

Cellular kinetics of an allergic-type response in a sheep mammary gland model of inflammation

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Summary

Background Tissue recruitment of eosinophils and activated lymphocytes is a characteristic feature of allergic reactions. However, little is known about the involvement of specific adhesion molecules in the traffic of leucocytes during the allergic response.

Objective To use a sheep mammary infusion model to characterize the kinetics of cell recruitment and expression of cellular adhesion molecules and activation markers on eosinophils and lymphocytes involved in an allergic-type response.

Methods Mature non-lactating ewes were primed and challenged by direct infusion of the mammary glands with nematode larvae. Using a non-invasive method of saline infusion and 'milking' of the glands, large numbers of inflammatory cells were repeatedly sampled over 10 to 96 h following their migration into the mammary gland lumen, and analyzed by 2-colour flow cytometry.

Results Leucocyte recruitment into the mammary lumen was characterized by two separate phases involving an acute neutrophilic response at 10 h post-challenge, followed by a dramatic reduction in neutrophils and appearance of eosinophils and activated lymphocytes. From 48 h post-challenge, eosinophils were predominant and represented 40 to 65% of leucocytes in the mammary lavage (MAL). Increases in activated CD4⁺ T cells and $\gamma\delta^+$ T cells were also observed at this time-point. The kinetics of expression of cell surface molecules on eosinophils and lymphocytes in blood and MAL were compared during the course of the allergic-type reaction. Adhesion molecule expression on lymphocytes was modulated following allergen challenge and an activation of MAL vs. blood lymphocytes was seen during the later stages of the allergic response. Eosinophil expression of VLA-4 and L-selectin was down-regulated compared with blood at all time-points examined. There were high levels of expression of CD11b and CD44 on eosinophils during the early compared to the late-phase of the allergic reaction.

Conclusion These results indicate the existence of two separate mechanisms of eosinophil recruitment during the allergic inflammatory response.

Keywords allergic inflammation, allergy, eosinophils, leucocyte migration, mammary gland, sheep
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Introduction

Eosinophils are a major pro-inflammatory leucocyte cell type involved in allergic diseases such as asthma [1–3]. Eosinophilia is also typical of helminth infections and there is strong evidence to indicate that the cellular and molecular response to parasite antigens closely mimics the immune response seen in allergic inflammation [4]. Infusion of ruminant mammary glands has been used previously to investigate the host immune response to parasite-derived stimuli [5–8]. The mammary infusion model allows non-invasive and repeated sampling of inflammatory cells following tissue migration, which can then be compared directly with non-migrated cell populations in peripheral blood [7]. In addition, as inflammatory cells recovered from mammary lavage (MAL) have crossed both endothelial and epithelial

barriers they resemble the bronchoalveolar lavage (BAL) cells recovered during lung inflammations such as asthma.

The kinetics of an allergic reaction are divided into an early- and late-phase response involving the orchestrated recruitment and activation of a variety of leucocyte cell types, including mast cells, eosinophils and lymphocytes. In asthma, the early phase is dominated by the presence of activated mast cells and basophils, while there is a profound influx of eosinophils and Th2 CD4⁺ T cells seen in the late-phase response [9]. Cytokines, chemokines and other inflammatory mediators secreted by cells in the inflamed tissue drive leucocyte recruitment via activation signals and modulation of adhesion molecule expression. The up-regulation of activation markers and changes in the expression of adhesion molecules on lymphocytes appear a characteristic feature of the allergic response [10,11]. An increase in the adhesion molecules ICAM-1, VLA-4 and LFA-1 on lymphocytes and eosinophils recruited into the airways has also been reported in a murine model of pulmonary inflammation [12]. However, little is known about the involvement of specific

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adhesion molecules in the recruitment of leucocytes during the course of an allergic response and few studies have investigated changes in the kinetics of cell surface antigen expression on lymphocytes and eosinophils during allergic reactions.

Using the sheep mammary infusion model, the aim of this study was to characterize the kinetics of cell recruitment and expression of cellular adhesion molecules and activation markers on eosinophils and lymphocytes involved in an allergic-type response both before (blood) and after (mammary lavage) tissue migration.

Materials and methods

Animals

Mature non-lactating merino ewes were purchased from a commercial farm and treated with the anthelmintic Nilverm (Cooper's Animal Health, North Ryde, Australia) to eliminate existing parasites prior to the experiment. They were housed in pens and fed Barastoc pellets (Pakenham, Australia). Sheep were primed by 4 to 5 weekly infusions of the mammary glands with 5000 live exsheathed L3 *Haemonchus contortus* (*Hc*) larvae [7] (see below), then rested for 3 to 4 weeks prior to the experimental challenge.

