

RESEARCH ARTICLE

Open Access



Dynamics of IL-4 and IL-13 expression in the airways of sheep following allergen challenge

Bahar Liravi¹, David Piedrafita², Gary Nguyen¹ and Robert J. Bischof^{1,3*}

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

* Correspondence: rob.bischof@hudson.org.au

¹Biotechnology Research Laboratories, Department of Physiology, Monash University, Clayton 3800VIC, Australia

³The Ritchie Centre, Hudson Institute of Medical Research, Clayton 3168 VIC, Australia

Full list of author information is available at the end of the article

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 μ g in 5 ml) at weekly intervals over 3 weeks (ie. three challenges over 3 weeks) using a flexible fibre-optic endoscope (Model FG-16 \times , 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

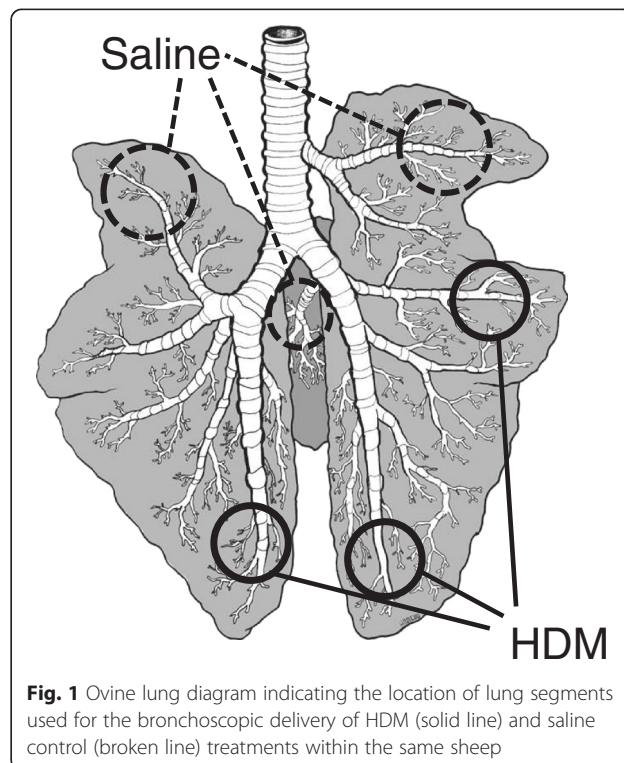


Fig. 1 Ovine lung diagram indicating the location of lung segments used for the bronchoscopic delivery of HDM (solid line) and saline control (broken line) treatments within the same sheep

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 DiffTM 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 %

Table 1 Details of reagents used in ovine-specific cytokine ELISAs

ELISA	Antibody (Ab)/cytokine details	Clone/type	Dilution	Source
IL-4	<i>rov</i> 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	<i>rov</i> IL-6 (cytokine standard)	-	-	CAB ^d
	Anti-ovine IL-6 mAb (coating)	4B6	1:400	Epitope ^e
	Anti-ovine IL-6, pAb (detecting)	rabbit pAb	1:5000	CAB
	Anti-rabbit Ig/HRP	-	1:1000	Dako
IL-10	<i>rbov</i> IL-10 (cytokine standard)	-	-	Moredun
	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	<i>rbov</i> 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
TNF α	<i>rov</i> TNF α (cytokine standard)	-	-	CAB
	Anti-ovine TNF α mAb ascites (coating)	6.09	1:200	CAB
	Anti-ovine TNF α 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 $\mu\text{g}/\text{ml}$; 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 \pm standard deviation (SD).

