

ORIGINAL ARTICLE

The innate response to peanut extract in ovine afferent lymph and its correlation with allergen sensitisation

Jenna L Van Gramberg¹, Robert J Bischof¹, Robyn E O’Hehir², Michael J de Veer^{1,3} and Els N Meeusen^{1,3}

The innate response generated after initial allergen exposure is crucial for polarising adaptive immunity, but little is known about how it drives an atopic or type-2 immune response. The present study characterises the response of skin-draining afferent lymph in sheep following injection with peanut (PN) extract in the presence or absence of aluminium hydroxide (AIOH) adjuvant. Lymph was collected and innate cell populations characterised over an 84 h time period. The innate response to PN extract in afferent lymph displayed an early increase in neutrophils and monocytes without any changes in the dendritic cell (DC) population. PN antigen was transported by neutrophils and monocytes for the first 36 h, after which time DCs were the major antigen trafficking cells. AIOH adjuvant gradually increased antigen uptake by DCs at the later time points. Following lymphatic characterisation, sheep were sensitised with PN extract by three subcutaneous injections of PN in AIOH, and the level of PN-specific immunoglobulin E (IgE) was determined. Sheep with higher levels of steady-state DCs in afferent lymph showed increased monocytic recruitment in afferent lymph and reduced PN-specific IgE following sensitisation. In addition, DCs from afferent lymph that had ingested PN antigen increased the expression of monocyte chemoattractant mRNA. The results of this study show that the innate response to PN extract involves a dynamic change in cell populations in the afferent lymph over time. In addition, DCs may determine the strength of the initial inflammatory cell response, which in turn may determine the nature of the antigen-specific adaptive response.

Immunology and Cell Biology advance online publication, 10 February 2015; doi:10.1038/icb.2015.4

A crucial step in the development of clinical allergies is the generation, in some ‘atopic’ individuals, of high levels of allergen-specific immunoglobulin E (IgE) to otherwise benign, non-infectious environmental antigens (allergens).^{1–3} IgE antibody is a hallmark of type-2 (which includes Th2) or atopic immune responses, and a predisposition to atopy in humans has been linked to both genetic and environmental factors with the innate immune system, the likely determining factor.^{3,4} However, exactly how the innate system is triggered by allergens and how it can skew the adaptive system to a type-2 response in some individuals is still not known.

Several animal models have been used to investigate the immune response after exposure to peanut (PN), one of the leading food allergens implicated with severe allergic reactions (reviewed in Van Gramberg *et al.*⁵). In a recently developed sheep model of PN allergy,⁶ ~50% of sheep responded with high IgE levels to PN extract, a proportion similar to that previously observed for house dust mite allergen.⁷ The availability of an outbred animal model that displays similar differential susceptibility to atopy as the human population may be useful to study the basis underlying atopic predisposition, using technologies and experimental procedures not possible in humans.

Upon initial exposure, antigen is transported from the tissues to the lymph nodes, via the afferent lymph. Afferent lymph is a rich source of innate immune cells leaving the tissue where antigen is present, and can be collected *in vivo* in real time using a unique lymphatic cannulation procedure in sheep.⁸ The present study characterises the innate cellular response in afferent lymph to PN allergen. As aluminium-based adjuvants are commonly used to accelerate sensitisation to allergens in animal models, administration of PN extract in the presence or absence of aluminium hydroxide (AIOH) was compared. In addition, the innate response in afferent lymph was correlated with the ability of individual sheep to raise an IgE response to subsequent immunisation with PN extract.

RESULTS

Afferent lymphatic flow rates and cell migration before and after injection with PN extract

Afferent lymph draining from the prefemoral skin area flowed at a relatively constant cell number (Figure 1b) and at a flow rate of 4–5 ml h^{–1}, although there was some variation between animals (Figure 1a). There were no significant differences observed after injection of PN with either saline (PN-saline) or AIOH adjuvant (PN-AIOH) over the 84 h course of the experiment.

¹Biotechnology Research Laboratories, Department of Physiology, Monash University, Clayton, VIC, Australia and ²Department of Allergy, Immunology and Respiratory Medicine, Alfred Hospital and Monash University, Melbourne, VIC, Australia

³These authors contributed equally to this work.

Correspondence: Dr MJ de Veer, Biotechnology Research Laboratories, Department of Physiology, Monash University, Clayton, Building 13f, VIC 3800, Australia.

E-mail: michael.deveer@monash.edu

Received 3 April 2014; revised 30 November 2014; accepted 2 December 2014

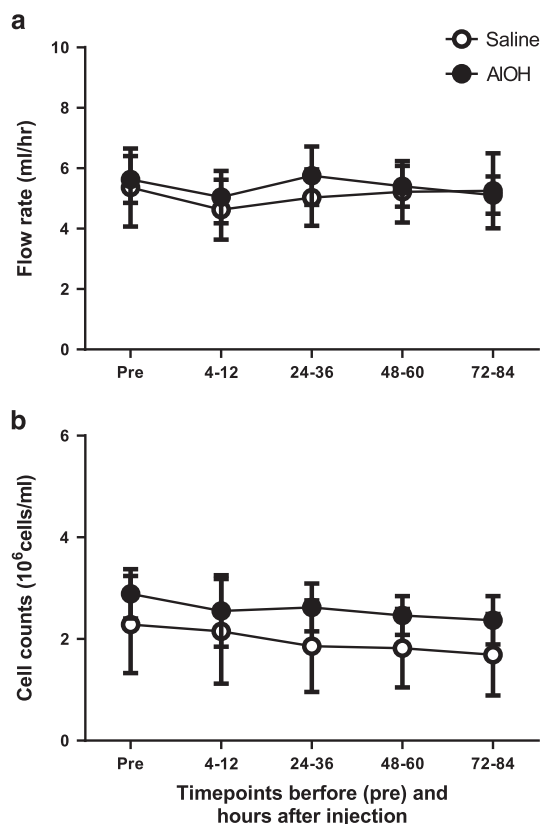


Figure 1 Lymphatic parameters following PN injection. Changes over time in afferent lymphatic flow rate (a) and total cell number (b) before (pre) and after injection with PN-saline (clear, $n=4$) or PN-AIOH (black, $n=11$). Data are expressed as mean $\pm$ s.e.m. for each treatment group.