Mammary gland challenge and collection of leucocytes

Mammary infusions were performed using a 10-mL syringe fitted with a blunted 22-gauge needle. The tip of the needle was gently rotated into the teat canal followed by infusion of the L3 larvae suspended in 5 mL sterile pyrogen-free saline (PFS) (Baxter, Melbourne, Australia). Control challenges included the infusion of PFS or larvae-free PFS alone. At 10, 24, 48 and 96 h post-challenge, mammary lavage (MAL) cell suspensions were gently 'milked' from the mammary glands after the infusion of 8 mL sterile PFS. On ice, MAL cells were washed twice with 1% bovine serum albumin (BSA; fraction V) (Trace Biosciences, Melbourne, Australia) in phosphate-buffered saline (PBS) prior to immunostaining. Immediately preceding the collection of MAL cells, 20 mL blood was drawn from the jugular vein of sheep into a plastic tube containing EDTA (BDH; Merck, Kilsyth, Australia). Red blood cells were lysed with the addition of Tris-buffered ammonium chloride (170 mM Tris, 160 mM NH₄Cl, pH 7.2) and leucocytes washed twice with 1% BSA/PBS, then stored on ice prior to immunostaining. Cytospots of MAL cells and blood smears were prepared and stained with Wright's stain (Sigma, Castle Hill, Australia) for differential leucocyte cell counts.

Flow cytometry

Monoclonal antibodies (mAbs) against sheep cell surface molecules (Table 1) were generated at the Centre for Animal Biotechnology or generously supplied by Dr Charles Mackay (Basel Institute, Basel, Switzerland) and Dr Chris Howard (Institute for Animal Health, Compton, UK) [7,13]. The mAb SBU-3 does not react with sheep leucocytes and was used as a negative control.

MAL and peripheral blood leucocytes were enumerated using a Coulter counter[®] (Coulter Electronics, Luton, UK) and resuspended to 2 to 3 × 10⁷ cells/mL in wash buffer (1% BSA/0.05% azide/PBS) on ice. Cells were pre-incubated with 5% normal sheep serum and 5% fetal calf serum for 10 min (on ice), then

Table 1. Monoclonal antibodies against ovine cell surface and adhesion molecules

Molecule	Clone	Source
CD1	20-27	CAB
CD2	36F	CAB
CD4	44-97 + 44-38	CAB
CD5	25-91	CAB
CD8	38-65	CAB
CD45R	20-96	CAB
WC1	19-19	CAB
WC2/γδ-TCR	86D	Basel
CD45	1.11.32	CAB
CD25 (IL-2 receptor α chain)	9-14	CAB
MHC class II	49-1	CAB
LFA-1	F10-150	Basel
CD11b	CC-126	Compton
CD44	25-32	CAB
VLA-4 (α4β1-integrin)	FW3218-1	Basel
L-selectin	Du1-29	Basel
β1-integrin	FW347-11	Basel
β7-integrin	FIB30	Basel
Fetal epithelial cell	SBU-3	CAB

CAB = Centre for Animal Biotechnology; Basel = Basel Institute of Immunology; Compton = Institute for Animal Health.

transferred in 50-μL aliquots to a 96-well V-bottomed plate. To each well, 50 μL of mAb (undiluted culture supernatant) was added and cells were incubated for 30 min, then centrifuged and washed three times with wash buffer prior to incubation with fluorescein (FITC)-conjugated sheep anti-mouse F(ab')₂ immunoglobulins (Silenus, Melbourne, Australia). All staining incubations were performed at 4 °C on a Dynatech microshaker (Selby's, Melbourne, Australia). After further washes, cells were pre-incubated with 5% normal mouse serum in wash buffer for 10 min prior to the secondary staining using a biotinylated anti-CD4 mAb (Table 1), followed by three washes and incubation with a streptavidin-phycoerythrin (PE) -conjugate (Biosource Int., Camarillo, CA, USA). Cells were then washed as before, fixed in 3% formaldehyde in PBS and analysed on a FACSCalibur[®] instrument (Becton-Dickinson, Mountain View, CA, USA) using CA software (Becton-Dickinson).

Statistical analyses

The Student's *t*-test was used to determine whether there was any significant difference (*P* < 0.05) between means of the sample groups.

