

An investigation of the impact of the location and timing of antigen-specific T cell division on airways inflammation

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Summary

It is widely accepted that allergic asthma is orchestrated by T helper type 2 lymphocytes specific for inhaled allergen. However, it remains unclear where and when T cell activation and division occurs after allergen challenge, and whether these factors have a significant impact on airways inflammation. We therefore employed a CD4-T cell receptor transgenic adoptive transfer model in conjunction with laser scanning cytometry to characterize the location and timing of T cell division in asthma *in vivo*. Thus, for the first time we have directly assessed the division of antigen-specific T cells *in situ*. We found that accumulation of divided antigen-specific T cells in the lungs appeared to occur in two waves. The first very early wave was apparent before dividing T cells could be detected in the lymph node (LN) and coincided with neutrophil influx. The second wave of divided T cells accumulating in lung followed the appearance of these cells in LN and coincided with peak eosinophilia. Furthermore, accumulation of antigen-specific T cells in the draining LN and lung tissue, together with accompanying pathology, was reduced by intervention with the sphingosine 1-phosphate receptor agonist FTY720 2 days after challenge. These findings provide greater insight into the timing and location of antigen-specific T cell division in airways inflammation, indicate that distinct phases and locations of antigen presentation may be associated with different aspects of pathology and that therapeutics targeted against leukocyte migration may be useful in these conditions.

Keywords: asthma, immune regulation, T cells

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Introduction

Allergic asthma is characterized by chronic eosinophilic airway inflammation, believed to be orchestrated by T helper type 2 (Th2) lymphocytes specific for inhaled allergen [1], that can be generated by the introduction of antigen into the airways [2,3]. The development of specific cell-mediated pulmonary immunity requires an afferent phase, during which inhaled antigens are captured, processed and presented to T cells in the context of major histocompatibility complex II on antigen-presenting cells (APCs) [4] and an efferent phase, where T cells traffic through lymph nodes (LN) to sites of inflammation. Thus T cells are potentially involved in two phases of airway inflammation.

The recirculation of T cells through secondary lymphoid organs and peripheral non-lymphoid tissues is fundamental to the maintenance of immune surveillance. Naive T cells recirculate through secondary lymphoid sites until they are

stimulated by cognate antigen conveyed there from tissues by APCs [5,6]. During T cell activation, naive T cells appear to be imprinted with a template of the site in which they were initially stimulated [7]. These T cells then differentiate to acquire a tissue-specific homing phenotype via expression of different receptor and signalling molecules, which facilitate return to that site [8]. However, it is still unclear exactly where and when T cell activation and division occurs after allergen challenge, and whether this has an impact on airways inflammation.

Previous studies have shown that depletion of CD4⁺ T cells by administration of antibody clearly inhibited allergen-induced airway eosinophilia and airway hyperresponsiveness (AHR) [9,10]. It has also been shown that CD4⁺ T lymphocytes are critical for the development of airway epithelial changes and subepithelial fibrosis as well as AHR in a murine model of chronic asthma [11]. Although the studies noted above demonstrate that T cells

play an important role in asthma pathogenesis, many of the studies looking at T cell activation or division have analysed results by flow cytometry or by looking at peripheral blood lymphocytes [12–14], and have not characterized these parameters *in situ*.

Within the lung an extensive network of dendritic cells (DCs) line the conducting airways and interstitium and are capable of presenting inhaled antigen to naive and memory T cells *ex vivo* [15–18]. These cells are also capable of maturation and migration to the T cell areas of the local draining LN (DLN) and are thought to be essential for mature DCs to interact with and prime naive recirculating T cells. Indeed, a recent study by Idzko *et al.* [19] showed that inhalation of FTY720 at the time of allergen challenge inhibits Th2-mediated features of asthma by altering the function of lung DCs, suggesting that this was a result of inhibiting the migration of lung DCs to the draining mediastinal LN [19]. This complements previous work from this group, showing that allergen inhalation causes ATP activation leading to recruitment of lung myeloid DCs that induce Th2 responses in DLN, highlighting the importance of DCs in asthma [20]. However, no studies have analysed directly the kinetics and location of the division of antigen-specific T cells upon airways challenge.

We therefore employed a CD4-T cell receptor (TcR) adoptive transfer model in conjunction with laser scanning cytometry (LSC) [21] to analyse *in situ* division of antigen-specific T cells in the lung and DLN in a model of airways inflammation. We found that accumulation of divided T cells in lung tissue occurred in two distinct waves. The first wave occurred at day 1 post-airways challenge, before T cell division was detected in the DLN. The second wave of T cell division in the lung appeared following division of T cells in the DLN and corresponded with peak eosinophilia. These observations indicated that distinct phases and locations of antigen presentation may be associated with different aspects of pathology. In support of this hypothesis, we demonstrated that administration of FTY720, a drug which inhibits cell migration, after the first wave of T cell division in the lung abolished the second wave of division and inhibited pathology.

Materials and methods

Animals

BALB/c (H-2^{d/d}) mice were purchased from Harlan-Olac (Oxon, UK). Mice homozygous for the cOVA peptide^{323–339}/I-A^d-specific DO11.10 TcR transgene (detected using the clonotypic monoclonal antibody KJ1-26) on the BALB/c background [22] were used as donors. All animals were specified pathogen-free and were maintained at the University of Glasgow Central Research Facilities in accordance with local and UK Home Office regulations.

Preparation of cell suspensions for adoptive transfer

Peripheral LN (PLN) (axillary, brachial, inguinal, cervical), mesenteric LN and spleens from DO11.10 BALB/c mice were pooled and prepared as single cell suspensions, by passing through a Nitex sieve (Cadisch Precision Meshes, London, UK) using a syringe plunger, and washed in sterile RPMI-1640 (Invitrogen Life Technologies, Paisley, UK). The percentage of KJ1-26⁺ CD4⁺ DO11.10 T cells was determined by flow cytometric analysis as described below and made up to required volume in phosphate-buffered saline (PBS).

Ovalbumin model of airway inflammation

Transgenic T cells (3×10^6) in 200 µl were injected intravenously (i.v.) into age-matched naive BALB/c recipients on day –1. The mice were then immunized with an intraperitoneal (i.p.) injection of 100 µg chicken ovalbumin (OVA) (OVA, Fraction V obtained from Sigma-Aldrich, Poole, UK) in a 1% alum suspension (Brenntag Biosector, Frederikssund, Denmark) made up to a volume of 200 µl, on days 0, 7 and 14. Mice were anaesthetized i.p. with 250 µl avertin (1:1 w/v solution of 2,2,2-tribromoethanol in *tert*-amyl alcohol, Sigma-Aldrich) and challenged intranasally in 30 µl PBS with 50 µg OVA or PBS alone on day 21. Mice were euthanized at various time-points by administration of a lethal dose of avertin.