The percentage of neutrophils recruited into the afferent lymph increased 4–12 h after injection, with a 3.6-fold change ($0.71 \pm 0.20\%$) in the PN-saline group and a 6.8-fold change ($3.03 \pm 1.15\%$) in the PN-AIOH group (Figure 2a). Monocyte numbers also peaked during this period, with the PN-saline group numbers only slightly increasing ($0.23 \pm 0.10\%$), while they were ~ 3 -fold higher ($0.59 \pm 0.17\%$) in the PN-AIOH group, reaching statistical significance compared with preinjection values. Monocyte levels remained elevated for up to 36 h after injection in the PN-AIOH group; however, by 48 h after injection, cell numbers had returned to basal resting levels (Figure 2a).

Lymphocyte and dendritic cell (DC) levels remained relatively constant in both the PN-saline and PN-AIOH groups over the time course, with mean values ranging from 85–91% to 7–10%, respectively (Figure 2a). Ovine afferent lymph is known to consist of two distinct DC subsets, SIRP α^{low} (signal regulatory protein- α) and SIRP α^{high} DCs (Figure 2b).⁹ The predominant subset in afferent lymph in this study was SIRP α^{high} DCs, roughly accounting for 70% of total DCs, with SIRP α^{low} DCs making up the remaining 30%; this ratio was consistent over the time course of the experiment in both the PN-saline and PN-AIOH groups.

Transport of antigen by innate cells in afferent lymph

Neutrophils and monocytes were the predominant cell types that trafficked antigen into afferent lymph in the early response following injection of fluorescein isothiocyanate-labelled PN with both saline and AIOH (Figure 3a). At the 4–12 h time point, there was an increase

in the number of PN-positive neutrophils per 10 000 cells in the PN-saline group (0.81 ± 0.54) and this increase was more pronounced and highly significant in the PN-AIOH group (51.03 ± 26.82) (Figure 3a). Monocyte trafficking of PN antigen displayed similar trends with maximal antigen transport also occurring at 4–12 h after injection. When PN was injected with saline, 0.82 ± 0.24 monocytes in every 10 000 lymphatic cells contained the antigen while a significant increase in the number of antigen-positive monocytes (2.61 ± 1.12) was observed following PN injection in AIOH (Figure 3a). By 24–48 h after vaccination, both PN-positive neutrophils and monocytes had dropped considerably, although they remained the predominant cells carrying antigen.

After 36 h, AIOH induced a significant increase in PN antigen-positive DCs with 5.35 (48 h) and 5.47 (72 h) DCs out of every 10 000 cells within afferent lymph found to contain the PN antigen (Figure 3a). In contrast, PN injected with saline resulted in only 1.02 (48 h) and 1.87 (72 h) PN-positive DCs per 10 000 afferent lymph cells (Figure 3a). These differences were not associated with an increase in DC numbers migrating into the afferent lymph (see Figure 2a). DCs were the only cells to display a significant difference in antigen uptake between the PN-saline and PN-AIOH groups at the 48–60 h time points (Figure 3a).

SIRP α^{high} DCs were the predominant subtype involved in PN antigen uptake and trafficking to the local draining lymphatics, with no significant differences seen between the PN-saline and PN-AIOH groups (Figure 3b). SIRP α^{high} DCs also appeared to be more efficient at capturing PN-Ag compared with SIRP α^{low} DCs, as indicated by a higher percentage of SIRP α^{high} DCs being antigen positive at all time points (Figure 3c).

Correlation of innate lymph cell populations with subsequent IgE responses

To gain an insight into aspects of the innate immune system possibly involved in a predisposition to atopy, correlations were calculated between the innate cells present in afferent lymph before or after a first PN injection and the ability of individual sheep to generate PN-specific IgE after four subsequent immunisations with PN-AIOH. To correct for variable nonspecific reactivity against PN, IgE responses were calculated as the IgE ratio of individual animals before and after immunisation (see Supplementary Table 2 and Van Gramberg *et al.*⁶). The only significant correlation between PN-specific IgE ratio and lymph cell populations before injection was a significant negative correlation with DC numbers (Table 1; $P=0.006$). After PN injection, the negative correlation with DCs was maintained at 24 h and extended to the monocyte population at 6–24 h. Interestingly, after injection monocyte levels at 6–24 h correlated positively with the pre-DC levels. There was no significant correlation between neutrophils and IgE ratio or pre-DCs at any time point (Table 1).