Results

Cellular kinetics in response to mammary gland challenge

Sheep were initially primed with repeated mammary infusions of *Hc* larvae and then rested for 3 to 4 weeks. The collection of mammary lavage (MAL) leucocytes at 5 days following each of the weekly infusions revealed a progressive increase in both cell number and eosinophil percentage recovered (data not shown), as reported previously [7,8].

Primed sheep were given a single mammary challenge with *Hc* larvae and peripheral blood and MAL leucocytes were collected over the next 10 to 96 h. The kinetics of the appearance of

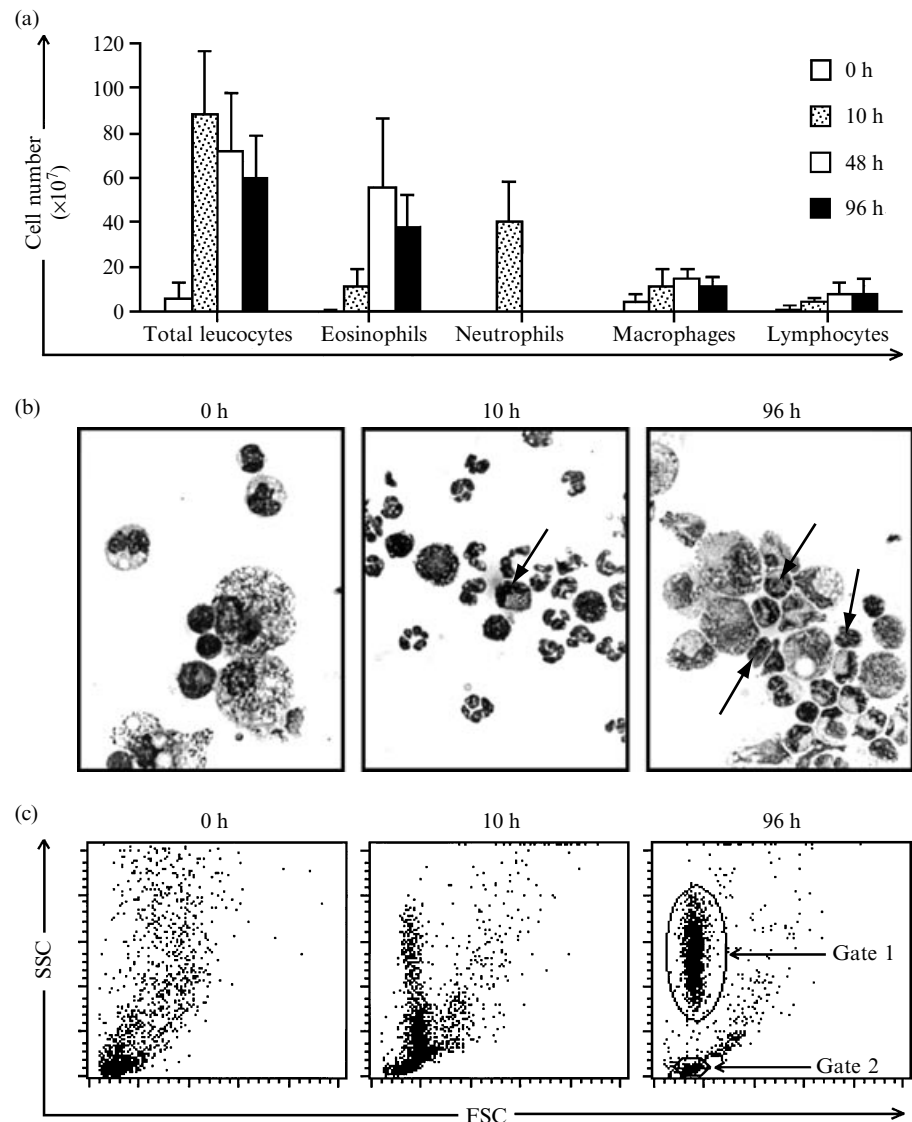


Fig. 1. Kinetics of leucocyte traffic into the MAL following mammary gland challenge. (a) MAL cell numbers collected from unchallenged mammary glands (0 h) and at 10 h, 48 h and 96 h post-challenge (mean $\pm$ SD; $n = 3$). (b) Representative cytospot preparations of MAL cells stained with Wright's stain showing the appearance (10 h) and prominence (96 h) of eosinophils (arrows) in the MAL after mammary gland challenge. (c) Representative forward scatter (FSC) and side scatter (SSC) profile of leucocytes (2500 total events shown) in the unchallenged mammary gland (0 h) and at 10 h and 96 h post-challenge. Eosinophils (Gate 1) and lymphocytes (Gate 2) can be easily distinguished on the basis of their SSC profile.