Results

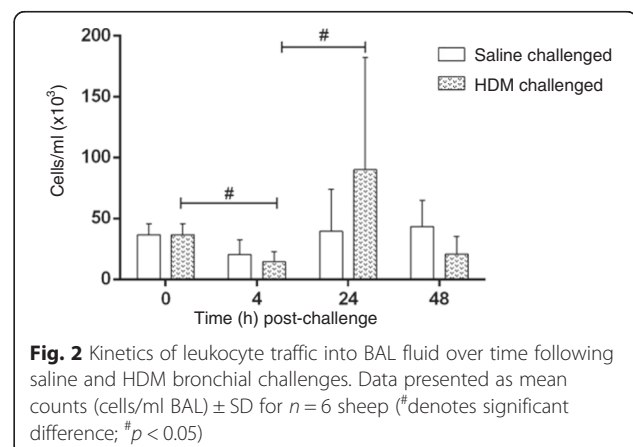
Analysis of BAL cells following airway allergen challenge

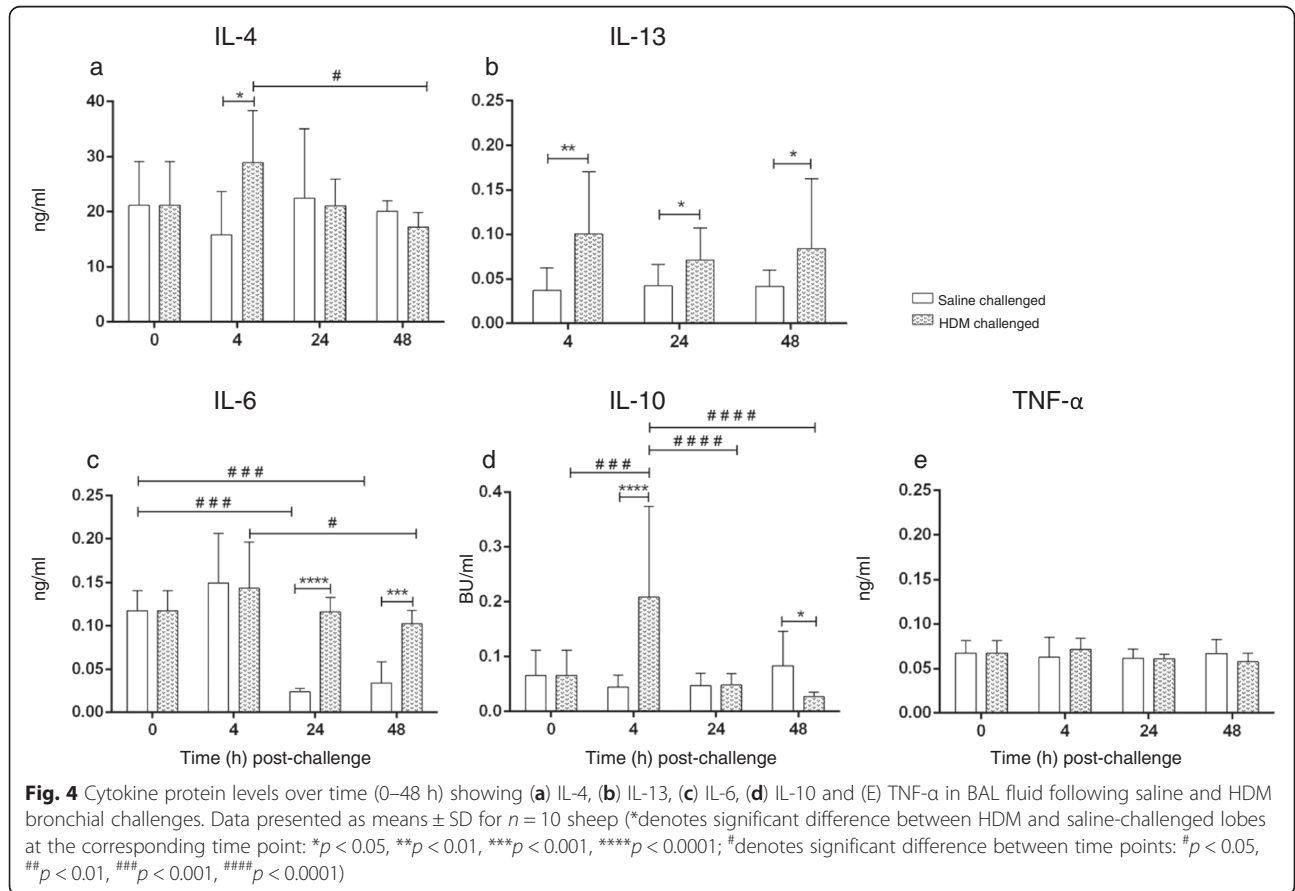
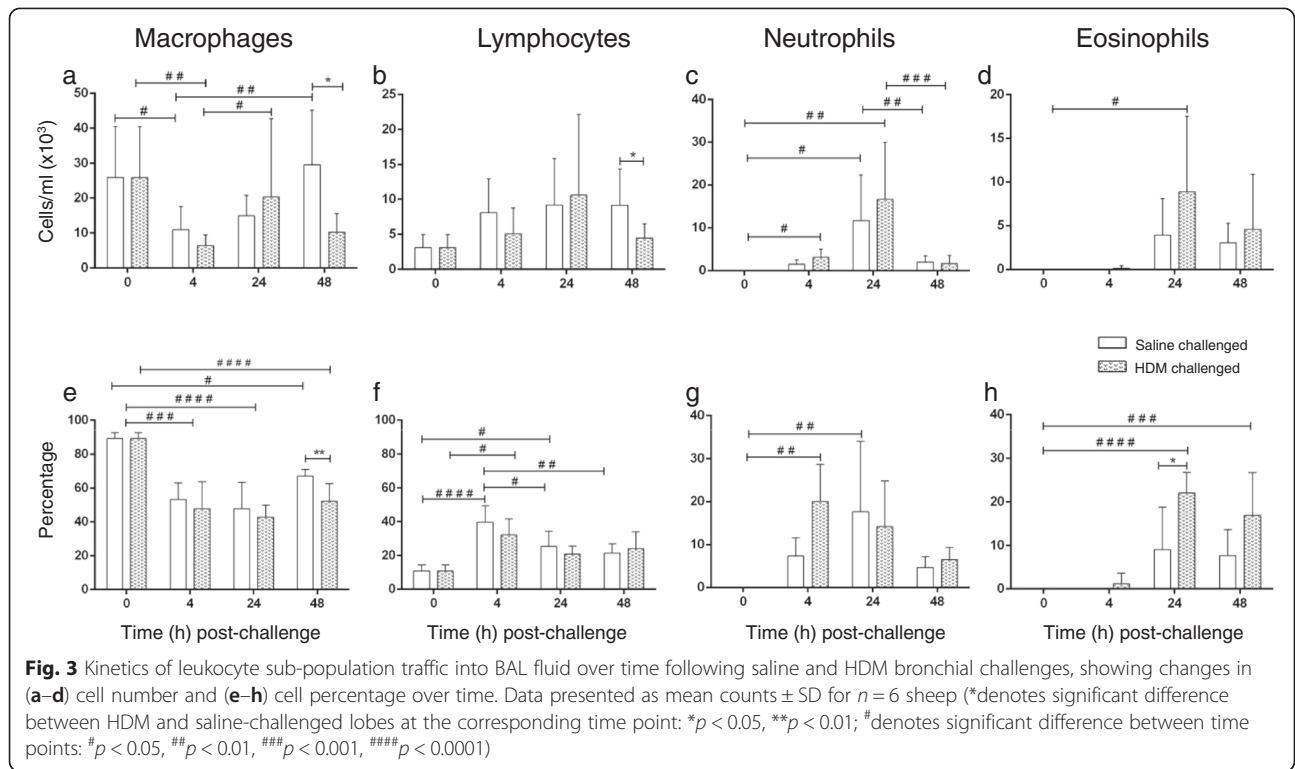
BAL leukocyte numbers from HDM- and saline- challenged lungs before and at 4, 24 and 48 h post-challenge are shown in Fig. 2. In saline-challenged lobes there was little change in leukocyte numbers across the different time points. Conversely, in HDM-challenged lung lobes there was initially a decline 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





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).