Chemokine expression in DCs before and after PN injection

To examine whether DCs were able to influence monocyte recruitment in response to PN or AIOH, the expression of monocytic chemokines was investigated (CCL2, CCL4 and CCL8). Afferent DCs were sorted before injection (pre) and at 30 h after injection and distinguished based on antigen positivity. Before injection, sorted DCs expressed very low levels of monocyte chemotactic cytokines; however, by 30 h after injection, PN antigen-positive DCs displayed higher expression of all chemokines (two out of three sheep tested) (Figure 4) compared with PN-negative DCs. Interestingly, upregulation of these chemokines was seen in both saline and AIOH groups, indicating that

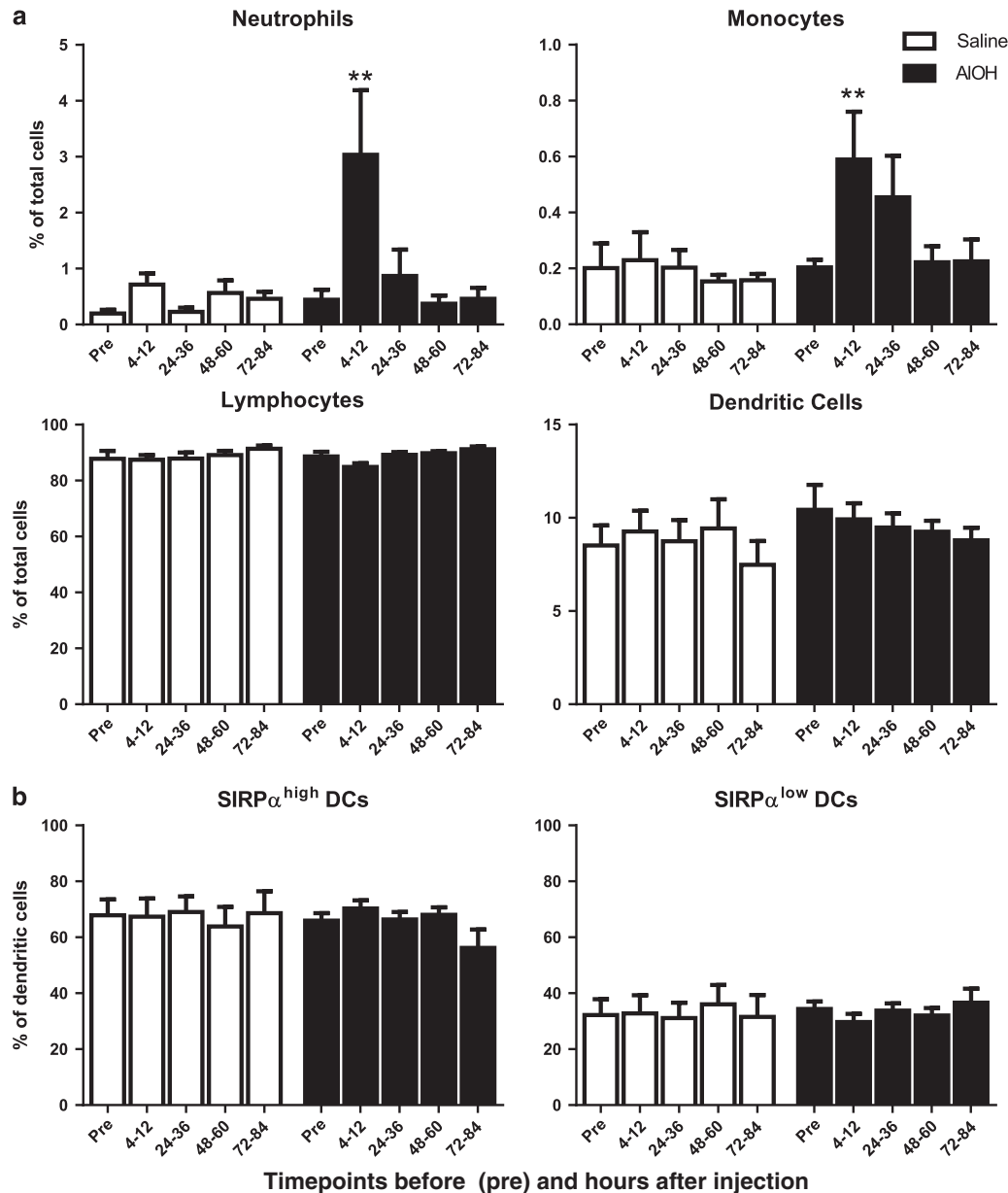


Figure 2 Changes in afferent lymph cell populations. Cell types before (pre) and after injection with PN-saline (clear, $n=4$) or PN-AIOH (black, $n=11$). The percentage of neutrophils, monocytes, lymphocytes and DCs in afferent lymph (a), and the percentage of DCs that expressed high or low levels of SIRP α , respectively (b). Data are expressed as mean $\pm$ s.e.m. for each treatment group. **Significant difference in comparison with baseline (pre) levels, $P<0.01$.

DCs may be capable of responding to PN antigen alone, in the absence of an adjuvant.

DISCUSSION

Intradermal injection of PN extract with or without the aluminium adjuvant (AIOH) induced a temporal change in innate cell populations in the draining afferent lymph, characterised by an early (4–12 h) increase in neutrophils and monocytes. The addition of AIOH boosted the number of these cell populations and extended the elevated monocyte numbers to 24–36 h without changing the lymph flow. However, neither immunisation increased the numbers of DCs in afferent lymph over the 84 h time period. Studies in mice have consistently shown that aluminium-adsorbed immunisations significantly increased DC numbers in the draining lymph nodes.^{10,11}

Our present and previous studies,^{12–14} showing no change in DC numbers in afferent lymph, would suggest that any increase in lymph node DCs would be derived from the blood rather than the tissue.