leucocytes in the MAL following mammary challenge is presented in Fig. 1. The lumen of the resting mammary gland comprised mainly macrophages and lymphocytes. However, within 10 h of stimulation there was a rapid influx of inflammatory cells into the lumen of the challenged gland, representing a 10- to 20-fold increase in total cells ($6.0 \pm 7.2 \times 10^7$ vs. $88.2 \pm 28.2 \times 10^7$) recovered from the gland (Fig. 1a). The cells recovered from the mammary washes at 10 h consisted predominantly of neutrophils with a lesser but significant recruitment of eosinophils and macrophages (Figs 1a–c). By 48 h post-challenge there was a dramatic decline in neutrophil recruitment and eosinophils became the predominant cell type in the MAL (Fig. 1a), increasing by 48 to 96 h to represent 40 to 65% of MAL leucocytes recovered (Figs 1b and c). Macrophages and lymphocytes comprised the remaining MAL leucocytes from 48 h post-challenge. There was no evidence of larval invasion into the mammary tissue during the course of the experiment. However, attachment of leucocytes (mainly eosinophils) to the larval surface was evident from the first time-point examined (10 h post-infusion) and there was a decline in larval viability with time, possibly due to eosinophil-

mediated killing as shown previously [8]. Interestingly, mammary gland challenge with PFS or larvae-free PFS also induced a rapid and acute neutrophilic response but without the appearance of eosinophils in the MAL (data not shown).

Analysis of blood and MAL lymphocyte sub-populations following mammary challenge

Following incubation with mAbs (Table 1), small-medium lymphocytes in blood and MAL samples were gated on the basis of FSC and SSC characteristics for FACS analysis (Fig. 1c). Cells analysed were all found to be leucocytes (CD45⁺). Lymphocytes in peripheral blood consisted of CD4⁺ ($23.2 \pm 5.1\%$), CD8⁺ ($15.4 \pm 3.0\%$) and $\gamma\delta$ ⁺ ($20.4 \pm 6.1\%$) T cell and CD45R⁺ ($39.5 \pm 11.2\%$) (mainly B cell) sub-populations. There was little change seen in the proportions of the lymphocyte sub-populations in peripheral blood at any of the time points following larval challenge. In contrast, there was a dramatic increase in the percentage (Fig. 2a) and number (Fig. 2b) of CD4⁺ T cells in the MAL between the 10 h and 48 to 96 h time-points, accompanied by an increase in the ratio of CD4:CD8 lymphocytes. At 48 h post-challenge