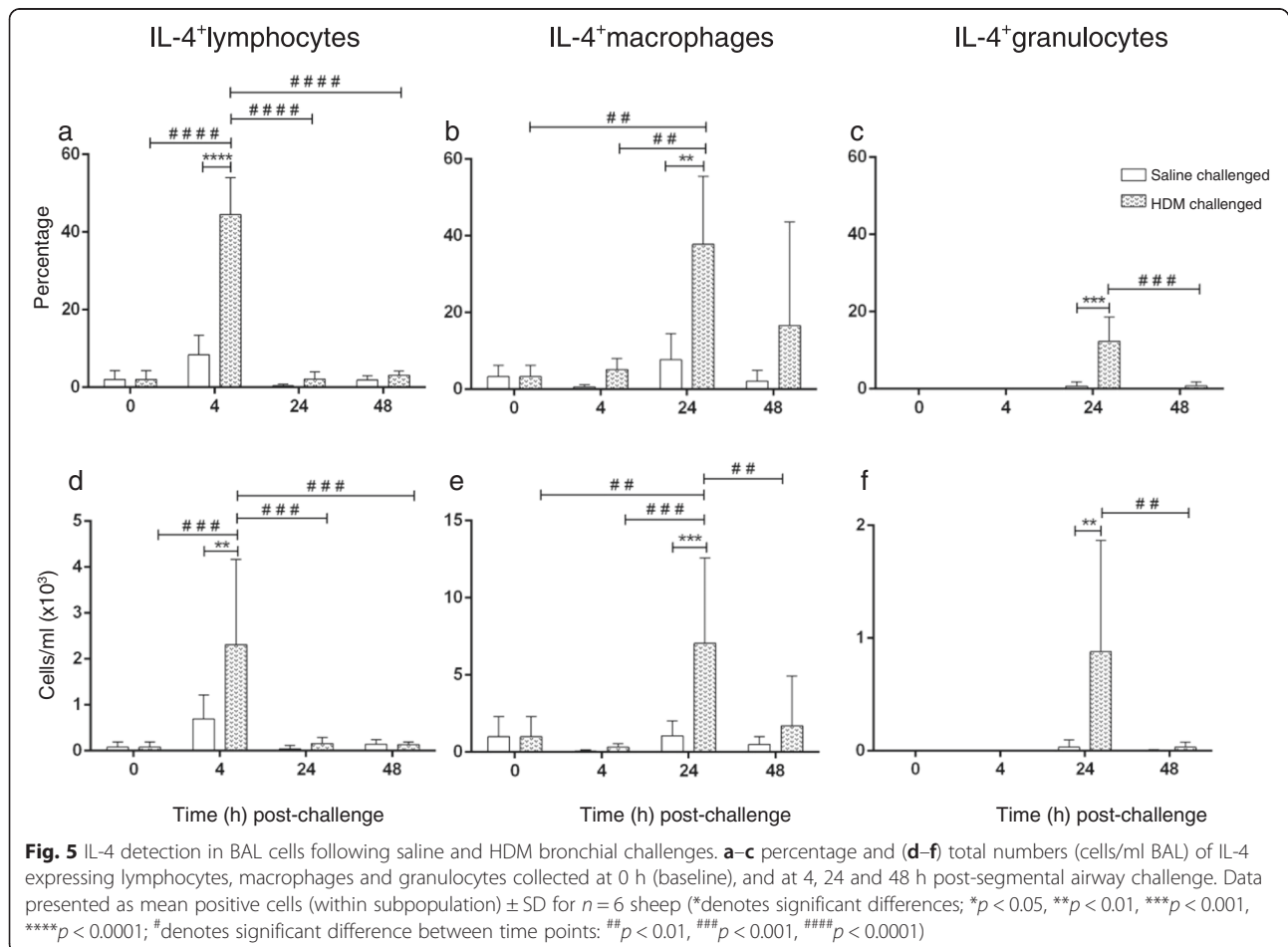
Intracellular expression of IL-4 and IL-13 in BAL cells