Neutrophils and monocytes constitute the vast majority of cells transporting PN antigen into the afferent lymph during the first 38 h after injection. After this time, uptake of antigen by DCs increased significantly but only in the PN-AIOH-injected group. This is consistent with a previous study showing an increased uptake of OVA-coated beads by afferent lymph DCs at these later time points¹² and seems to be unique to the aluminium adjuvant as it has not been observed with other adjuvant systems we have investigated.^{13,14} It may be that this characteristic is related to the type-2-inducing properties generally associated with the aluminium adjuvants.¹⁵ As soluble antigen has been shown to disappear from

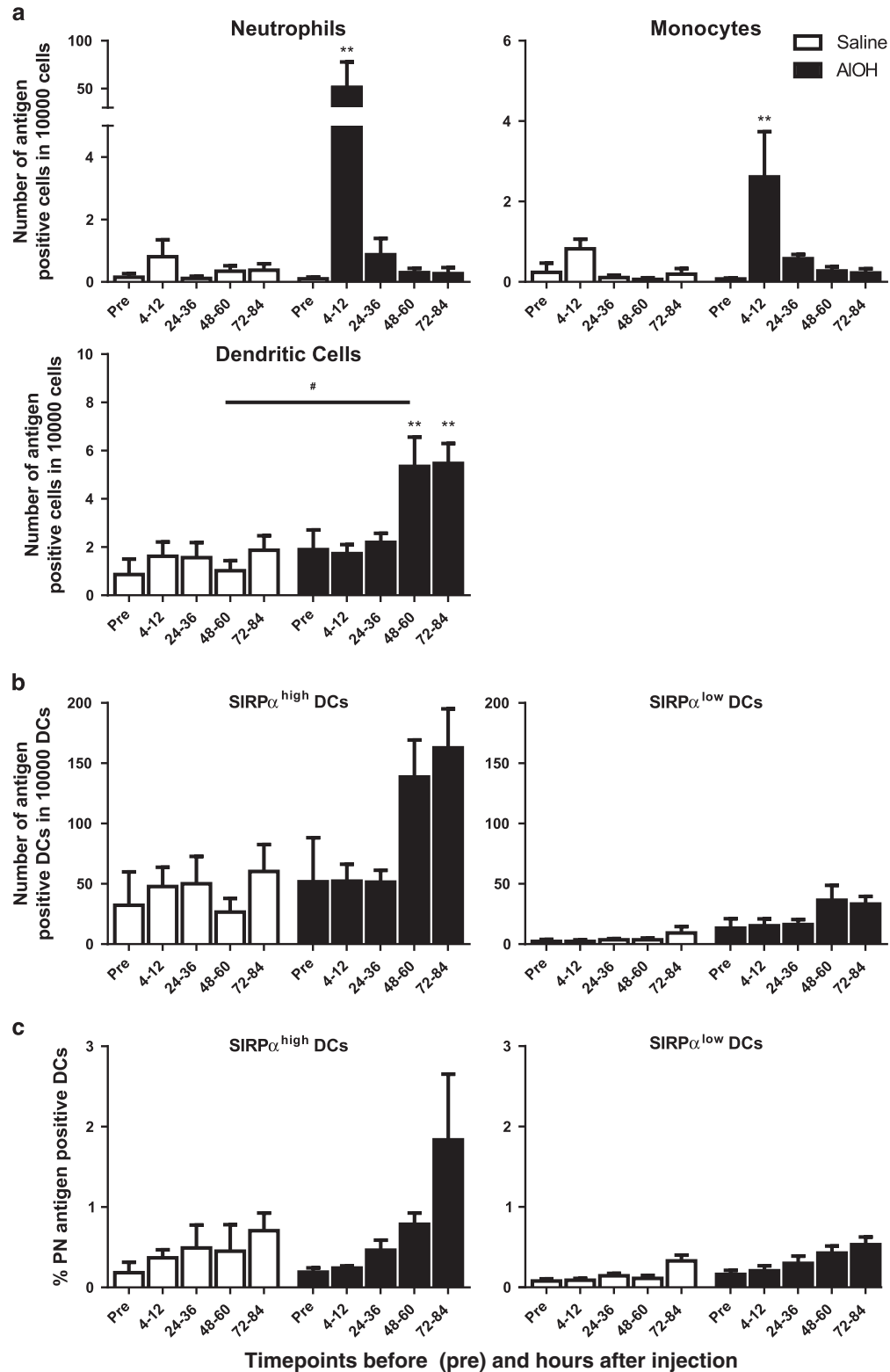


Figure 3 Antigen transport by different innate cell populations in afferent lymph. Antigen uptake before (pre) and after injection with PN-saline (clear, $n=4$) or PN-AIOH (black, $n=11$). The number of PN-Ag-positive cells in 10000 cells was determined for neutrophils, monocytes and DCs (a). The number of PN-Ag-positive DCs per 10000 DCs is shown in (b) for SIRP α^{high} and SIRP α^{low} DCs. (c) The percentage of SIRP α^{high} and SIRP α^{low} DCs that were PN-Ag positive at each time point. Data expressed as mean $\pm$ s.e.m. for each treatment group. *A significant difference in comparison with baseline (pre) levels and #significant differences between groups, ** $P<0.01$ and # $P<0.05$.

Table 1 Correlations between PN sensitisation and innate cell populations in afferent lymph before (pre) and after injection of PN extract

	Pre	6 h	24 h	48 h	72 h
<i>IgE ratio vs innate cell populations</i>					
DCs	-0.89	0.07	-0.82	0.07	-0.64
	0.006	0.453	0.017	0.453	0.069
Monocytes	-0.5	-0.8	-0.8	0.0	-0.3
	0.133	0.017	0.024	0.518	0.297
Neutrophils	0.36	-0.29	-0.11	0.68	0.29
	0.222	0.278	0.420	0.055	0.278
<i>Pre-DC vs other innate cell populations</i>					
Monocytes	0.75	0.93	0.79	0.21	0.18
	0.066	0.007	0.048	0.662	0.713
Neutrophils	-0.21	0.57	0.21	-0.50	-0.14
	0.662	0.200	0.662	0.267	0.783

Abbreviations: DC, dendritic cells; Ig, immunoglobulin; PN, peanut. Spearman's rank correlation coefficient and *P*-values are shown. Bold numerals indicate significance, *P* < 0.05.

afferent lymph by 24 h,¹² it is likely that the later transport of PN antigen by DCs is in the form of ingested antigen bound to aluminium particles.