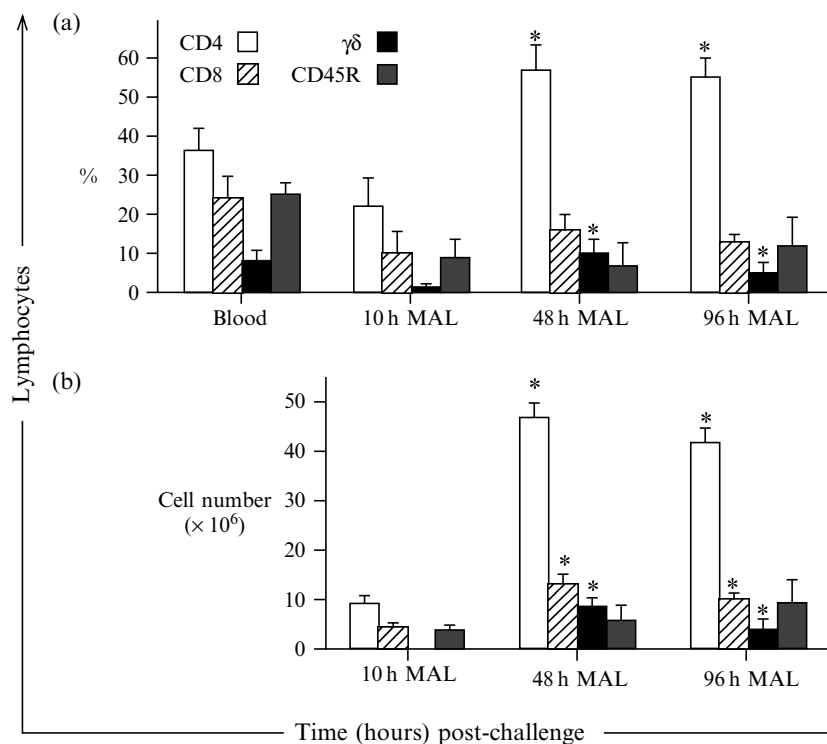


Fig. 2. Changes in lymphocyte sub-populations in blood and MAL after mammary challenge (mean $\pm$ SD; $n = 3$ sheep). Lymphocytes were gated out on FSC and SSC characteristics (Gate 2, Fig. 1c) and 10 000 events analysed. (a) Percentage of all lymphocytes. (b) Cell number. *Denotes significant difference compared with the 10 h time-point ($P < 0.05$).

there was also an increase in the $\gamma\delta^+$ T cell sub-population (Figs 2a and b) and the number of CD8⁺ T cells (Fig. 2b) in the MAL.

Lymphocyte activation and adhesion molecule expression after mammary gland challenge

Based on the expression of the activation markers CD25 (IL-2R) and MHC class II, there was little variation in the activation status of blood lymphocytes following larval challenge (Fig. 3a). However, there was a marked increase in the percentage of MAL lymphocytes expressing IL-2R and MHC class II at 48 and 96 h post-challenge compared with MAL lymphocytes at 10 h and blood lymphocytes at all stages (Fig. 3a). This activation was seen for both the CD4⁺ and CD4⁻ MAL lymphocytes (data not shown).

Two-colour immunofluorescence staining revealed that the CD4⁺ lymphocytes in blood and MAL expressed the adhesion molecules VLA-4, β 1-integrin, β 7-integrin, CD44, L-selectin and LFA-1 but showed little expression of CD11b (data not shown). The percentage of CD4⁺ T cells expressing L-selectin in blood and MAL showed no change with time after mammary challenge; however, there was a decline in the percentage of L-selectin⁺ CD4⁺ T cells in MAL compared with blood from 48 h post-challenge (Fig. 3b). LFA-1 expression was constitutive on blood and MAL CD4⁺ T cells; however, the intensity of LFA-1 expression (Fig. 3b) was significantly greater on MAL than on blood CD4⁺ T cells over all time-points examined.

Expression of cell surface molecules on blood and MAL eosinophils

Eosinophils in peripheral blood and MAL samples could be clearly distinguished in a mixed leucocyte sample by flow

cytometry on the basis of their characteristic forward-scatter (FSC) and side-scatter (SSC) (Fig. 1c). In sheep, eosinophils are depicted as a population of cells with medium FSC (size) and high SSC (granularity) relative to other leucocytes in blood and in MAL samples (Fig. 1c) [7]. Examination of Wright's-stained cytoplots of cells sorted in this gate confirmed the purity of eosinophils (>98%; not shown). After staining of blood and MAL leucocytes with a panel of mAbs, eosinophils were gated out for analysis of cell surface marker expression.

There was a slight increase in the percentage of eosinophils in peripheral blood of primed sheep compared with unprimed controls (data not shown), in agreement with our earlier observation [7]. As seen in Fig. 4, these blood eosinophils clearly expressed the adhesion molecules VLA-4, L-selectin, CD11b and CD44, with little change in the level of expression of these markers during the course of the experiment. Blood eosinophils also expressed LFA-1 and very low levels of the β 1- and β 7-integrins (data not shown). There was a significant reduction in the mean fluorescence intensity (MFI) of VLA-4 on MAL eosinophils compared with blood eosinophils at all time-points examined (Fig. 4a). There was also a reduction in both the percentage and MFI of L-selectin on MAL eosinophils after mammary challenge (Fig. 4b) and levels remained lower than blood at all stages. CD11b (Fig. 4c) and CD44 (Fig. 4d) were constitutively expressed by blood and MAL eosinophils at all stages examined; however, at 10 h post-challenge the MFI for CD11b and CD44 was significantly elevated on eosinophils after passing into the mammary gland lumen and then gradually declined to blood levels. No expression of the activation markers IL-2R or MHC class II was observed at any stage on eosinophils in either blood or MAL (data not shown).