FTY720 treatment

Mice were treated with 0.1 mg/kg FTY720. Mice were anaesthetized i.p. with 250 µl avertin and 0.1 mg/kg FTY720 (Cayman Chemical, Ann Arbor, MI, USA) in 50 µl PBS was delivered intranasally on day 2 post-airways challenge (day 23 of experiment).

Bronchoalveolar lavage

Immediately after termination with avertin, blood was collected by cardiac puncture. Bronchoalveolar lavage (BAL) was harvested in two 0.8 ml aliquots of fluorescence activated cell sorter buffer [2% fetal calf serum (FCS), 0.05% sodium azide in PBS]. Individual BAL samples were kept on ice before centrifugation at 400 g for 5 min. The supernatants were collected and volumes measured before storage at –20°C until assayed for cytokines. The cell pellets were resuspended in 1 ml of PBS and counted in a haemocytometer. Cytospin preparations were prepared in a Cytospin (Thermo Shandon, Runcorn, UK) and were stained with Rapi-diff II (Triangle Biomedical Sciences, Durham, NC, USA). Blinded differential cell counting was performed using standard morphological criteria as described previously [23].

Flow cytometry

Draining LN, lung and BAL fluid were harvested the day before airway challenge and between days 1 and 7 after

airway challenge. Cell suspensions were prepared as described above and analysed by flow cytometry for CD4 and antigen-specific T cells, detected using the monoclonal antibody KJ1-26, as described previously [24]. Where lungs were analysed, the thoracic cage was opened and the lungs were separated carefully from surrounding tissue by blunt dissection and removed *en bloc* with the heart. For flow cytometry, lungs were processed as described for LN.

Enzyme-linked immunosorbent assay

To detect OVA-specific immunoglobulin (Ig)E in serum samples, Immulon 2 plates (Costar; Corning, NY, USA) were coated with OVA (20 µg/ml) in PBS at 4°C overnight. Plates were then washed at least three times with PBS/Tween 0.05% (Sigma-Aldrich) before being blocked with PBS-FCS 10% (v/v) for 1 h at 37°C. Plates were washed and incubated with diluted serum samples for 3 h at 37°C before further washing. IgE levels in serum were determined by incubation with biotinylated anti-IgE (1/8000; BD Pharmingen, Oxford, UK) for 1 h at 37°C. Plates were then washed and incubated with Extravidin-horseradish peroxidase (HRP) (1/1000; Sigma-Aldrich) for 1 h at 37°C then washed again and tetramethylbenzidine (TMB) microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was added to stop the reaction. Light absorbance was read on a plate reader at 630 nm.

Cytokine analysis

Immulon 4 HBX plates (ThermoShandon) were coated with capture antibody in enzyme-linked immunosorbent assay (ELISA) coating buffer and incubated overnight. Plates were washed with ELISA wash buffer before incubating in 200 µl/well ELISA blocking buffer for 37°C for 1 h. After washing, standards at known concentrations and samples were added in duplicate and incubated for 2 h. The plates were washed again and detection (biotinylated) antibodies added and incubated for a further 1 h. Streptavidin–HRP conjugate (Extravidin; Sigma) was then added after further washes and incubated for 30 min before washing and addition of TMB substrate (Kirkegaard & Perry Laboratories) for up to 30 min or until sufficient blue colour had developed. Optical density was then read on an MRX II microplate reader (Dynex Technologies, Worthing, UK) at 630 nm or at 405 nm if the reaction was stopped by addition of an equal volume of stop solution (KPL). A standard curve was generated from the standard data allowing the calculation of cytokine concentrations in samples.

Immunofluorescence staining for KJ1-26⁺ T cells, B220⁺ B cells and proliferating cell nuclear antigen

Sections were stained as described previously [25]. Briefly, PLN were frozen in liquid nitrogen in octreotide (OCT) embedding medium (Miles Diagnostic Division, Elkhart, IN,

USA) and stored at –70°C. Sections (8 µm) were cut, mounted on microscope slides (Shandon, Leicestershire, UK), and stained immediately with biotinylated anti-KJ1-26 and FITC-anti-B220 (BD Pharmingen) and anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz, Heidelberg, Germany), for 30 min, washed, and then incubated with streptavidin–HRP for 30 min. After washing, the cells were treated with biotinylated tyramide (TSA Biotin system; PerkinElmer Life Sciences, Milano, Italy) for 10 min, washed, and then incubated with streptavidin Alexa Fluor 647 (Molecular Probes, Invitrogen, Paisley, UK) for 30 min. Finally, slides were washed three times and mounted in Vectashield (Vector Laboratories, Peterborough, UK) for analysis by LSC.

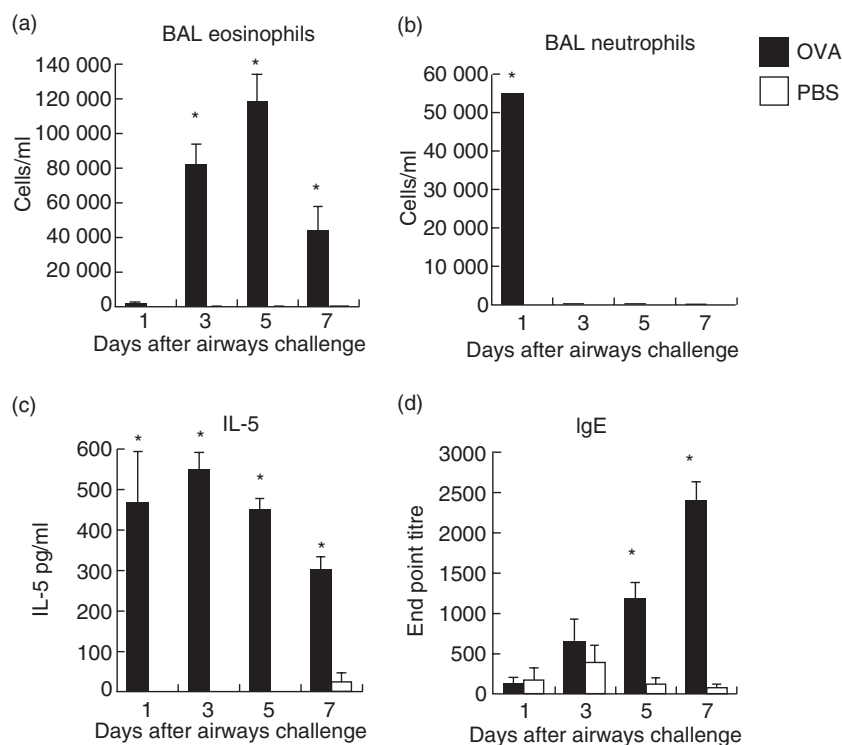
Laser scanning cytometry

Sections were imaged using a LSC equipped with argon, helium–neon and ultraviolet lasers (Compucyte, Cambridge, USA) and data were analysed using the WinCyt software (Improvision, Coventry, UK). LSC combines the identification of fluorescently labelled cells in tissue sections with the ability to quantify these cells within anatomical regions within the section [26]. A computer-controlled microscope stage allows the precise localization and quantification of cells identified by their fluorescence following laser excitation. By setting a threshold intensity of KJ1-26 staining, antigen-specific T cells can be identified and enumerated within a tissue section or within predefined regions within the section. CD4⁺ PCNA⁺ T cells were assessed and gated as described previously [27].

