

ORIGINAL PAPER

T-helper type 2-dependent early recruitment of antigen non-specific CD4⁺ T cells in experimental asthma

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Summary

Background Following antigen challenge, adoptively transferred antigen-specific CD4⁺ T cells induce allergic airway inflammation, comprised primarily of an increase in lymphocytes and eosinophils.

Objective Our goal was to better understand the contribution of the GATA-3 transcription factor to the ability of adoptively transferred T cells to induce airway inflammation in the Brown Norway rat model of adoptively transferred asthma.

Methods We transduced antigen-stimulated CD4⁺ T cells with recombinant retroviruses encoding enhanced green fluorescent protein (EGFP) only or EGFP and the GATA-3 transcription factor. Each population of transduced cells was adoptively transferred to naïve recipients that were then challenged with antigen. Airway inflammatory responses were then quantified.

Results Our data indicate that T cells transduced with retroviruses encoding GATA-3 expressed high levels of GATA-3 protein as well as T-helper type 2 cytokines. Following adoptive transfer and airway antigen challenge, these gene-modified T cells induced robust inflammatory responses in the lungs and draining lymph nodes. Increased numbers of total inflammatory cells and eosinophils were recovered in the bronchoalveolar lavage fluid (BALF). In addition, the number of antigen non-specific CD4⁺ T cells recovered in the BALF as well as the lung and draining lymph nodes was enhanced in recipients of GATA-3 overexpressing T cells following antigen challenge. Nevertheless, the transduced CD4⁺ T cells comprised only a small percentage of the population of CD4⁺ T cells infiltrating the lung and were not detectable at all in the draining lymph nodes.

Conclusion These data provide evidence that GATA-3 plays a significant role in the ability of antigen-specific T cells to amplify allergic inflammatory responses *in vivo* by promoting the recruitment of endogenous antigen non-specific T cells to the lung.

Keywords adoptive transfer, asthma, GATA-3, inflammation, retroviral transduction, T cell

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Introduction

CD4⁺ T cells producing the T-helper type 2 (Th2) cytokines, IL-4, IL-5, IL-9 and IL-13, play a central role in allergic asthma, a disease characterized by eosinophilic airway inflammation and mucus production accompanied by airway hyper-responsiveness [1, 2]. Adoptive transfer of antigen-specific CD4⁺ T cells is sufficient to induce allergic airway inflammation following antigen challenge. The magnitude of inflammation following T cell adoptive transfer is similar to that produced in actively sensitized animals, providing evidence that CD4⁺ T cells

are sufficient for the induction and maintenance of inflammation in experimental asthma [3]. Phenotypically, the cells responsible for transfer of allergic responses are activated, antigen-specific T cells that express high levels of CD4 and produce Th2 cytokines [3–7]. Following culture with antigen, adoptive transfer of remarkably few CD4⁺ T cells is sufficient to induce inflammation following antigen challenge in the Brown Norway (BN) rat [8]. While several million T cells expressing a transgenic T cell receptor that recognizes ovalbumin (OVA) (from DO11.10 mice) are typically transferred in murine models [9, 10] fewer than 1200 Ag-specific CD4⁺ T cells may be

sufficient to induce a robust airway eosinophilic inflammatory response in experimental asthma in the mouse [3].

The ability of adoptively transferred CD4⁺ T cells to induce asthma pathogenesis is due in part to the production of Th2 cytokines that then regulate chemokine and cytokine production and the recruitment of other effector cells to the lung [10–12]. The production of IL-4, IL-5 and IL-13 is regulated by the transcription factor GATA-3 [13–17]. In addition to inducing Th2 cytokine expression, GATA-3 autoactivates its own expression by a positive feedback mechanism [18]. Significantly, the expression of GATA-3 is increased in atopic asthmatics [19–21]. Moreover, in experimental asthma, disruption of GATA-3 expression or activity inhibits airway eosinophilia, Th2 cytokine expression and airway hyper-responsiveness [22, 23].

We have used an adoptive transfer model in the BN rat to investigate the role of GATA-3, and its capacity to increase Th2 cytokine production, in the ability of adoptively transferred T cells to induce airway inflammation in experimental allergic asthma. Our data show that adoptively transferred T cells overexpressing GATA-3, although present in small numbers in the airways, induce robust lymphocytic and eosinophilic inflammatory responses within days of antigen challenge. These data suggest that antigen-specific T cells overexpressing GATA-3 and Th2 cytokines have the ability to amplify allergen-specific inflammatory responses in the airways by enhancing the recruitment of endogenous T lymphocytes.