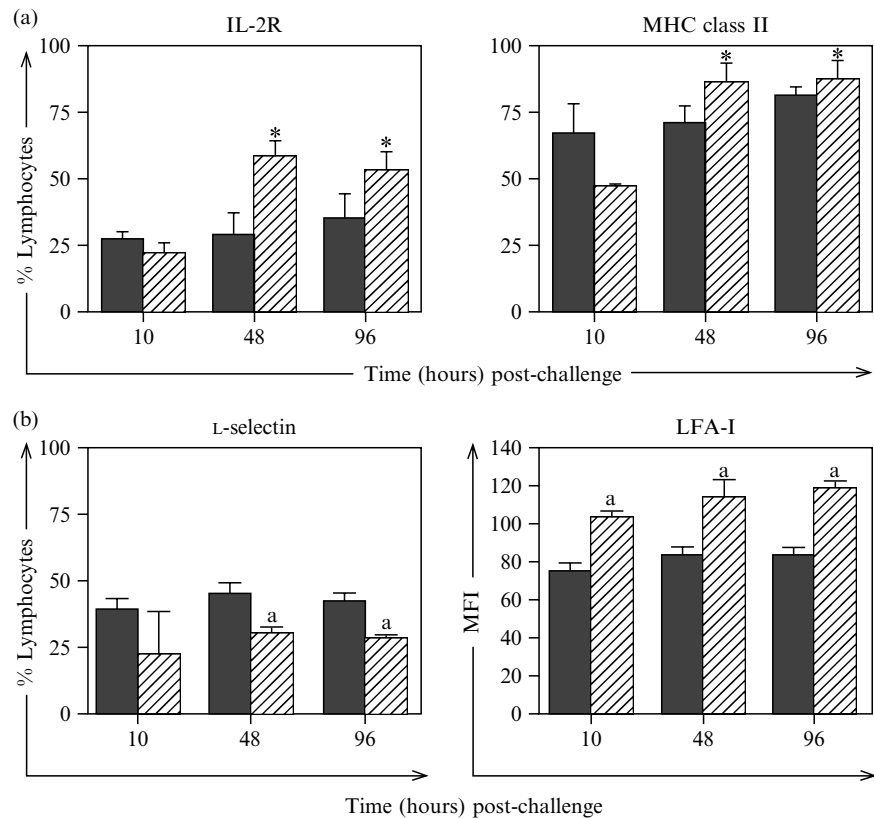


Fig. 3. Activation of lymphocytes after mammary gland challenge. Lymphocytes were gated out on FSC and SSC characteristics (Gate 2, Fig. 1c) and 10 000 events analysed. (a) Expression of the activation markers IL-2R and MHC class II on lymphocytes in blood (closed bars) and MAL (hatched bars) following mammary gland challenge. *Denotes significant difference compared with the 10 h time point ($P < 0.05$). (b) Kinetics of expression of adhesion molecules L-selectin (percentage) and LFA-1 (mean fluorescence intensity; MFI) by blood and MAL CD4⁺ lymphocytes. ^aDenotes significant difference between blood and MAL values ($P < 0.05$). Data presented are means $\pm$ SD from a group of three sheep.

Discussion

An increase in number and persistence of eosinophils is a common pathological feature of both allergic disease, including allergic asthma, and helminth infections. Priming and challenge of sheep mammary glands with helminth larvae elicits a cellular response dominated by the appearance of eosinophils in the mammary gland lumen [7]. This approach was used in the present study to investigate the cellular kinetics and changes in the expression of cell surface markers and adhesion molecules on eosinophils and lymphocytes during an allergic-type response. While there are obvious functional and physiological differences between the mammary gland and the lung, inflammatory cells recovered in the MAL and BAL have traversed both endothelial and epithelial barriers and may therefore show similar migration characteristics.

The response to intramammary larval challenge was characterized by an acute infiltration of predominantly neutrophils into the lumen of the gland within 10 h of stimulation. A small but significant percentage of eosinophils, lymphocytes and macrophages were also detected at this earliest time-point. This was followed by a dramatic decline in neutrophils and a second wave of leucocyte recruitment involving large numbers of eosinophils as well as lymphocytes and macrophages. Eosinophils were the predominant leucocyte cell type in the MAL of sensitized sheep from 24 h post-challenge. The recruitment of neutrophils followed by eosinophils and lymphocytes in the mammary infusion model is in agreement with other experimental models of allergic disease [12,14,15], as well as

human allergic reactions [16,17], where a distinct antigen-induced late-phase eosinophilia is reported. In contrast to eosinophils, acute neutrophilia was observed after the first parasite infusion, indicating that it is part of a non-specific response. Such a response to antigen has been reported in airway studies in *Ascaris*-sensitive dogs [18] and may be induced by the release of IL-8 and mast cell-derived mediators [17]. In the present study, release of mediators by mast cells sensitized with parasite-specific IgE through previous exposure to nematodes on pasture may have resulted in neutrophil recruitment. However, the fact that the acute neutrophil response was equally induced by the infusion of sterile pyrogen-free saline argues for a non-specific recruitment and also precludes the possible involvement of bacterial-derived toxins such as LPS in this system.