Preparation of bone marrow DCs

The DCs were prepared from bone marrow (BM) as described previously [28]. Cell suspensions were obtained from femurs and tibias of female BALB/c mice. The BM cell concentration was adjusted to 5×10^5 cells/ml and cultured in six-well plates (Corning Costar) in complete RPMI-1640 and 10% FCS (Labtech International, Ringmer, UK) containing 10% of culture supernatant from X63 myeloma cells transfected with mouse granulocyte–macrophage colony-stimulating factor (GM-CSF) cDNA. Fresh medium was added to the cell cultures every 3 days. On day 6, DCs were harvested and cultured. This technique generated a large number of CD11c⁺ DC largely free from granulocyte and monocyte contamination, as described previously [28]. DCs were then left unpulsed or treated with antigen (5 mg/ml OVA (Worthington Biochemical, Reading, UK) or 5 µg/ml OVA323–339 peptide (Sigma-Genosys, Dorset, UK) for 6 h. In imaging experiments, DCs were fluorescently labelled with Cell Tracker 5,6-carboxy-succinimidyl-fluorescein-ester (CFSE; Invitrogen) [29] and 1×10^6 DCs were injected subcutaneously in the footpad of recipient animals and the draining popliteal LN imaged 20 h later.

Fig. 1. Characterization of adoptive transfer model. 3×10^6 unprimed transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunized with 100 μ g OVA/alum 1%, by i.p. injection, three times, 7 days apart, followed by one intranasal challenge 7 days later with 50 μ g OVA or PBS. Mice were sacrificed at various time points after airways challenge (days 1, 3, 5, 7). BAL fluid was sampled, and cytospin preparations were analysed for the presence of eosinophils (a) and neutrophils (b). BAL IL-5 production (c) and OVA-specific serum IgE levels (d) were measured in the same mice. Values are represented as mean $\pm$ standard error of the mean, $n = 3$ per group and are representative of three similar experiments (* $P < 0.05$).



Statistics

Results are expressed as mean $\pm$ standard error of the mean. Significance was tested using Student's unpaired *t*-test. A value of $P < 0.05$ was regarded as significant.

Results

Characterization of adoptive transfer model

The airways model consists of the transfer of OVA-specific T cells into naive BALB/c mice, followed by three OVA/alum immunizations 7 days apart. Mice then received an intranasal challenge of OVA or PBS 7 days later. This model results in significant BAL eosinophilia in the OVA, although not the PBS, treatment group (Fig. 1a). A transient influx of neutrophils was seen at day 1 post-airways challenge in the OVA treatment group (Fig. 1b). As shown in clinical and other murine models of airways inflammation, eosinophilia was associated with an increase in BAL interleukin (IL)-5 production in the OVA challenge group (Fig. 1c) and antigen-specific serum IgE levels (Fig. 1d).

Dividing antigen-specific T cells in lung and DLN

When cells were quantified in DLN sections, there were few dividing antigen-specific T cells present at day 1 post-airways challenge. However, by day 3 after challenge, PCNA-positive

antigen-specific T cells could be detected in the OVA group, indicating that they had undergone division within the preceding 20 h [30]. In contrast, the PBS challenged animals showed little evidence of cell division (Fig. 2a). The percentage of double-positive (KJ1-26⁺ PCNA⁺) cells declined after the peak at day 3 post-airways challenge to days 5 and 7. In lung sections, an initial burst of T cell division was seen at day 1 post-airways challenge in the OVA group; again, very little division was seen in the PBS-treated mice (Fig. 2b). The number of divided antigen-specific T cells increased in the OVA group to day 3 and peaked at day 5 post-airways challenge, declining by day 7. Figure 2c and d illustrates antibody staining for OVA-specific T cells (KJ1-26) and PCNA. Representative tissue section for OVA-challenged mice from 72 h post-airways challenge are shown for DLN (c) and lung (d). Green staining in the LN represents B cell follicles and auto-fluorescent structure in lung tissue, PCNA-expressing cells are blue and KJ1-26 Tg T cells are red. Antigen-specific T cells expressing PCNA, i.e. recently divided cells, are coloured pink. Interestingly, when these data were analysed in parallel with BAL neutrophil and eosinophils it can be seen that the initial division of T cells in the lung corresponds to the transient neutrophil influx seen in the BAL. It is also of note that the peak T cell division in the lung corresponds to the peak of BAL eosinophilia. Thus, accumulation of antigen-specific T cells occurs in two distinct waves, the early wave occurring in the lung, followed by division in the DLN, with the second wave in the lung coinciding with peak eosinophilia.