Materials and methods

Animals

Inbred BN rats aged 10–12 weeks were obtained from Harlan Laboratories (Harlan-UK, Bicester, Oxon, UK). All animal protocols complied with Canadian Council on Animal Care guidelines and were approved by the McGill University Animal Care Committee.

Antibodies and cytokines

Allophycocyanin (APC)-conjugated mouse anti-rat CD4, mouse anti-rat CD25, mouse anti-rat CD62L, mouse anti-rat IL-4, mouse anti-rat IFN- γ , APC-conjugated anti-mouse IgG, phycoerythrin (PE)-conjugated anti-mouse IgG and recombinant rat IL-2 were obtained from BD Biosciences (Oakville, ON, Canada). Murine anti-rat CD134 (OX40) was purchased from Cedarlane (Burlington, ON, Canada). Fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-green fluorescent protein (GFP) antibody was purchased from Abcam (Cambridge, MA, USA). Mouse anti-rat GATA-3 antibody (HG3-31) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Production of recombinant retroviruses

Retroviruses were harvested from Phoenix-Eco packaging cells stably transfected with the pAP2 retroviral vector, which encodes enhanced GFP (EGFP) only [24], or its derivative containing the full-length GATA-3 cDNA isolated from rat TIB-236 cells and cloned upstream of the IRES-EGFP cassette in pAP2. Recombinant retroviruses, concentrated 10-fold by centrifugal filtration, had titres on NIH3T3 fibroblasts in the range of $5\text{--}10 \times 10^6$ CFU/mL [25]. Stimulated splenocytes were transduced with the control retrovector encoding EGFP only (control) or the GATA-3 retrovector encoding both GATA-3 and EGFP (GATA-3).

Antigen sensitization, splenocyte isolation and retroviral transduction

BN rats (ages 10–12 weeks) were sensitized intraperitoneally (i.p.) with a suspension of 100 μ g of OVA (Sigma-Aldrich, St Louis, MO, USA) and 1 mg of aluminium hydroxide (EM Industries, Gibbstown, NJ, USA). Animals were killed 14 days later and spleens harvested and passed through a cell strainer to obtain single-cell suspensions. Red blood cells were removed using red blood cell lysis buffer (Sigma-Aldrich). To enhance the proliferation and thus retroviral transduction of OVA-specific T cells [8, 26], total splenocyte populations were cultured in DMEM (Invitrogen Canada Inc., Burlington, ON, Canada) containing 200 μ g/mL OVA, 100 U/mL IL-2, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine (Invitrogen) at 37 °C and 5% CO₂. Following 48 h of culture with OVA, cells were resuspended in retroviral supernatants containing 6 mg/mL of hexadimethrine bromide (polybrene) (Sigma-Aldrich) and IL-2 (100 U/mL). Cells and retroviruses were then centrifuged at $1,000 \times g$ for 1 h. A total of four spin infections were carried out over the course of 48 and 72 h after the last spin, cells were assessed for EGFP expression using a fluorescence-activated cell sorter (FACS) Calibur flow cytometer (BD Biosciences) and CELLQUEST Software (BD Biosciences). Five days following the last spin, CD4⁺ cells were purified using magnetic cell sorting (MACS; Miltenyi Biotec Inc., Auburn, CA, USA) by negative selection and live, EGFP⁺ cells were sorted for adoptive transfer on a MoFlo flow cytometer (Cytomation, Fort Collins, CO, USA). Alternatively, cells were maintained in culture for 2 weeks in order to assess cytokine expression.

