenge of atopic asthmatic subjects. J Allergy Clin Immunol. 2000;106(5):880–6.

59. Thorburn AN, Hansbro PM. Harnessing regulatory T cells to suppress asthma: from potential to therapy. Am J Respir Cell Mol Biol. 2010;43(5):511–9.

60. McAleer JP, Kolls JK. Directing traffic: IL-17 and IL-22 coordinate pulmonary immune defense. Immunol Rev. 2014;260(1):109–14.

61. Lu TX, Muniz A, Rotenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol. 2009;182(8):4994–5002.

62. Collison A, Herbert C, Siegle JS, Mattes J, Foster PS & Kumar RK. Inhibition of house dust mite-induced allergic airways disease by antagonism of microRNA-145 is comparable to glucocorticoid treatment. J Allergy Clin Immunol. 2011;128(1):160–7.

63. Hallstrand TS, Hackett TL, Altemeier WA, Matute-Bello G, Hansbro PM & Knight DA. Airway epithelial regulation of pulmonary immune homeostasis and inflammation. Clin Immunol. 2014;151(1):1–15.