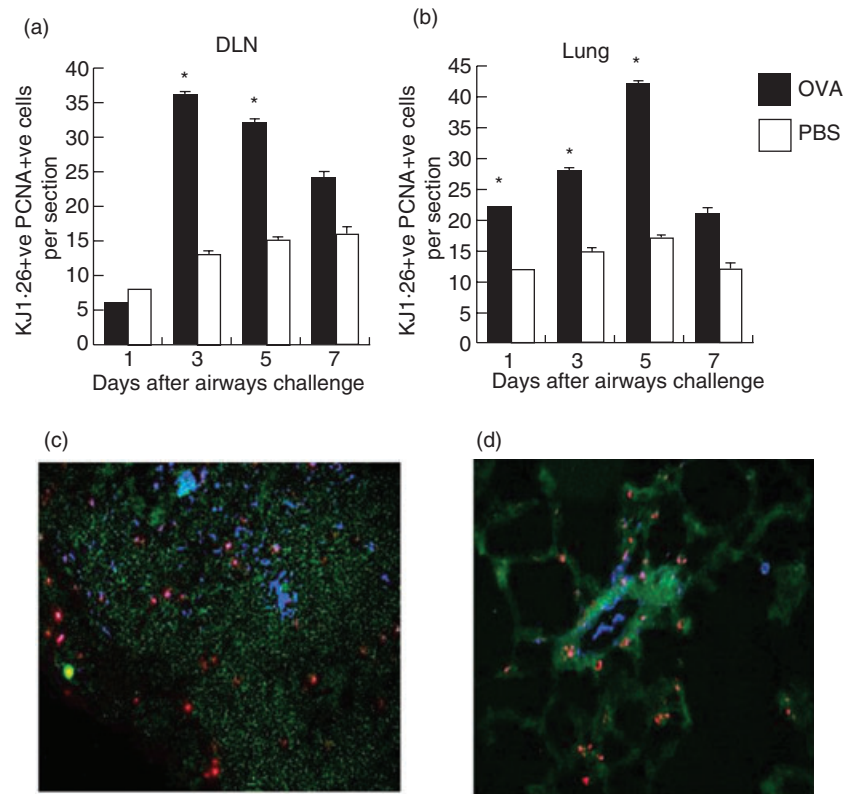


Fig. 2. Quantification of dividing antigen-specific T cells in lung and draining lymph node by laser scanning cytometry (LSC). Mice were challenged as in Fig. 1, with OVA or PBS. LN and lung sections were stained for OVA-specific T cells (KJ1-26) and PCNA. Double-positive cells were quantified by LSC in DLN (a) and lung (b) tissue sections. Representative tissue section for OVA challenged mice from 72 hours post airways challenge are shown for DLN (c) and lung (d). Green represents B220 in DLN section and autofluorescence in lung section. OVA-specific T cells are red, PCNA-positive cells are blue and double-positive cells are pink. Values are represented as mean $\pm$ standard error of the mean, $n = 3$ per group (* $P < 0.05$).

Effect of FTY720 on inflammatory markers

To determine whether modulation of the timing and location of the division of antigen-specific T cells can influence the outcome of disease progression, mice were treated after the airways challenge with FTY720, which has been shown to sequester lymphocytes from blood and spleen selectively and reversibly into secondary lymphoid organs, preventing their migration to the sites of inflammation.

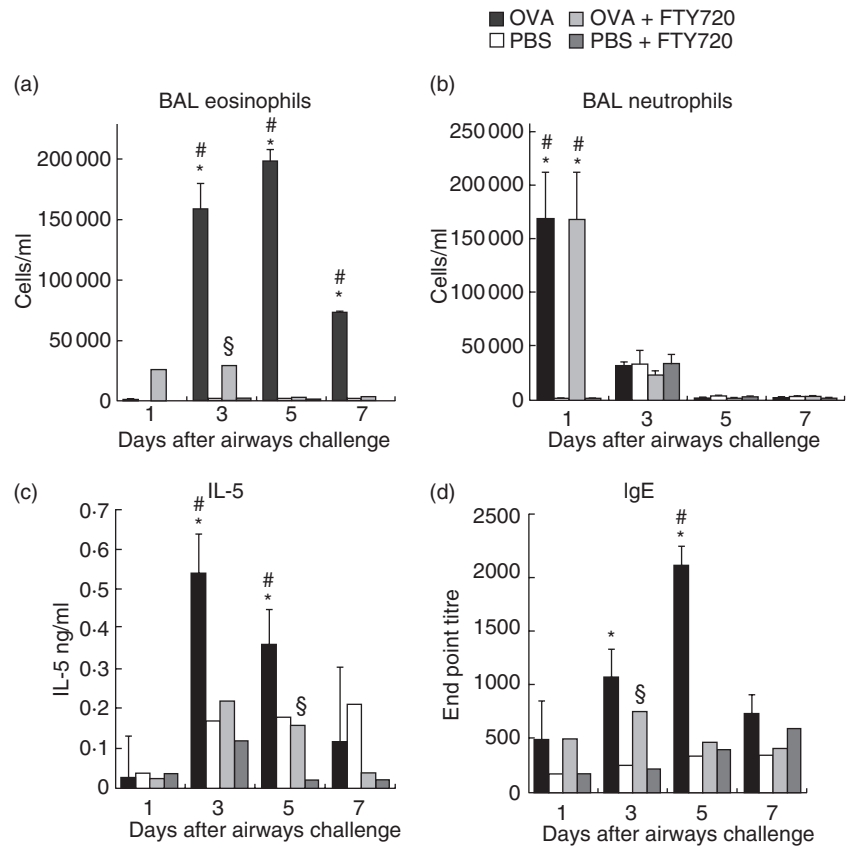
The airways inflammation model was performed as described above and FTY720 administered 2 days after the airways challenge, allowing initial antigen presentation in the lung to occur, as demonstrated in Fig. 2. As seen previously, airway challenge with OVA resulted in significant BAL eosinophilia compared with the PBS airway challenge (Fig. 3a). When FTY720 was given after the OVA airways challenge an initial small rise in BAL eosinophilia was observed, although this was much lower and more transient than that seen with the non-drug-treated group. When total BAL cell counts were calculated there was a significant increase in cells at day 3 post-airways challenge in the OVA and OVA plus FTY720 groups compared with PBS controls. The cell number continued to rise in the OVA group at day 5, declining by day 7; however, this accumulation of cells was not apparent in the OVA plus FTY720-treated group (data not shown). The transient influx of neutrophils was seen in both the OVA and OVA plus FTY720 treatment groups, as expected (Fig. 3b). BAL fluid was assessed for IL-5 and interferon (IFN)- γ

production. As shown previously, OVA airways challenge resulted in a significant increase in IL-5 production at day 3 post-challenge compared with the PBS-treated mice. However, when FTY720 was given after the airway challenge, no difference was seen between the OVA and PBS groups (Fig. 3c). IFN- γ production was not detected in any groups (data not shown). Antigen-specific serum IgE levels were also measured over the time-course. As expected, the OVA-challenged mice had a significant increase in antigen-specific IgE 3 days post-airways challenge which was not apparent in any other groups. When mice were treated with FTY720, this increase in IgE was not observed in the OVA or PBS groups (Fig. 3d). When non-antigen-specific T cells ($CD4^+ KJ1-26^-$) were analysed by flow cytometry there was no difference in their accumulation in the lung between OVA *versus* OVA + FTY720 (data not shown).