Analysis of GATA-3 and cytokine expression in transduced cells

To detect GATA-3 expression following retroviral transduction, splenocytes were fixed in 1% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) and incubated with primary GATA-3 antibody, followed

by APC-conjugated anti-mouse IgG. GATA-3 protein expression was subsequently analysed using a FACS Calibur flow cytometer. As a control, cells were stained with APC-conjugated anti-mouse IgG only. The expression of GATA-3 mRNA was assessed by RT-PCR using RNA isolated from transduced splenocyte populations and primers derived from the rat GATA-3 sequence (forward primer: 5'-CGA AAG ACG TCT CCC CAG AC-3' and reverse primer: 5'-AGC GTT TGC AAA GGT AGT GC-3'). Briefly, total RNA was extracted from splenocytes, and 1 µg was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen), which was then PCR amplified (2 min at 95 °C, followed by 30 s at 94 °C, 30 s at 56 °C, 1 min 30 s at 72 °C for 35 cycles, and then 10 min at 72 °C). PCR products were resolved by gel electrophoresis and visualized on a FluorChem 8000 Imaging System (Alpha Innotech, San Leandro, CA, USA). The expressions of IL-4, IL-5 and the housekeeping gene cyclophilin A were also assessed by RT-PCR using RNA isolated from transduced splenocyte populations as described previously [27].

Cytokine expression in transduced splenocytes was also examined by intracellular cytokine staining using the BD Cytofix/Cytoperm intracellular cytokine staining kit (BD Biosciences) as per the manufacturer's instructions. Briefly, cells were stimulated with 1 µg/mL of recombinant rat CD3 in the presence of Golgi stop for 8 h. Cells were then fixed, permeabilized and stained for IL-4 and IFN-γ. Cytokine expression was then determined following analysis by flow cytometry.

Adoptive transfer and antigen challenge (Fig. 3a)

In order to increase the homing of OVA-specific CD4⁺ T cells to the lungs, naïve recipient rats were intubated and intratracheally challenged with an aerosol of 5% OVA or PBS for 5 min 24 h before adoptive transfer. The next day, 10⁶ FACS-sorted CD4⁺ EGFP⁺ T cells, obtained from sensitized donor rats, were injected i.p. into anaesthetized BN recipient rats. Animals were challenged immediately after receiving T cells as above with 5% OVA or PBS for 5 min. Rats were killed 72 h following challenge with a lethal dose of sodium pentobarbital, and a bronchoalveolar lavage (BAL) was performed. Subsequently, the right lung was inflated and fixed in 10% formalin for 24 h, after which it was embedded in paraffin for immunofluorescence. The left lung was homogenized into a cell suspension for flow cytometry analysis. The spleens and draining (mediastinal) lymph nodes were also harvested and homogenized to form single-cell suspensions for flow cytometry.

Cytospin and flow cytometry analysis

For analysis of inflammatory infiltrates, cells in BAL fluid were cytospun onto microscope slides and differential cell

counts were performed using Diff Quick (Dade Behring Inc., Newark, DE, USA). Cells from the BAL fluid were also stained with APC-conjugated anti-rat CD4 or murine anti-CD134 (OX40) and APC-conjugated goat anti-mouse IgG, respectively, and the surface expression of CD4 and OX40 was quantified on a FACS Calibur flow cytometer (BD Biosciences) using CELLQUEST software (BD Biosciences).

ELISA

Rat IL-4, IFN-γ and IL-13 were quantified using ELISA kits from BD Biosciences and Biosource (Medicorp Inc., Montreal, QC, Canada), respectively, and performed as per the manufacturer's instructions.

Immunofluorescence and histology of lung tissue

Lung tissues were inflated, fixed in 10% buffered formalin and embedded into paraffin blocks from which 5-µm-thick sections were made. To assess airway inflammation, lung sections were stained with haematoxylin and eosin (H&E) and examined using light microscopy. To detect EGFP, tissue sections were permeabilized using PBS containing 0.5% Triton X-100 for 20 min and then stained using goat polyclonal FITC-conjugated anti-GFP antibody (Abcam) overnight at 4 °C. Nuclei were counterstained using Hoechst 33342 (Molecular Probes, Invitrogen). Slides were mounted using PermaFluor mounting medium (Shandon, Pittsburg, PA, USA) and examined using a fluorescence microscope (Olympus BX 51 Fluorescence Microscope System, Olympus America, Melville, NY, USA). Images were then analysed using Image-Pro Plus 4.5 software (Media Cybernetics Inc., Silver Spring, MD, USA).

Statistical analysis

Data are presented as the mean ± SEM. Data were analysed using Student's *t*-test (two samples, assuming unequal or equal variances). *P* < 0.05 was considered to be significant. Data are from a minimum of three experiments using a total of five to seven animals per group.