Most studies examining the basis for predisposition to allergy have looked at qualitative differences in innate cell populations between allergic and non-allergic individuals, including differences in innate pattern recognition receptors.^{16–19} The present results show a negative correlation between the number of DCs in afferent lymph of naive sheep and the generation of an IgE response after subsequent sensitisation with PN extract, suggesting that polarisation towards a type-2 immune response may depend on quantitative rather than qualitative differences in the DC population. DC numbers in afferent lymph vary between sheep but remain relatively constant within individual sheep under steady-state conditions (de Veer, unpublished), and this may account for the genetic factor associated with allergy predisposition. However, DC numbers in the tissue have been shown to increase upon microbial stimulation,^{16,20} indicating that the environmental factors may also contribute to DC numbers and subsequent susceptibility to allergy.

In the present study, the number of DCs in steady-state afferent lymph showed a highly significant positive correlation with the increase in afferent lymph monocytes 6–24 h after injection of PN extract. This may indicate that DC activation induced the recruitment and migration of monocytes into afferent lymph. This agrees with the increase in chemokine expression by DCs that have ingested PN antigen. Monocytes have recently been defined as independent surveillance and effector cells producing high levels of type-1-inducing cytokines,^{20,21} and we previously reported increased levels of proinflammatory gene expression in afferent lymph cells at the peak of monocyte recruitment.²² Taken together, these results are consistent with a scenario where steady-state DC numbers determine the level of monocyte recruitment in tissue and afferent lymph. Subsequent secretion of immunomodulatory signals by monocytes upon arrival in the lymph nodes may create an environment conducive to the generation of an adaptive type-1 or type-2 response, depending on the strength of the proinflammatory signals. The later arrival of antigen-loaded DCs into the lymph node may provide the

cognate antigen-specific signals to the T cells within this polarising environment.

METHODS

Preparation of PN extract

Crude PN extract was prepared from unsalted, dry-roasted PNs as described.^{6,23} Briefly 10 g of PNs were crushed and then delipidated with 25 ml acetone, followed by five washes with 25 ml diethyl ether. The pellet was filtered and dried for 10 min under vacuum, ground to a fine powder using liquid nitrogen, suspended in 30 ml phosphate-buffered saline and incubated overnight rotating at 4 °C. The extract was further centrifuged at 1000 g and 20 000 g to obtain a clear supernatant, dialysed against phosphate-buffered saline, filter-sterilised and protein concentration determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Fluorescein isothiocyanate-labelling of PN was performed as described.¹²

Pseudoafferent lymphatic cannulation and PN injections

Pseudoafferent lymphatic cannulation was performed in a two-stage surgical procedure, as described previously.¹² Briefly, merino sheep had both prefemoral lymph nodes surgically removed at 1 year of age and left for ~1 year to allow the afferent lymphatics to reanastomose with the efferent lymphatic duct. In a second surgery, a 0.96 mm × 0.58 mm heparin-coated polyvinyl chloride cannula was inserted into the pseudoafferent (previously efferent) lymphatic duct and exteriorised to allow for the collection of afferent lymph fluid and cells draining the area from the site of injection. Sheep were randomly assigned to two groups: PN-saline (*n* = 4) or PN-PN-AIOH (*n* = 11), and 1 ml injections were administered into the prefemoral drainage area as 10 individual intradermal injections of 100 µl. Each 1 ml injection volume consisted of 100 µg of fluorescein isothiocyanate-conjugated PN extract prepared in 950 µl sterile saline with or without 50 µl of AIOH adjuvant (Rehydralgel LV-AIOH; Reheis, Berkeley Heights, NJ, USA). The handling of animals and experimental procedures were all approved by the Monash University Animal Ethics Committee in accordance with the relevant licensing agreement.

Analysis of afferent lymph and cells

Afferent lymph was collected in sterile 50 ml falcon tubes containing 0.05 IU heparin (Pfizer, West Ryde, NSW, Australia) and 20 µl of 100 × cell culture penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) for a period of no more than 2 h before removal and storage at 4 °C. Lymph cells were washed in 10 ml ice-cold FACS wash (5% horse serum, 2 mM EDTA in phosphate-buffered saline) before being resuspended in 1 ml red cell lysis buffer (0.093 mM NH₄Cl with 0.1 M Tris (pH 7.2)) for 3 min, followed by two washes with ice-cold FACS wash. For flow cytometry analysis, 3 × 10⁶ per ml lymph cells were resuspended in 25 µl of FACS wash and 25 µl of antibody mix was added; for sorting, 1 × 10⁷ afferent lymph cells were resuspended in 75 µl FACS wash and 75 µl of antibody mix was added. Following washes, cells were resuspended at 1 × 10⁷ cells per ml FACS wash for sorting. The antibodies used were: anti-MHC II-Pacific Blue (dilution 1/2000) (clone 49.1, locally produced), CD14-Alexa Fluor 647 (dilution 1/200) (clone TUK4; Sapphire Bioscience, Waterloo, NSW, Australia), anti-CD172a (SIRP-α) (dilution 1/250) (clone DH59B; VMDR, Pullman, WA, USA) and anti-mouse IgG1 coupled to phycoerythrin (dilution 1/1000; Caltag Laboratories, Burlingame, CA, USA). Samples were analysed on an LSR2 Flow Cytometer (BD Biosciences, San Jose, CA, USA) and sorted on an Influx cell sorter (BD Biosciences). Flow cytometry gating strategies can be found in Supplementary Figure 1. All flow cytometry experiments were performed on fresh afferent lymph cells.