Effect of FTY720 on accumulation of divided antigen-specific T cells in lung and DLN

To investigate whether there was any relationship between the reduction in these parameters and T cell behaviour we next assessed their location and division *in situ*. As before, LN and lung sections were stained for PCNA, KJ1-26 and double-positive cells and quantified by LSC. In the DLN there was no accumulation of antigen-specific T cells at day 1 post-airways challenge in any of the treatment groups (Fig. 4a). As seen previously, there were increased numbers

Fig. 3. Effect of FTY720 in accumulation of divided antigen-specific T cells and pathology. Mice were challenged as Fig. 1 and treated with 2.5 µg FTY720 or PBS. Other mice were treated as in Fig. 1 with no FTY720 plus OVA or PBS. Mice were sacrificed at various time points (days 1, 3, 5, 7). BAL fluid was sampled, and cytopins preparations were analysed for the presence of eosinophils (a) and neutrophils (b). BAL IL-5 production (c) and OVA-specific serum IgE levels (d) were measured in the same mice. Values are represented as mean $\pm$ standard error of the mean, $n = 5$ per group and are representative of two similar experiments (* $P < 0.05$ OVA versus PBS, # $P < 0.05$ OVA versus OVA plus FTY720, § $P < 0.05$ ova plus FTY720 versus PBS plus FTY720).



of divided T cells present at day 1 post-airways challenge in the lung in the OVA-treated group compared with the PBS group. This was mirrored in the OVA and PBS groups with FTY720 treatment (Fig. 4b). The peak accumulation of divided antigen-specific T cells in the DLN of the OVA-treated group occurred at day 3 post-airways challenge, which was not seen in any of the other groups, suggesting that FTY720 prevented division of antigen-specific T cells or that newly divided cells were unable to migrate to this site. As shown previously, there was a second wave of T cell accumulation seen in the lung at day 5 post-airway challenge in the

OVA-treated group. This was not observed in the FTY720 treatment groups.

Effect of FTY720 on accumulation of antigen-specific T cells in LN

It has been hypothesized that FTY720 may modify the potential of DCs to stimulate T cells by affecting the maturation of DCs [31] or affecting the DC–T cell interactions [32]; previous studies have shown that local administration of FTY720 at the time of airways challenge inhibited the

Fig. 4. Quantification of dividing antigen-specific T cells in lung and draining lymph node by laser scanning cytometry. Mice were challenged as Fig. 1 and treated with 2.5 µg FTY720 or PBS. Other mice were treated as in Fig. 1 with no FTY720 plus OVA or PBS. Mice were sacrificed at various time points (day 1, 3, 5, 7). DLN and lung sections were stained for OVA-specific T cells (KJ1-26) and PCNA. Double-positive cells were quantified by laser scanning cytometry. Values are represented as mean $\pm$ standard error of the mean, $n = 5$ per group (* $P < 0.05$ OVA versus PBS, # $P < 0.05$ OVA plus FTY720, § $P < 0.05$ OVA plus FTY720 versus PBS plus FTY720).

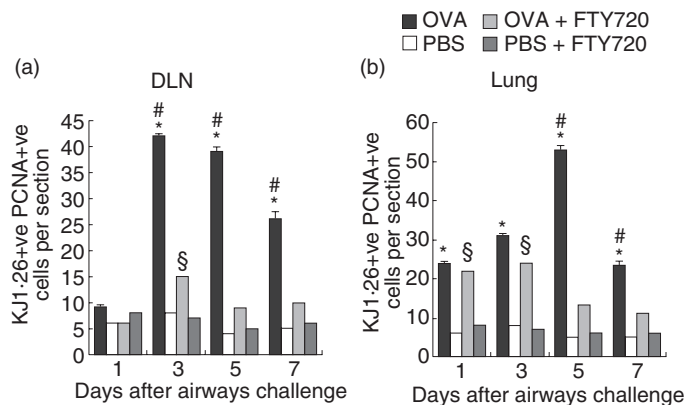
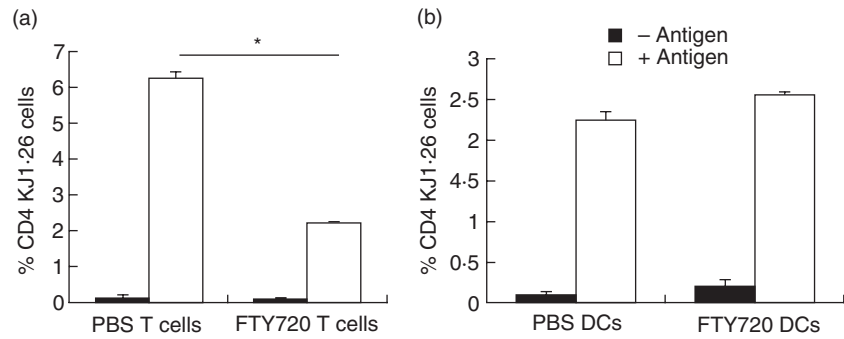


Fig. 5. Effect of FTY720 on accumulation of antigen-specific T cells in lymph nodes. Antigen-specific T cell or BMDCs were treated with FTY720 *in vitro*. T cells were transferred intravenously into naïve mice with or without antigen pulsed DCs, and BMDCs were injected subcutaneously in the footpad with or without antigen pulsing into the same mice. The accumulation of OVA-specific T cells was measured in the draining popliteal LN (a and b) at 20 hours post transfer by flow cytometry. Values are represented as mean $\pm$ standard error of the mean, $n = 5$ per group (* $P < 0.05$).



migration of DCs to DLN, and pretreatment of DCs with FTY720 prior to transfer in an airways model inhibits Th2-mediated asthma by altering the function of lung DCs [19].

To determine whether FTY720 had an effect on DCs and T cells and their ability to interact with each other *in vivo*, both cell types were treated with the drug *in vitro* and transferred into naïve mice and the DLN was removed for the analysis of antigen-specific T cells. It was found that there were few T cells found in the DLN of all treatment groups when given DCs without antigen, while antigen-pulsed DCs produced a large and significant increase in T cell number. FTY720 pretreated T cells had a reduced accumulation following transfer of antigen-pulsed DCs with FTY720 in the DLN compared with sham-treated T cells (Fig. 5a). Interestingly, when antigen-presenting DCs were pretreated with FTY720 there was no difference in T cell accumulation to the LN compared with the control group (Fig. 5b).