Results

Retroviral transduction of antigen-stimulated splenocytes

To overexpress GATA-3 in CD4⁺ T cells destined for adoptive transfer, we established methodology to produce high-titre retroviral preparations that efficiently transduce BN rat splenocyte CD4⁺ T cells cultured with the antigen, OVA [8, 25]. OVA-stimulated splenocytes were transduced efficiently by the control retrovirus as well as that encoding GATA-3 (Fig. 1a). As we have shown previously [8, 25], when total splenocytes are cultured with antigen, CD4⁺ T cells are preferentially transduced

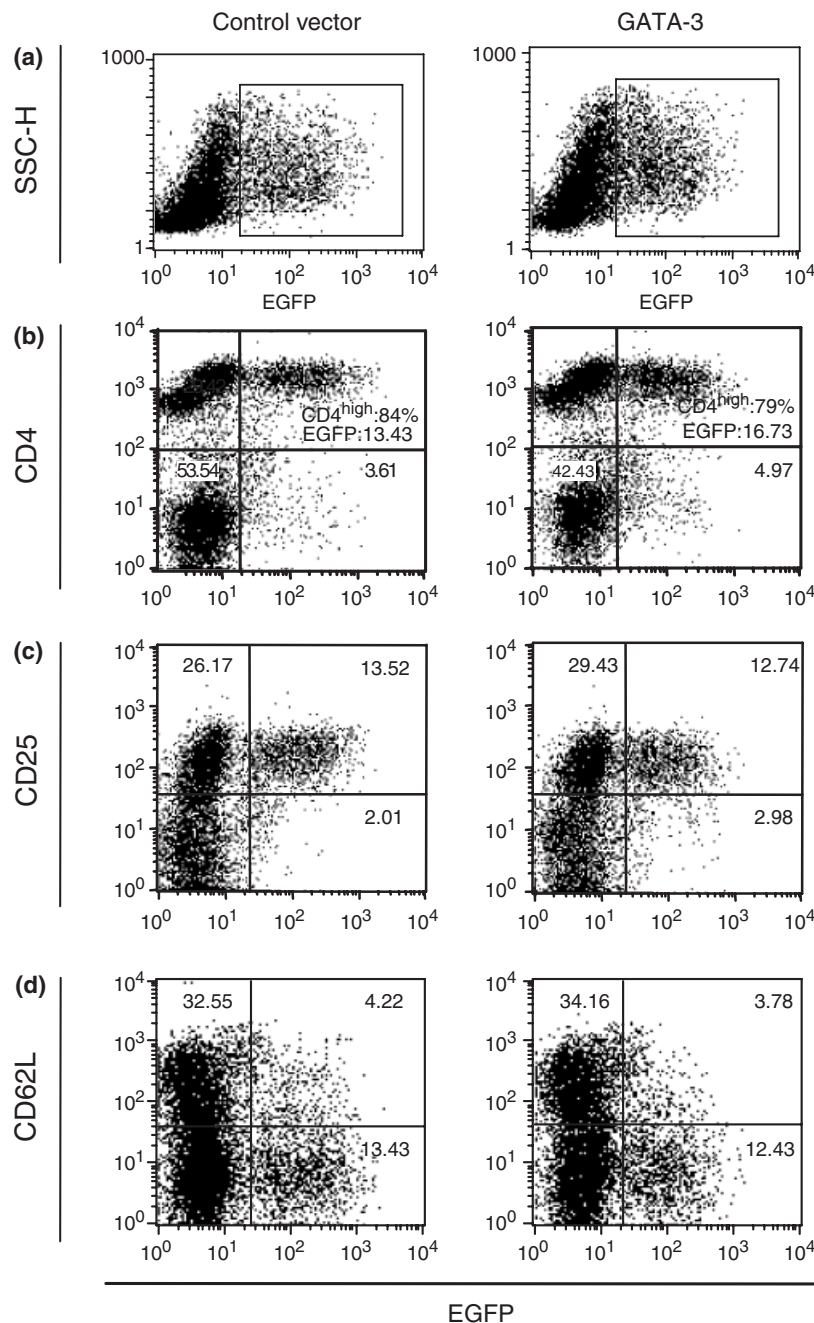


Fig. 1. Recombinant retroviruses selectively transduce CD4⁺ T cells in splenocyte cultures harvested from ovalbumin (OVA)-sensitized donors. (a) OVA-stimulated splenocytes were retrovirally transduced with a control retrovirus or a retrovirus encoding GATA-3. Enhanced green fluorescent protein (EGFP) expression was monitored using FACS analysis. (b) Total splenocyte populations were stained with allophycocyanin (APC)-conjugated anti-CD4 to identify CD4⁺ T cells. (c, d) Transduced cells were also stained with anti-CD25 (c) or anti-CD62L (d) to determine the level of activation 48 h after the last transduction. All data obtained from FACS were analysed by gating on live cells and are representative of at least three independent experiments. FACS, fluorescence-activated cell sorter.