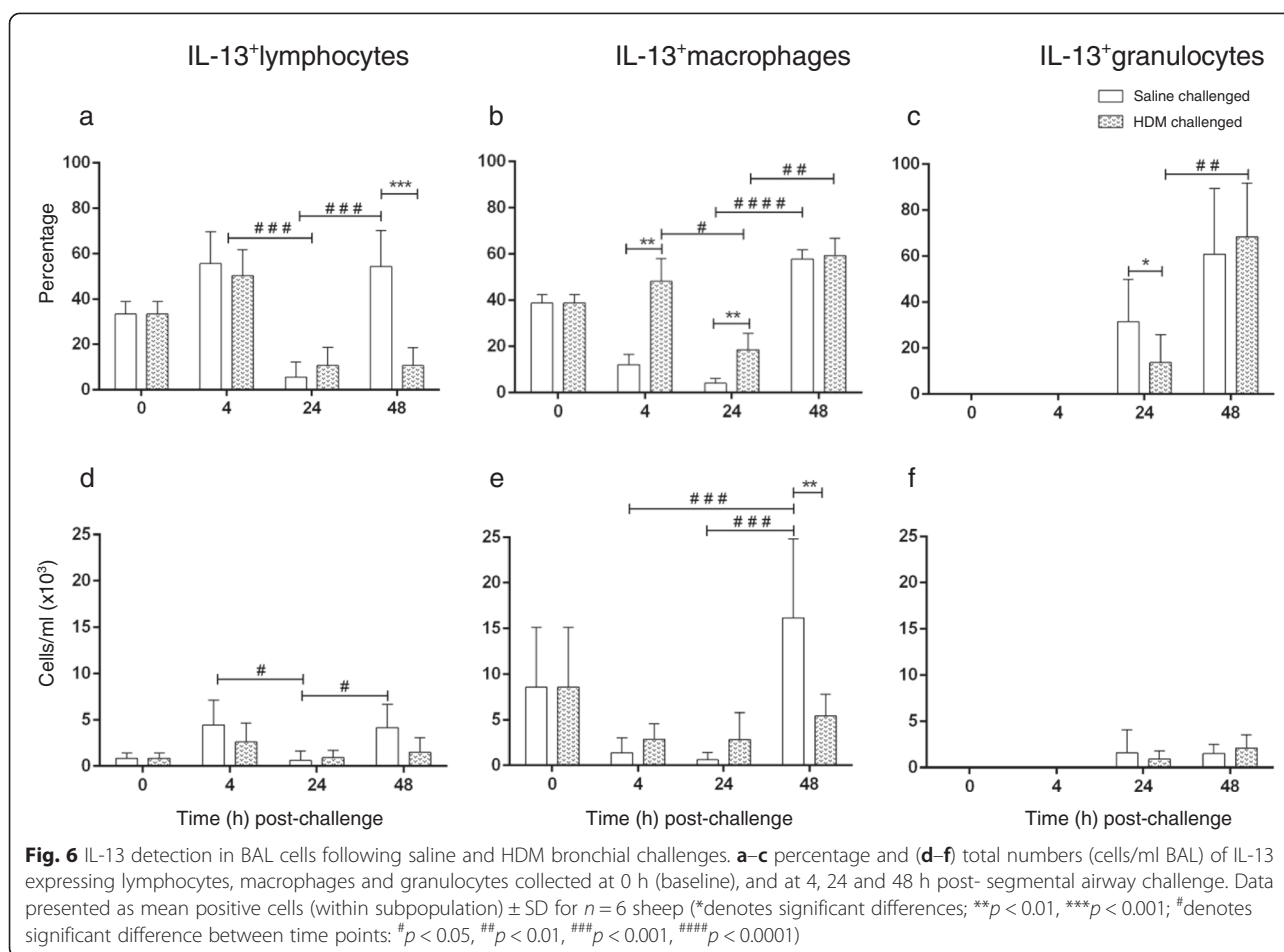
BAL cell subpopulations were identified in the pre- (0 h) and post-challenge (4 h, 24 h, 48 h) BAL samples, with gating based on forward- and side- light scatter (FSC, SSC) properties as reported elsewhere [23]. BAL cell subpopulations were then examined for intracellular IL-4 and IL-13 expression and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