The mechanism by which eosinophils and lymphocytes selectively migrate to sites of inflammation is still not fully understood. Leucocyte traffic in allergic inflammation is mediated by the cell surface expression of adhesion molecules involved in binding to endothelia and epithelia. A number of cell adhesion molecules appear to be important in the recruitment of leucocytes to the lumen of the allergic lung. For example, studies have described enhanced expression of the adhesion molecules LFA-1, ICAM-1 VLA-4 and L-selectin on BAL lymphocytes [12, 19] and VLA-4, LFA-1 and ICAM-1 expression on BAL eosinophils [12]. In our studies, VLA-4 and L-selectin were found to be expressed by the majority of eosinophils in the blood and MAL but their level of expression was much reduced in the MAL. We also found that the majority

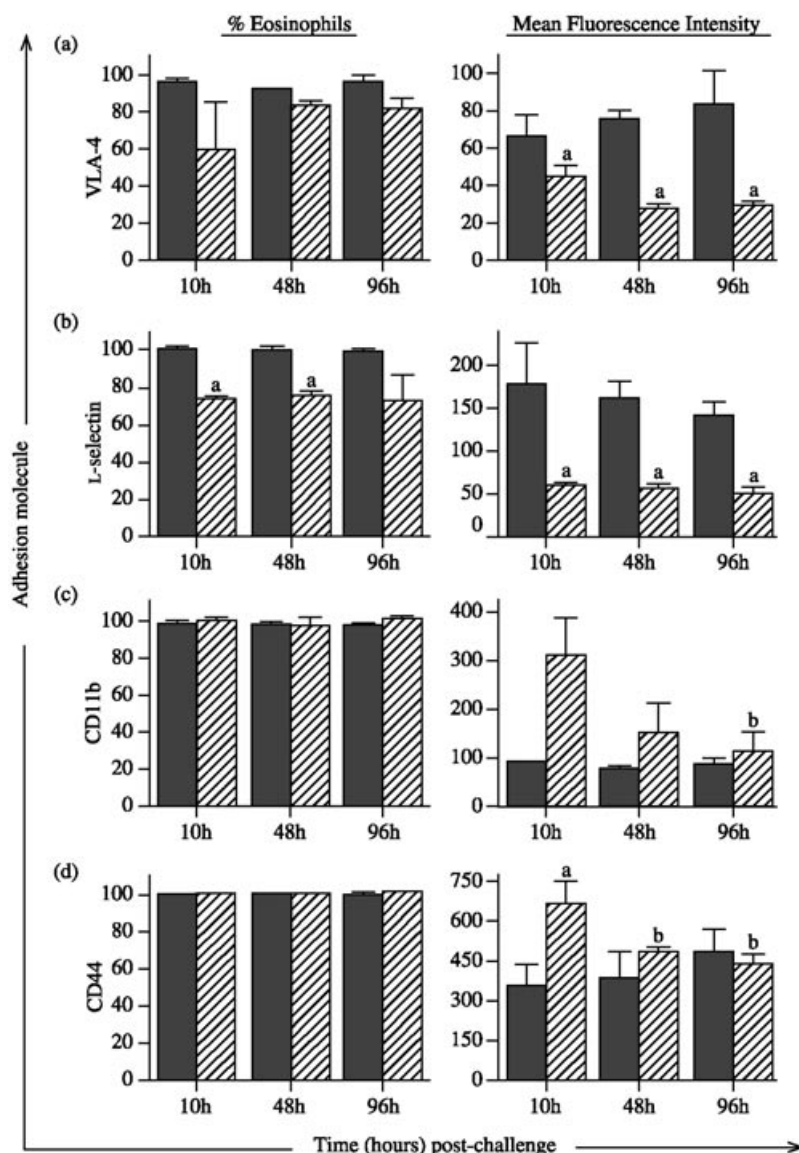


Fig. 4. Kinetics of adhesion molecule expression on eosinophils in blood (closed bars) and MAL (hatched bars) following mammary gland challenge (mean $\pm$ SD; n = three sheep). (a) VLA-4; (b) L-selectin; (c) CD11b; (d) CD44. Eosinophils were gated on FSC and SSC characteristics (Gate 1, Fig. 1c) and 10 000 events analysed. ^aDenotes significant difference between blood and MAL values ($P < 0.05$); ^bdenotes significant difference compared with 10 h time-point ($P < 0.05$).