Real-time PCR on sorted DCs

For analysis of RNA, sorted afferent lymph DCs were resuspended in 350 µl of Qiazol (Qiagen, Valencia, CA, USA) and stored at -80 °C until required. Samples were thawed and RNA was extracted using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. RNA quantity was measured using a Nanodrop (Nanodrop Technologies, Wilmington, DE, USA). cDNA was synthesised from RNA using a Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Primer 3 software was used to design primers

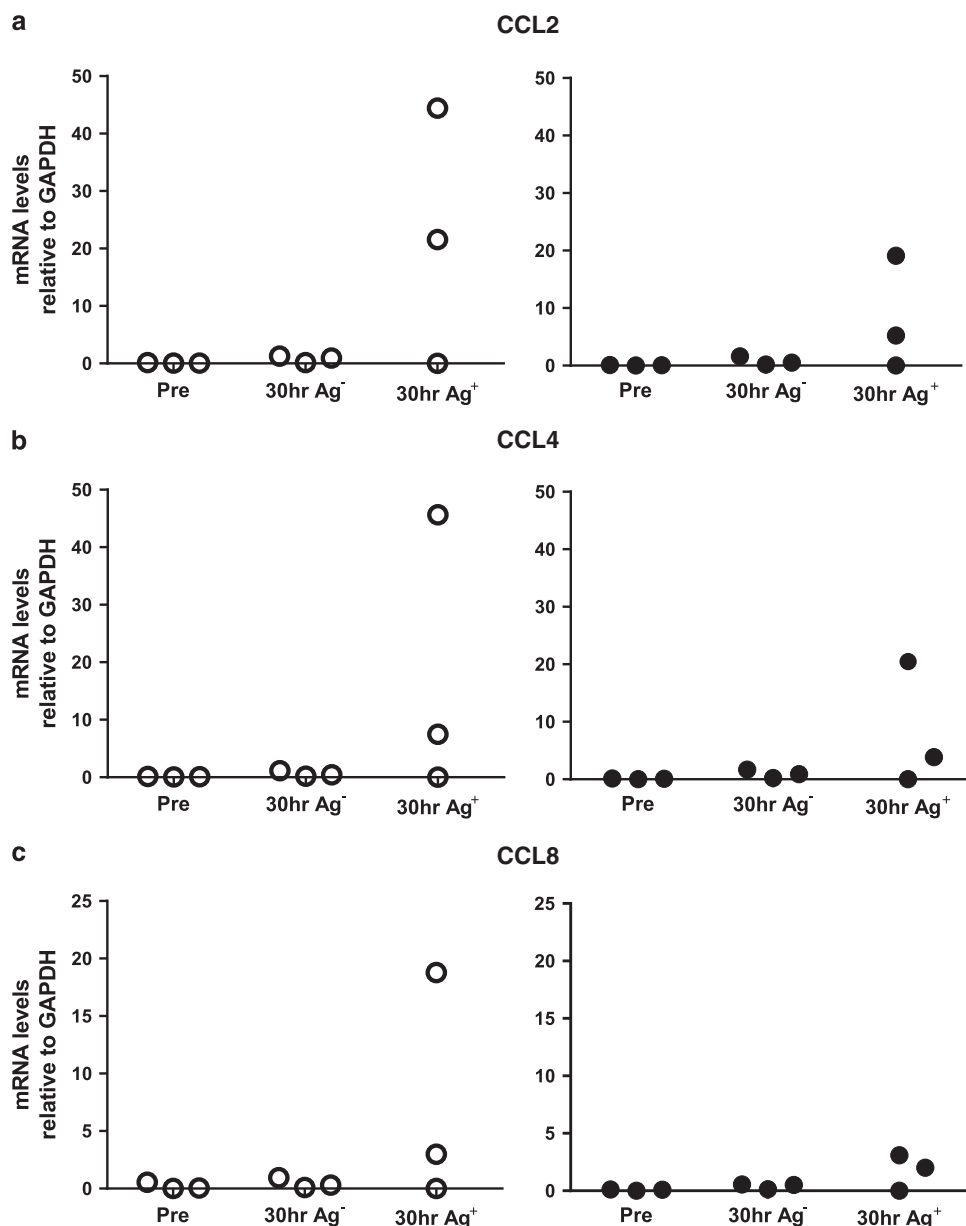


Figure 4 Changes in monocyte chemotactic cytokine gene expression in afferent lymph DCs. DCs sorted on the basis of antigen (PN-FITC) positivity before (pre) and 30 h after injection with either PN-saline (clear, $n=3$) or PN-AIOH (black, $n=3$). Expression is reported for CCL2 (a), CCL4 (b) and CCL8 (c). FITC, fluorescein isothiocyanate.

(spanning an intron where possible) on annotated ovine mRNA sequences (Supplementary Table 1). Real-time PCR was performed on specific samples in triplicate using SYBR green master mix on an Eppendorf Realplex4 (Eppendorf, Hamburg, Germany). The housekeeping gene GAPDH was used to normalise gene expression.

Sensitisation and measurement of PN-specific IgE

Following the cannulation experiments, cannulae were removed and seven sheep were sensitised with PN by three subcutaneous injections in the foreleg at 2-week intervals and a fourth 'boost' injection after a rest period of 4 weeks. Each injection consisted of 100 μ g PN prepared in 950 μ l sterile saline with 50 μ l of AIOH adjuvant. Blood samples were collected 7 days after the boost injection and PN-specific IgE was determined by enzyme-linked immunosorbent assay of $(\text{NH}_4)_2\text{SO}_4$ -treated serum samples as described previously.^{6,7} All samples were tested in duplicate. Owing to the variable background reactivity to PN in an outbred sheep population, the level of PN sensitisation was

determined as the ratio of post/pre-PN-specific IgE serum level for each sheep (see Supplementary Table 2) and as described previously.⁶