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

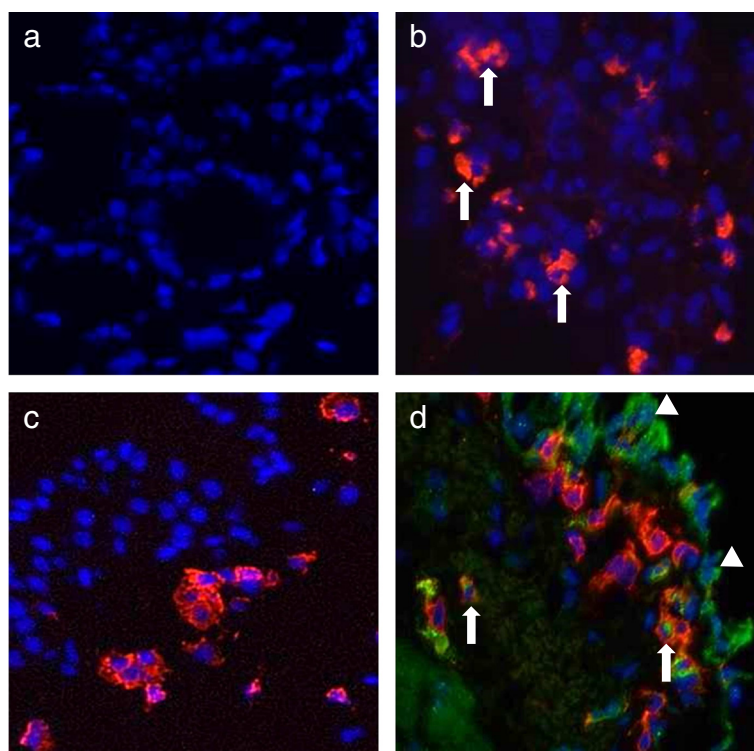


Fig. 7 Immunostaining of IL-4 and IL-13 in lung tissues following allergen challenge, showing (a) negative staining (isotype-matched control Ab), (b) IL-4⁺ cells (red, arrows), (c) CD45⁺ leukocyte (red) staining, and (d) CD45⁺ (red) and IL-13⁺ (green, arrows) staining; the arrow shows an IL-13 expressing leukocyte (IL-13⁺CD45⁺) and the arrow head shows an IL-13 expressing non-leukocyte (IL-13⁺CD45⁻). Representative sections were taken from the same lung lobe of a sheep, collected post-mortem at 48 h post-HDM challenge. All slides were counterstained with DAPI (blue) when mounted in Mowiol (Original magnification $\times 400$)

models such as dog, sheep, horse and non-human primates, although it appears Type 2 or Th₂ immune pathways 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 expression of IL-4 and IL-13 in allergen-challenged airways, with the goal to extend our understanding of Th₂-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 Th₂ cytokines followed by the recruitment of neutrophils, lymphocytes and macrophages in this model, precedes the late phase allergic response associated with the appearance of eosinophils 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 repeated 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. Lymphocytes, most likely CD4⁺ T cells [23], were a major source of IL-4 in the immediate hours (4 h) after allergen challenge, 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 bronchial epithelium also serves as a cellular source for IL-13 in sheep airway tissues following allergen challenge, similar 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 TNF α [56]. In the present study TNF α 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

¹Biotechnology Research Laboratories, Department of Physiology, Monash University, Clayton 3800VIC, Australia. ²School of Applied and Biomedical Sciences, Federation University, Churchill 3842 VIC, Australia. ³The 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