(Fig. 1b). The majority of the transduced cells (>80%) expressed high levels of CD4 (Fig. 1b) as well as the activation markers, CD25 (Fig. 1c) and OX40 (data not shown) and low levels of CD62L (CD62L^{low}), a marker commonly expressed by naïve T cells (Fig. 1d).

We assessed the expression of GATA-3 in splenocyte populations by flow cytometry 48 h after retroviral transduction. As predicted, compared with cells transduced with control retrovector, cells transduced with the GATA-3 retrovector expressed higher levels of GATA-3 protein

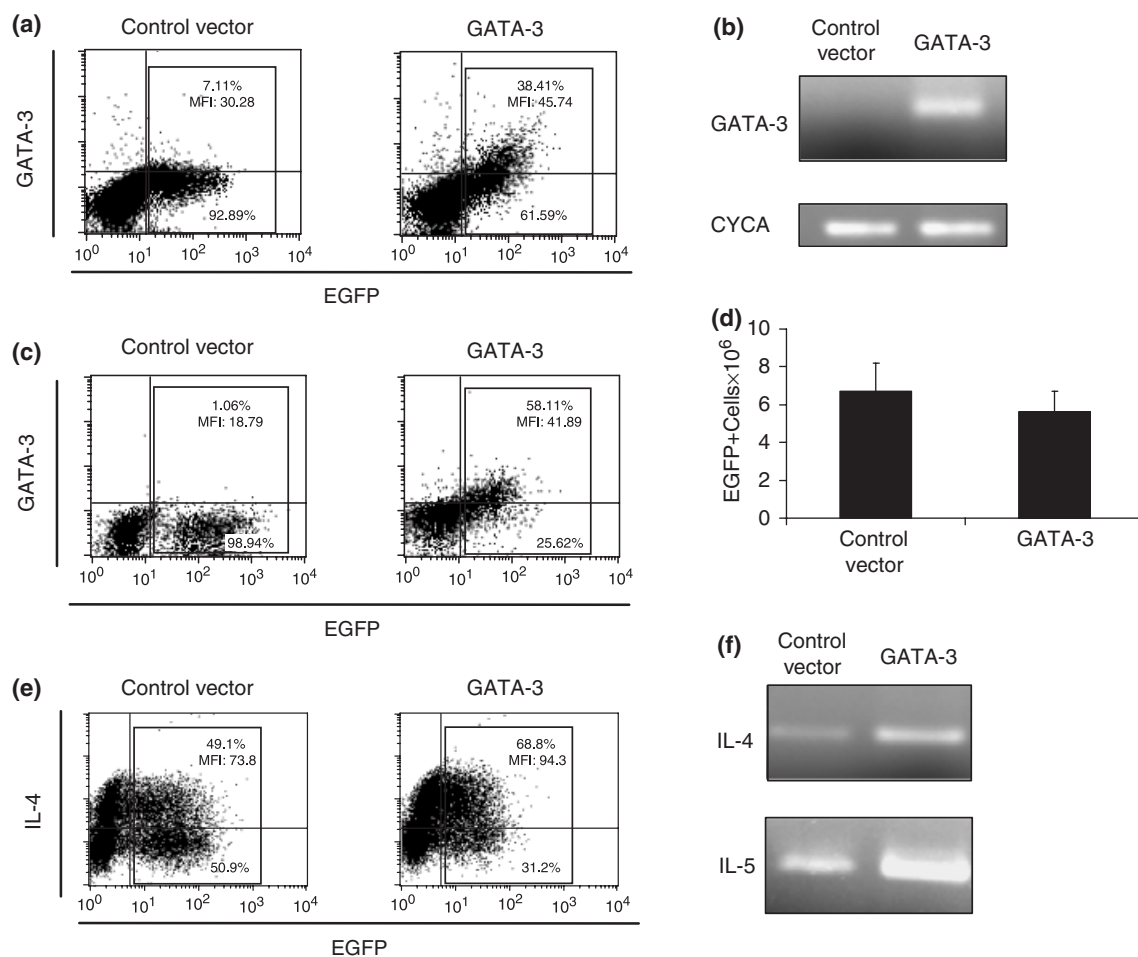


Fig. 2. Retroviral vector encoding GATA-3 increases the expression of GATA-3 and T-helper type 2 (Th2) cytokines in transduced splenocytes. (a) Two days after transduction, splenocytes were fixed, permeabilized and stained with a primary anti-GATA-3 antibody and a secondary allophycocyanin (APC)-conjugated antibody. GATA-3 and EGFP protein expressions were then assessed by flow cytometry. (b) RT-PCR was performed to assess the expression of GATA-3 and cyclophilin mRNA in transduced splenocytes. (c) Transduced splenocytes were cultured for 2 weeks, fixed, permeabilized and stained with GATA-3 antibody as in (a). GATA-3 and EGFP expressions were then assessed by flow cytometry. (d) EGFP⁺ CD4⁺ T cells were quantified before adoptive transfer of splenocyte populations transduced with control retrovirus or retrovirus encoding GATA-3. (e) Splenocytes transduced with the control retrovirus or retrovirus encoding GATA-3 were activated with anti-CD3 in the presence of Golgistop. Intracellular cytokine staining allowed detection of IL-4 in both EGFP⁺ and EGFP⁻ cells. Analysis of cell populations was performed on gated, live cells. (f) RT-PCR was performed to detect the expression of IL-4 and IL-5 mRNA in transduced splenocytes. mRNA was the same as in (b) with cyclophilin as the control. Data shown are representative of at least two independent experiments. EGFP, enhanced green fluorescent protein.