Discussion

Although it is known that T cells are present in inflamed airways and the DLN [33], it has been difficult to determine exactly where, how and when they interact with the rest of immune system. Adoptive transfer of Th2 cells into naïve mice followed by aerosol challenge has demonstrated clearly that Th2 cells are recruited into the lung and are critical for inducing the characteristic hallmarks of asthma, including AHR, eosinophilia and mucus production [34]. Many studies have assessed the behaviour of T cells in airways inflammation by flow cytometry and immunohistochemistry in murine models [35–40]. These elegant studies have shown that once sensitized, T cells migrate back to the airway to the site of antigen presentation under the influence of chemokines [41], and these T cells become producers of a range of cytokines that further enhance antigen-induced proliferation and maturation [42]. Many of these studies have used murine systems where T cells have been primed systemically before specific airways challenge. Sensitization via the arguably more relevant and physiological airway alone has also been employed and shown to induce tolerance, immunity or allergy depending on a number of factors, including antigen dose, form and frequency of exposure

[43]. For example, pulmonary infection with influenza in mice induces strong immunity after a vigorous phase of T cell activation and proliferation in the DLN [44,45], while multiple doses of soluble antigen delivered via the nasal route generally induce tolerance, also after a vigorous T cell response in the DLN [46–50]. Allergic sensitization of the airways can also be induced by multiple inhaled doses or systemic immunization via the i.p. route [51,52]. However, there has not been a detailed study of the location and kinetics of the antigen-specific T cells *in situ* in challenged airway and DLN. A recent study examining CD8 T cells after allergen challenge in the lung employed flow cytometry to demonstrate that CD8⁺ T cells specific for inhaled allergens are generated in the DLN and able to suppress airway inflammation via their interaction with respiratory DCs and production of IL-12 in the lung [40], but this did not detail the localization of T cells directly *in situ*. A greater understanding of these critical events in initiating, maintaining and potentially regulating the course of airway inflammation will be important for design and application of new therapeutic approaches. As such, we have employed the adoptive transfer of TcR Tg T cells in the classical murine model of airways inflammation in conjunction with LSC to dissect the importance of dividing T cells in the lung and DLN of airways inflammation.

The adoptive transfer model used in this study allows the identification of antigen-specific T cells transferred into mice, which are primed and then given an airways challenge to induce airways pathology. The use of LSC allows the quantification of recently divided antigen-specific T cells *in situ* providing a novel model to study these parameters. When T cell division was quantified by LSC it was found that the initial division of T cells in the lung corresponded with the transient neutrophil influx. The presence of neutrophils has been shown to be associated with severity of asthma symptoms [53,54] and to drive chemotaxis of T cells [55], suggesting that their presence in the BAL early in the response may cause the recruitment of activated T cells to the site of inflammation. In the DLN, there was little T cell division at the early time-point; however, by day 3 post-airways challenge there was a peak number of double-positive (PCNA⁺ KJ1-26⁺) cells at this site. At day 5 post-

challenge, there was another wave of antigen-specific T cell division seen in the lung, which corresponded with peak eosinophilia. The early T cell division is presumably caused by uptake and presentation of antigen by resident DCs, which have been shown to be present in the lung [56]. The location of the lung DC network, situated immediately above and beneath the basement membrane of the upper and lower airways, enables these cells to sample the epithelium for inhaled antigens [18,57,58]. It has also been shown in experimental models of respiratory virus infection that a population of memory T cells are established in the lung parenchyma and the lung airways [59,60]. Airway resident memory T cells contribute significantly to recall responses by providing immediate effector activity at the site of antigen entry [61], showing that there is local antigen presentation in the lung. T cell–APC interactions can occur in the lung and that these interactions are functionally important in recall [62].

To investigate further the different phases and locations of antigen presentation shown in the airways model we employed FTY720, which acts via the sphingosine 1-phosphate receptor to sequester lymphocytes selectively and reversibly, but not monocytes or granulocytes to secondary lymphoid organs, preventing their migration towards sites of inflammation [63–65]. FTY720 has also been shown to be an effective drug in the treatment of experimental asthma [19,66]. Although these studies showed reduced airways inflammation, it remains unclear as to which cells are affected by FTY720 treatment, with one study suggesting that the reduced airways inflammation was a result of inhibition of DC migration to DLN and subsequently not activating T cells, and the other showing reduced infiltration of antigen-specific T cells to the lung. These previous studies have administered FTY720 at the time of airways challenge before responses in the lungs have been established. In the current study we administered FTY720 locally, 2 days after the initial airways challenge, where we have shown that airways inflammation and T cell division was already established. It was found that FTY720 prevented the recruitment of eosinophils to the lung and the production of IL-5 and antigen-specific IgE. We demonstrated further that FTY720 inhibited the accumulation of antigen-specific T cells in the DLN and lung seen previously in the model, suggesting that the drug may have prevented division of these cells or that newly divided cells were unable to migrate to the site. The effect of FTY720 on T cells has been studied in various disease models including airway inflammation. In a model of allergic diarrhoea, FTY720 inhibited the migration of primed pathogenic CD4⁺ T cells into the intestine [67]. Similarly, treatment of oxazolone-induced colitis with FTY720 reduced the pathology and associated Th2 cytokines and markers, GATA-3 and T1/ST2 [68]. In many allograft studies the effect of FTY720 has been shown to be by reduced infiltration of antigen-specific T cells in the DLN and lymphoid tissue [69,70]. These suggested effects are complemented by the data in our current

study, where FTY720 treatment reduces airways pathology in an established model through an effect on the accumulation of dividing antigen-specific T cells in the lung and DLN.

Our results suggest that airways inflammation is ameliorated with administration of FTY720 through an effect on accumulation of divided antigen-specific T cells in the DLN and lung tissue. To determine if FTY720 was having a direct effect on T cells or by altering DC function and subsequent T cell responses as suggested previously by some studies [64,66,68,71], we pretreated T cells or DCs with FTY720 *in vitro*, transferred them into naive mice and analysed the effect on antigen-specific T cell accumulation in the DLN *in vivo*. Pretreatment of T cells with FTY720 led to less accumulation in LN, with no difference in accumulation when DCs were pretreated with the drug. This supports further the proposal that FTY720 affects migration of antigen-specific T cells to LN and is acting directly primarily on T cells rather than through an effect on DCs. T cell circulation is crucial in localized immune responses, such as airways diseases, as it ensures that antigen-specific T cells have the optimal opportunity to encounter their cognate antigen in any region of the body, whether it be the lung or the DLN. As shown in this study and others, FTY720 is able to prevent activated T cells from homing to the peripheral site of antigen, reduce expansion of antigen-specific T cells [70] and lead to amelioration of inflammatory disease. These studies, in conjunction with the current data, suggest that FTY720 affects the migration and division of antigen-specific T cells and may be a novel strategy for preventing allergic diseases.