- Hall S, Agrawal DK. Key mediators in the immunopathogenesis of allergic asthma. *Int Immunopharmacol*. 2014;23(1):316–29.
- Murdoch JR, Lloyd CM. Chronic inflammation and asthma. *Mutat Res*. 2010;690(1–2):24–39.
- Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol*. 2008;8(3):183–92.
- Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy*. 2008;38(6):872–97.
- Kishimoto T. IL-6: from its discovery to clinical applications. *Int Immunol*. 2010;22(5):347–52.
- Rincon M, Irvin CG. Role of IL-6 in asthma and other inflammatory pulmonary diseases. *Int J Biol Sci*. 2012;8(9):1281–90.
- Finkelman FD, Boyce JA, Vercelli D & Rothenberg ME. Key advances in mechanisms of asthma, allergy, and immunology in 2009. *J Allergy Clin Immunol*. 2010;125(2):312–8.
- Daher S, Santos LM, Sole D, De Lima MG, Nasipitz CK & Musatti CC. Interleukin-4 and soluble CD23 serum levels in asthmatic atopic children. *J Investig Allergol Clin Immunol*. 1995;5(5):251–4.
- Walker C, Kaegi MK, Braun P & Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol*. 1991;88(6):935–42.
- Shi H, Qin S, Huang G, Chen Y, Xiao C, Xu, H et al. Infiltration of eosinophils into the asthmatic airways caused by interleukin 5. *Am J Respir Cell Mol Biol*. 1997;16(3):220–4.
- Herrick CA, Bottomly K. To respond or not to respond: T cells in allergic asthma. *Nat Rev Immunol*. 2003;3(5):405–12.
- Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev*. 2004;202:175–90.
- Humbert M, Durham SR, Kimmitt P, Powell N, Assoufi B, Pfister R et al. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J Allergy Clin Immunol*. 1997;99(5):657–65.
- Kroegel C, Julius P, Matthys H, Virchow JcJ, Luttmann W. Endobronchial secretion of interleukin-13 following local allergen challenge in atopic asthma: relationship to interleukin-4 and eosinophil counts. *Eur Respir J*. 1996;9(5):899–904.
- 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.
- Grinig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science*. 1998;282(5397):2261–3.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL & Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science*. 1998;282(5397):2258–61.
- Blanchard C, Mishra A, Saito-Akei H, Monk 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.
- De Boever EH, Ashman C, Cahn AP, Locantore NW, Overend P, Pouliquen J 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):989–96.
- Piper E, Brightling C, Niven R, Oh C, Faggioni R, Poon K et al. A phase II placebo-controlled study of talrolinimab in moderate-to-severe asthma. *Eur Respir J*. 2013;41(2):330–8.
- Collie DD. Comparative, complementary and relevant: the immunological basis of ovine lung allergic responses. *Clin Exp Allergy*. 2003;33(3):282–6.
- Meeusen 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(4):101–6.
- Bischof RJ, Snibson K, Shaw R, Meeusen EN. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. *Clin Exp Allergy*. 2003;33(3):367–75.
- Snibson KJ, Bischof RJ, Slocombe RF, Meeusen EN. Airway remodelling and inflammation in sheep lungs after chronic airway challenge with house dust mite. *Clin Exp Allergy*. 2005;35(2):146–52.
- Kirschvink N, Reinhold P. Use of alternative animals as asthma models. *Curr Drug Targets*. 2008;9(6):470–84.
- Allen JE, Bischof RJ, Sucie Chang HY, Hirota JA, Hirst SJ, Inman MD et al. Animal models of airway inflammation and airway smooth muscle remodelling in asthma. *Pulm Pharmacol Ther*. 2009;22(5):455–65.
- Miller LA, Hurst SD, Coffman RL, Tyler NK, Stovall MY, Chou DL et al. Airway generation-specific differences in the spatial distribution of immune cells and cytokines in allergen-challenged rhesus monkeys. *Clin Exp Allergy*. 2005;35(7):894–906.
- Bischof RJ, Snibson KJ, Van Der Velden J, Meeusen EN. Immune response to allergens in sheep sensitized to house dust mite. *J Inflamm (Lond)*. 2008;5:16.
- Hope JC, Kwong LS, Entrican G, Wattedegera S, Vordermeier HM, Sopp P & Howard CJ. Development of detection methods for ruminant interleukin (IL)-12. *J Immunol Methods*. 2002;266(1–2):117–26.