of T lymphocytes expressed VLA-4 and there was clearly a reduction in the percentage of MAL T cells expressing L-selectin compared with blood. Down-regulation of these adhesion molecules may be due to enzymatic cleavage from the cell surface as shown for L-selectin (but not for VLA-4) following leucocyte-endothelial cell interaction [20]. The importance of VLA-4 for the selective migration of eosinophils and lymphocytes to sites of inflammation is well established [21,22]. In both a rat [23] and guinea-pig [24] model of allergic asthma it has been shown that *in vivo* blockade of cells expressing VLA-4 using an α 4-integrin-specific mAb abrogates the allergic airway response. Similar findings have also been reported in a sheep asthma model where several approaches have been successful in blocking VLA-4 dependent adhesion and activation mechanisms [25–27]. In the present study VLA-4 expression on eosinophils, in contrast to L-selectin, was lower at 48 to 96 h compared with 10 h, suggesting that VLA-4 mediated transmigration may be more critical for the later stages of the allergic response.

Activated eosinophils release cytokines, reactive oxygen intermediates and other inflammatory mediators that help to orchestrate the inflammation and tissue destruction at the allergic site. Eosinophil activation is driven by cytokines and other soluble mediators, as well as through adhesion to the endothelium and subsequent transmigration into inflamed tissue. We confirm our previous observation [7] that sheep eosinophils in blood and MAL lack some of the cell surface markers reported on eosinophils in other species [1,28] such as the classical activation markers MHC class II and the IL-2 receptor (IL-2R). Such discrepancies may be due to species differences or reflect different experimental conditions for harvesting inflammatory cells. Up-regulation of the cell surface antigens CD11b [29], CD44 [30] and CD69 [30] has been linked with the activation of eosinophils in response to IL-5, eotaxin and other inflammatory stimuli at the allergic site [29,31,32]. Interestingly, our studies of the kinetics of CD11b and CD44 expression revealed their up-regulation on MAL eosinophils only in the early stages after allergen challenge, followed by a reduction to levels

observed in blood. Such changes may reflect an altered cytokine/chemokine profile during the course of the allergic response in the mammary gland.

The late-phase allergic response and the associated eosinophilia is believed to be strongly regulated by CD4⁺ T cells and the release of Th2 cytokines such as IL-4, IL-5 and IL-13 [31,33,34]. In the present study, CD4⁺ T cells were the predominant lymphocytes in the MAL at all time points after mammary gland challenge. There was a marked increase in the number and percentage of activated MAL lymphocytes at 48 to 96 h compared with 10 h post-challenge. From 48 h post-challenge, the majority of lymphocytes in the MAL were found to be activated, consistent with previous reports where T lymphocytes derived from BAL in humans [35,36] and rodents [37,38] have been shown to be mainly activated compared with peripheral blood. An activation (IL-2R expression) of MAL versus blood lymphocytes at 48 to 96 h but not at 10 h post-challenge seems to confirm the largely non-specific, possibly mast cell mediated, nature of the initial neutrophil-dominated response, while the subsequent recruitment of predominantly eosinophils into the mammary lumen may be mediated by signals derived from antigen-specific CD4⁺ T cells. The differential expression of adhesion/activation molecules (CD11b and CD44) on MAL eosinophils at 10 h compared with later time-points supports the existence of two separate mechanisms of recruitment of inflammatory cells during the course of an allergic-type response. The sudden disappearance of neutrophils at 48 h post-challenge may be associated with the increase in $\gamma\delta^+$ T cells in the MAL at this time as $\gamma\delta^+$ T cells have been shown to control neutrophil migration [39].

In conclusion, this study has demonstrated that the cellular kinetics of the leucocytic response in mammary glands after larval challenge is closely aligned to an allergic reaction such as asthma. The kinetics of expression of cell surface antigens on eosinophils and lymphocytes during the course of an allergic response in blood and following their transmigration into the mammary lumen indicate that sequential non-specific and specific mechanisms may orchestrate the recruitment of inflammatory cells during an allergic response.

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