In conclusion, the current study has directly assessed the contribution of the division of antigen-specific T cells *in situ* in the lung and DLN in a murine model of airways inflammation using LSC. We demonstrated that accumulation of antigen-specific T cells occurs in two distinct waves, initially in the lung, followed by division in the DLN. Furthermore, accumulation of antigen-specific T cells in the lung and DLN along with accompanying pathology was reduced following treatment with FTY720 after initial T cell division has been initiated. These observations indicate that distinct phases and locations of antigen presentation may be associated with different aspects of pathology. It was also noted that T cells pretreated with FTY720 showed reduced migration to DLN, whereas pretreated DCs did not, suggesting that FTY720 may affect accumulation of dividing T cells in lung and DLN by acting directly on T cells and not indirectly through DCs and subsequent T cell responses. These data provide an insight into the timing, location and functional significance of antigen-specific T cell division in airways inflammation and will be important for rational design and application of therapeutics targeted against leukocyte migration.

References

- 1 Krug N, Frew AJ. The Th2 cell in asthma: initial expectations yet to be realised. *Clin Exp Allergy* 1997; 27:142–50.

- 2 Henney CS, Waldman RH. Cell-mediated immunity shown by lymphocytes from the respiratory tract. *Science* 1970; **169**:696–7.
- 3 Ahlstedt S, Smedegard G, Nygren H, Bjorksten B. Immune responses in rats sensitized with aerosolized antigen. Antibody formation, lymphoblastic responses and mast cell and mucous cell development related to bronchial reactivity. *Int Arch Allergy Appl Immunol* 1983; **72**:71–8.
- 4 Xia W, Pinto CE, Kradin RL. The antigen-presenting activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. *J Exp Med* 1995; **181**:1275–83.
- 5 Bradley LM, Watson SR. Lymphocyte migration into tissue: the paradigm derived from CD4 subsets. *Curr Opin Immunol* 1996; **8**:312–20.
- 6 Mackay CR. Homing of naive, memory and effector lymphocytes. *Curr Opin Immunol* 1993; **5**:423–7.
- 7 Davenport MP, Grimm MC, Lloyd AR. A homing selection hypothesis for T-cell trafficking. *Immunol Today* 2000; **21**:315–7.
- 8 Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; **76**:301–14.
- 9 Nakajima H, Iwamoto I, Tomoe S *et al.* CD4+ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* 1992; **146**:374–7.
- 10 Gavett SH, Chen X, Finkelman F, Wills-Karp M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994; **10**:587–93.
- 11 Foster PS, Yang M, Herbert C, Kumar RK. CD4(+) T-lymphocytes regulate airway remodeling and hyper-reactivity in a mouse model of chronic asthma. *Lab Invest* 2002; **82**:455–62.
- 12 Krinzman SJ, De Sanctis GT, Cernadas M *et al.* T cell activation in a murine model of asthma. *Am J Physiol* 1996; **271**:L476–83.
- 13 Lara-Marquez ML, Deykin A, Krinzman S *et al.* Analysis of T-cell activation after bronchial allergen challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1998; **101**:699–708.
- 14 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**:935–42.
- 15 Schon-Hegrad MA, Oliver J, McMenamin PG, Holt PG. Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J Exp Med* 1991; **173**:1345–56.
- 16 Gong JL, McCarthy KM, Telford J, Tamatani T, Miyasaka M, Schneeberger EE. Intraepithelial airway dendritic cells: a distinct subset of pulmonary dendritic cells obtained by microdissection. *J Exp Med* 1992; **175**:797–807.
- 17 Holt PG, Schon-Hegrad MA, Oliver J. MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage populations. *J Exp Med* 1988; **167**:262–74.
- 18 Holt PG, Schon-Hegrad MA, Oliver J, Holt BJ, McMenamin PG. A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *Int Arch Allergy Appl Immunol* 1990; **91**:155–9.
- 19 Idzko M, Hammad H, van Nimwegen M *et al.* Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* 2006; **116**:2935–44.
- 20 Idzko M, Hammad H, van Nimwegen M *et al.* Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* 2007; **13**:913–9.
- 21 Connolly KM, Bogdanffy MS. Evaluation of proliferating cell nuclear antigen (PCNA) as an endogenous marker of cell proliferation in rat liver: a dual-stain comparison with 5-bromo-2'-deoxyuridine. *J Histochem Cytochem* 1993; **41**:1–6.
- 22 Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCR α thymocytes in vivo. *Science* 1990; **250**:1720–3.
- 23 Hutchison S, Choo-Kang BSW, Bundick RV *et al.* Tumour necrosis factor alpha blockade suppresses murine allergic airways inflammation. *Clinical and Experimental Immunology* 2008; **151**:114–22.
- 24 Smith KM, McAskill F, Garside P. Orally tolerized T cells are only able to enter B cell follicles following challenge with antigen in adjuvant, but they remain unable to provide B cell help. *J Immunol* 2002; **168**:4318–25.
- 25 Smith KM, Brewer JM, Rush CM, Riley J, Garside P. *In vivo* generated Th1 cells can migrate to B cell follicles to support B cell responses. *J Immunol* 2004; **173**:1640–6.
- 26 Pozarowski P, Holden E, Darzynkiewicz Z. Laser scanning cytometry: principles and applications. *Methods Mol Biol* 2006; **319**:165–92.
- 27 Grierson AM, Mitchell P, Adams CL *et al.* Direct quantitation of T cell signaling by laser scanning cytometry. *J Immunol Methods* 2005; **301**:140–53.
- 28 Lutz MB, Kutsch N, Ogilvie AL *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; **223**:77–92.
- 29 Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 1994; **171**:131–7.
- 30 Ghazvini S, Kroll S, Char DH, Frigillana H. Comparative analysis of proliferating cell nuclear antigen, bromodeoxyuridine, and mitotic index in uveal melanoma. *Invest Ophthalmol Vis Sci* 1995; **36**:2762–7.
- 31 van Rijt LS, Vos N, Willart M *et al.* Essential role of dendritic cell CD80/CD86 costimulation in the induction, but not reactivation, of Th2 effector responses in a mouse model of asthma. *J Allergy Clin Immunol* 2004; **114**:166–73.
- 32 Benvenuti F, Hugues S, Walmsley M *et al.* Requirement of Rac1 and Rac2 expression by mature dendritic cells for T cell priming. *Science* 2004; **305**:1150–3.
- 33 Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* 2001; **410**:101–5.
- 34 Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 1997; **186**:1737–47.
- 35 Mathew A, Medoff BD, Carafone AD, Luster AD. Cutting edge: Th2 cell trafficking into the allergic lung is dependent on chemoattractant receptor signaling. *J Immunol* 2002; **169**:651–5.
- 36 Xu B, Wagner N, Pham LN *et al.* Lymphocyte homing to bronchus-associated lymphoid tissue (BALT) is mediated by L-selectin/PNAd, α 4 β 1 integrin/VCAM-1, and LFA-1 adhesion pathways. *J Exp Med* 2003; **197**:1255–67.
- 37 Catron DM, Rusch LK, Hataye J, Itano AA, Jenkins MK. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. *J Exp Med* 2006; **203**:1045–54.