Statistical analysis

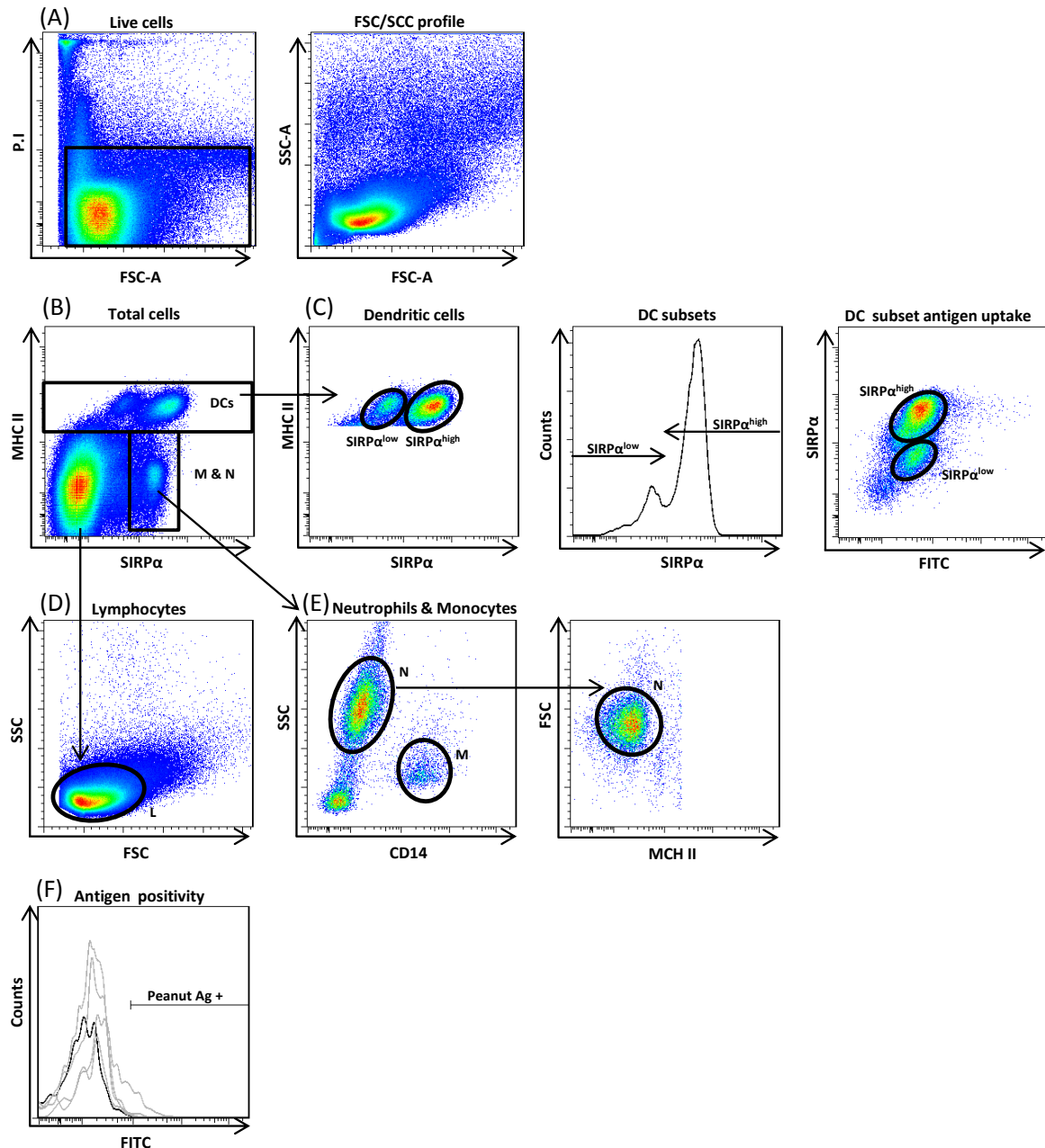
Means $\pm$ s.e.m. were calculated for each group ($n=4$ PN-saline and $n=11$ PN-AIOH). Significant differences were determined in comparison with baseline (pre) values using one-way repeated-measures analysis of variance with a Bonferroni's multiple comparison test. For comparisons between PN-saline and PN-AIOH groups across the time course, two-way repeated-measures analysis of variance with Bonferroni's multiple comparison test was used. For sorted cell differences, a Student's *t*-test was performed to calculate significance from preinjection. The dependence between PN-specific IgE ratios for individual sheep and the different lymph cell populations was calculated using Spearman's rank correlation coefficient. Significance was determined as the confidence interval $>95\%$ ($P<0.05$). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., CA, USA).

ACKNOWLEDGEMENTS

The research was funded by an Australian Research Council (ARC) Grant (to ENM and REO'H) and an APA scholarship (to JLVG).