- Kwong LS, Hope JC, Thom ML, Sopp P, Duggan S, Bembridge GP & Howard CJ. Development of an ELISA for bovine IL-10. *Vet Immunol Immunopathol*. 2002;85(3–4):213–23.
- Abeynaik L, Meeusen ENT, Bischof RJ. An ovine tracheal explant culture model for allergic airway inflammation. *J Inflamm (Lond)*. 2010;7:46–55.
- Bischof RJ, Meeusen EN. Cellular kinetics of an allergic-type response in a sheep mammary gland model of inflammation. *Clin Exp Allergy*. 2002;32(4):619–26.
- Gallelli L, Busceti MT, Vatrella A, Maselli R & Pelaia G. Update on anticytokine treatment for asthma. *Biomed Res Int*. 2013;2013:104315.
- Fahy JV. Type 2 inflammation in asthma—present in most, absent in many. *Nat Rev Immunol*. 2015;15(1):57–65.
- Fajt ML, Wenzel SE. Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. *J Allergy Clin Immunol*. 2015;135(2):299–310. quiz 311.
- Walsh GM. Anti-IL-4/-13 based therapy in asthma. *Expert Opin Emerg Drugs*. 2015;20(3):349–52.
- Ingram JL, Kraft M. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J Allergy Clin Immunol*. 2012;130(4):829–42. quiz 843–4.
- Abbas AR, Jackman JK, Bullens SL, Davis SM, Choy DF, Fedorowicz G et al. Lung gene expression in a rhesus allergic asthma model correlates with physiologic parameters of disease and exhibits common and distinct pathways with human asthma and a mouse asthma model. *Am J Pathol*. 2011;179(4):1667–80.
- Coffman RL, Hessel EM. Nonhuman primate models of asthma. *J Exp Med*. 2005;201(12):1875–9.
- Dewachi O, Joubert P, Hamid Q & Lavoie JP. Expression of interleukin (IL)-5 and IL-9 receptors on neutrophils of horses with heaves. *Vet Immunol Immunopathol*. 2006;109(1–2):31–6.
- Steinke JW, Borish L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir Res*. 2001;2(2):66–70.
- Collie DD, McLean N, Sallenave JM, Baker A, Blundell R, Milne E et al. Local lung responses following endobronchial elastase and lipopolysaccharide instillation in sheep. *Int J Chron Obstruct Pulmon Dis*. 2006;1(2):189–99.
- Collie DD, Baker A, Mauchline S, Porteous D & Mc Lachlan G. Ovine bronchoalveolar lavage cellularity: reproducibility and the effect of multiple repeated lavage. *Res Vet Sci*. 1999;67(2):137–40.
- Temann UA, Geba GP, Rankin JA & Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med*. 1998;188(7):1307–20.
- Moreira AP, Hogaboam CM. Macrophages in allergic asthma: fine-tuning their pro- and anti-inflammatory actions for disease resolution. *J Interferon Cytokine Res*. 2011;31(6):485–91.
- Loke P, Nair MG, Parkinson J, Guiliano D, Blaxter M & Allen JE. IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol*. 2002;3:7.
- Muller U, Stenzel W, Kohler G, Werner C, Polte T, Hansen G et al. IL-13 induces disease-promoting type 2 cytokines, alternatively activated

- 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 B, Timens W, Postma DS & Hylkema 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):129–44.
 61. Yang Z, Grinchuk V, Urban JF Jr, Bohl J, Sun R, Notari L et al. Macrophages as IL-25/IL-33-responsive cells play an important role in the induction of type 2 immunity. *PLoS One*. 2013;8(3), e59441.
 62. Willis CR, Siegel L, Leith A, Mohn D, Escobar S, Wannberg S et al. IL-17RA signaling drives airway inflammation and bronchial hyper reactivity in allergic asthma. *Am J Respir Cell Mol Biol*. 2015. doi:10.1165/rcmb.2015-0038OC.
 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.
 64. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *J Immunol*. 2009;182(8):4994–5002.
 65. 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. e4.
 66. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR et al. Requirement of bic/microRNA-155 for normal immune function. *Science*. 2007;316(5824):608–11.
 67. Malmhall C, Alawieh S, Lu Y, Sjostrand M, Bossios A, Eldh M & Radinger M. MicroRNA-155 is essential for T(H)2-mediated allergen-induced eosinophilic inflammation in the lung. *J Allergy Clin Immunol*. 2014;133(5):1429–38. 1438 e1-7.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