- 38 Durham SR, Till SJ, Corrigan CJ. T lymphocytes in asthma: bronchial versus peripheral responses. *J Allergy Clin Immunol* 2000; **106**:S221–6.
- 39 Kohlmeier JE, Woodland DL. Memory T cell recruitment to the lung airways. *Curr Opin Immunol* 2006; **18**:357–62.
- 40 Wells JW, Cowled CJ, Giorgini A, Kemeny DM, Noble A. Regulation of allergic airway inflammation by class I-restricted allergen presentation and CD8 T-cell infiltration. *J Allergy Clin Immunol* 2007; **119**:226–34.
- 41 Kallinich T, Schmidt S, Hamelmann E *et al.* Chemokine-receptor expression on T cells in lung compartments of challenged asthmatic patients. *Clin Exp Allergy* 2005; **35**:26–33.
- 42 Anderson GP. The immunobiology of early asthma. *Med J Aust* 2002; **177**:S47–9.
- 43 Wikstrom ME, Batanero E, Smith M *et al.* Influence of mucosal adjuvants on antigen passage and CD4+ T cell activation during the primary response to airborne allergen. *J Immunol* 2006; **177**:913–24.
- 44 Lawrence CW, Braciale TJ. Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection. *J Immunol* 2004; **173**:1209–18.
- 45 Roman E, Miller E, Harmsen A *et al.* CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J Exp Med* 2002; **196**:957–68.
- 46 Akbari O, Freeman GJ, Meyer EH *et al.* Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* 2002; **8**:1024–32.
- 47 Akbari O, Stock P, Meyer E *et al.* Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 2003; **9**:582–8.
- 48 Hoynes GF, Tan K, Corsin-Jimenez M *et al.* Immunological tolerance to inhaled antigen. *Am J Respir Crit Care Med* 2000; **162**:S169–74.
- 49 Sedgwick JD, Riminton DS, Cyster JG, Korner H. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today* 2000; **21**:110–13.
- 50 Unger WW, Hauet-Broere F, Jansen W, van Berkel LA, Kraal G, Samsom JN. Early events in peripheral regulatory T cell induction via the nasal mucosa. *J Immunol* 2003; **171**:4592–603.
- 51 Taube C, Dakhama A, Gelfand EW. Insights into the pathogenesis of asthma utilizing murine models. *Int Arch Allergy Immunol* 2004; **135**:173–86.
- 52 Harris NL, Holloway J, Fitzharris P *et al.* Tissue localization and frequency of antigen-specific effector CD4 T cells determines the development of allergic airway inflammation. *Immunol Cell Biol* 2005; **83**:490–7.
- 53 Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999; **160**:1532–9.
- 54 Martinez FD. Role of viral infections in the inception of asthma and allergies during childhood: could they be protective? *Thorax* 1994; **49**:1189–91.
- 55 Venuprasad K, Chattopadhyay S, Saha B. CD28 signaling in neutrophil induces T-cell chemotactic factor(s) modulating T-cell response. *Hum Immunol* 2003; **64**:38–43.
- 56 van Rijt LS, Lambrecht BN. Dendritic cells in asthma: a function beyond sensitization. *Clin Exp Allergy* 2005; **35**:1125–34.
- 57 Jahnsen FL, Moloney ED, Hogan T, Upham JW, Burke CM, Holt PG. Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge. *Thorax* 2001; **56**:823–6.
- 58 Lambrecht BN, Salomon B, Klatzmann D, Pauwels RA. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. *J Immunol* 1998; **160**:4090–7.
- 59 Hogan RJ, Usherwood EJ, Zhong W *et al.* Active antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J Immunol* 2001; **166**:1813–22.
- 60 Marshall DR, Turner SJ, Belz GT *et al.* Measuring the diaspora for virus-specific CD8+ T cells. *Proc Natl Acad Sci USA* 2001; **98**:6313–8.
- 61 Hogan RJ, Zhong W, Usherwood EJ, Cookenham T, Roberts AD, Woodland DL. Protection from respiratory virus infections can be mediated by antigen-specific CD4(+) T cells that persist in the lungs. *J Exp Med* 2001; **193**:981–6.
- 62 Kuipers H, Lambrecht BN. The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma. *Curr Opin Immunol* 2004; **16**:702–8.
- 63 Chiba K, Yanagawa Y, Masubuchi Y *et al.* FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J Immunol* 1998; **160**:5037–44.
- 64 Brinkmann V, Pinschewer D, Chiba K, Feng L. FTY720: a novel transplantation drug that modulates lymphocyte traffic rather than activation. *Trends Pharmacol Sci* 2000; **21**:49–52.
- 65 Yanagawa Y, Sugahara K, Kataoka H, Kawaguchi T, Masubuchi Y, Chiba K. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. *J Immunol* 1998; **160**:5493–9.
- 66 Sawicka E, Zuany-Amorim C, Manlius C *et al.* Inhibition of Th1- and Th2-mediated airway inflammation by the sphingosine 1-phosphate receptor agonist FTY720. *J Immunol* 2003; **171**:6206–14.
- 67 Kurashima Y, Kunisawa J, Higuchi M *et al.* Sphingosine 1-phosphate-mediated trafficking of pathogenic th2 and mast cells for the control of food allergy. *J Immunol* 2007; **179**:1577–85.
- 68 Daniel C, Sartory NA, Zahn N *et al.* FTY720 ameliorates oxazolone colitis in mice by directly affecting T helper type 2 functions. *Mol Immunol* 2007; **44**:3305–16.
- 69 Habicht A, Clarkson MR, Yang J *et al.* Novel insights into the mechanism of action of FTY720 in a transgenic model of allograft rejection: implications for therapy of chronic rejection. *J Immunol* 2006; **176**:36–42.
- 70 Xie JH, Nomura N, Koprak SL, Quackenbush EJ, Forrest MJ, Rosen H. Sphingosine-1-phosphate receptor agonism impairs the efficiency of the local immune response by altering trafficking of naive and antigen-activated CD4+ T cells. *J Immunol* 2003; **170**:3662–70.
- 71 Wang F, Tan W, Guo D, He S. Reduction of CD4 positive T cells and improvement of pathological changes of collagen-induced arthritis by FTY720. *Eur J Pharmacol* 2007; **573**:230–40.