- 1 Aalberse RC. Specific IgE and IgG responses in atopic versus nonatopic subjects. *Am J Respir Crit Care Med* 2000; **162**: S124–S127.
- 2 Jackola DR. Random allergen-specific IgE expression in atopic families: evidence for inherited 'stochastic bias' in adverse immune response development to non-infectious antigens. *Mol Immunol* 2007; **44**: 2549–2557.
- 3 Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol* 2002; **2**: 773–786.
- 4 Prescott SL. The influence of early environmental exposures on immune development and subsequent risk of allergic disease. *Allergy* 2011; **66**: 4–6.
- 5 Van Gramberg JL, de Veer MJ, O'Hehir RE, Meeusen EN, Bischof RJ. Use of animal models to investigate major allergens associated with food allergy. *J Allergy (Cairo)* 2013; **2013**: 635695.
- 6 Van Gramberg JL, de Veer MJ, O'Hehir RE, Meeusen EN, Bischof RJ. Induction of allergic responses to peanut allergen in sheep. *PLoS ONE* 2012; **7**: e51386.
- 7 Bischof RJ, Snibson K, Shaw R, Meeusen ENT. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. *Clin Exp Allergy* 2003; **33**: 367–375.
- 8 Neeland MR, Meeusen ENT, de Veer MJ. Afferent lymphatic cannulation as a model system to study innate immune responses to infection and vaccination. *Vet Immunol Immunopathol* 2013; **158**: 86–97.
- 9 Contreras V, Urien C, Guiton R, Alexandre Y, Vu Manh TP, Andrieu T *et al*. Existence of CD8alpha-like dendritic cells with a conserved functional specialization and a common molecular signature in distant mammalian species. *J Immunol* 2010; **185**: 3313–3325.
- 10 Kool M, Petrilli V, De Smedt T, Rolaz A, Hammad H, van Nimwegen M *et al*. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J Immunol* 2008; **181**: 3755–3759.
- 11 Mosca F, Trito E, Muzzi A, Monaci E, Bagnoli F, Iavarone C *et al*. Molecular and cellular signatures of human vaccine adjuvants. *Proc Natl Acad Sci USA* 2008; **105**: 10501–10506.
- 12 de Veer M, Kemp J, Chatelier J, Elhay MJ, Meeusen ENT. The kinetics of soluble and particulate antigen trafficking in the afferent lymph, and its modulation by aluminum-based adjuvant. *Vaccine* 2010; **28**: 6597–6602.
- 13 de Veer M, Kemp J, Chatelier J, Elhay MJ, Meeusen ENT. Modulation of soluble and particulate antigen transport in afferent lymph by monophosphoryl lipid A. *Immunol Cell Biol* 2012; **90**: 404–410.
- 14 de Veer M, Neeland M, Burke M, Pleasance J, Nathanielsz J, Elhay M *et al*. Cell recruitment and antigen trafficking in afferent lymph after injection of antigen and poly (I:C) containing liposomes, in aqueous or oil-based formulations. *Vaccine* 2013; **31**: 1012–1018.
- 15 Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol* 2009; **9**: 287–293.
- 16 Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012; **18**: 673–683.
- 17 Reider N, Reider D, Ebner S, Holzmann S, Herold M, Fritsch P *et al*. Dendritic cells contribute to the development of atopy by an insufficiency in IL-12 production. *J Allergy Clin Immunol* 2002; **109**: 89–95.
- 18 Schenten D, Medzhitov R. The control of adaptive immune responses by the innate immune system. *Adv Immunol* 2011; **109**: 87–124.
- 19 Vandenbulcke L, Bachert C, Van Cauwenberge P, Claeys S. The innate immune system and its role in allergic disorders. *Int Arch Allergy Immunol* 2006; **139**: 159–165.
- 20 Jakubick C, Gautier EL, Gibbins SL, Sojka DK, Schlitzer A, Johnson TE *et al*. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity* 2013; **39**: 599–610.
- 21 Soudja SM, Ruiz AL, Marie JC, Lauvau G. Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity* 2012; **37**: 549–562.
- 22 Burke ML, de Veer M, Pleasance J, Neeland M, Elhay M, Harrison P *et al*. Innate immune pathways in afferent lymph following vaccination with poly(I:C)-containing liposomes. *Innate Immunity* 2013; **20**: 501–510.
- 23 de Leon MP, Rolland JM, O'Hehir RE. The peanut allergy epidemic: allergen molecular characterisation and prospects for specific therapy. *Expert Rev Mol Med* 2007; **9**: 1–18.

The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (<http://www.nature.com/icb>)



Supplementary Figure 1. Flow cytometry gating plots for a representative sample of afferent lymph from one animal. **(A)** Preliminary gating for live cells using propidium iodide (PI) and the size (FSC) and granularity (SSC) profile of afferent lymph. **(B)** An example of total cell populations identified on MHC II and SIRP α expression. **(C)** Dendritic cells (DCs) were gated based on MHC II expression, with DC subsets further sub-divided into SIRP α^{low} and SIRP α^{high} DCs using histogram gates. **Dot plot of DC gate displaying antigen uptake in a representative 72 h sample.** **(D)** Lymphocytes (L) were classified based on a low FSC/SSC profile. **(E)** Neutrophils (N) and monocytes (M) were first gated on SIRP α expression. Neutrophils were classified based on a high SSC profile and low CD14, then confirmed by a low MHC II expression. Monocytes were determined by a low SSC profile and high CD14 expression. **(F)** Peanut antigen positivity (PN-Ag $^{+}$) in each cell type was determined based on FITC fluorescence greater than pre vaccination levels. **The plot shows a representative population of neutrophils with basal FITC fluorescence prior to injection (black line) and timepoints post injection (grey lines).** All plots are representative of one animal.

Supplementary Table 1. Primer Sequences for genes investigated with real time PCR.

Gene	Sequence (5'-3')
CCL2	Forward: GCTGTGATTTTCAAGACCATCCT
	Reverse: GGCGTCCTGGACCCATT
CCL4	Forward: CTGTTCTCCGGCACTCTCAG
	Reverse: AGGCTGCTGGTCTCGTAGTA
CCL8	Forward: ATCACCAACAGCCAGTGTCC
	Reverse: TTCGGGACTTTTGGTCCAGG
GAPDH	Forward: GTCCCCACCCCCAACGT
	Reverse: TCTCATCATACTTGGCAGGTTTCTC

Supplementary Table 2. Individual sheep peripheral blood IgE levels before (pre) and seven days after (post) the final PN sensitisation injection. All samples were tested in duplicate. Raw values shown in the table are mean OD 450nm values.

Sheep	Pre	Post	Ratio (post/pre)
1	0.138	0.172	1.243
2	0.173	0.278	1.609
3	0.121	0.240	1.983
4	0.127	0.243	1.921
5	0.251	0.321	1.277
6	0.113	0.133	1.173
7	0.286	0.830	2.902