(Fig. 2a). The expression levels correlated with the levels of EGFP expression, consistent with the translation of each protein from a single bicistronic mRNA. Similarly, the levels of mRNA encoding GATA-3 were also increased (Fig. 2b). GATA-3 overexpression was maintained in transduced cell populations for at least 2 weeks (Fig. 2c). Significantly, overexpression of GATA-3 did not alter the number of EGFP⁺ cells recovered following culture for 6 days (Fig. 2d), providing evidence that GATA-3 overexpression did not modify the proliferation and/or survival of transduced cells in the splenocyte cultures.

Because GATA-3 positively regulates Th2 cytokine expression, we assessed cytokine production in trans-

duced splenocyte populations. In cells transduced with retroviruses encoding GATA-3, 68.8% of the EGFP⁺ cells expressed IL-4 whereas in cells transduced with control retroviruses 49.1% of the EGFP⁺ cells expressed IL-4 (Fig. 2e). The mean fluorescence intensity of IL-4 expression in GATA-3 overexpressing cells was also increased. In contrast to IL-4 expression, IFN- γ was not detected in these cells (not shown). Finally, compared with cells transduced with the control virus, cells overexpressing GATA-3 also expressed higher levels of mRNA encoding IL-4 and IL-5 (Fig. 2f). Altogether, these data demonstrate that recombinant retroviruses can be used to generate primary rat CD4⁺ T cells overexpressing GATA-3 and Th2 cytokines.

Adoptively transferred T cells overexpressing GATA-3 induce enhanced airway inflammation and eosinophilia

To investigate the ability of these cells to regulate allergic responses *in vivo*, gene-modified CD4⁺EGFP⁺ cells were purified and delivered by an i.p. injection into naïve recipient rats using the protocol presented in Fig. 3a. To promote the recruitment of CD4⁺ T cells to the lung, animals were pre-challenged 24 h before adoptive transfer [8, 28]. Immediately following the delivery of T cells, animals were challenged with OVA or vehicle and 72 h post-challenge animals were killed and airway inflammation in BAL fluid and lungs was assessed. Although the CD4⁺ T cells had been activated and retrovirally transduced *in vitro*, they did not induce airway inflammation in recipient rats in the absence of OVA challenge (Fig. 3b). Moreover, compared with animals that received CD4⁺ T cells transduced with the control vector, a larger number of inflammatory cells were recovered in the BAL fluid from animals that received CD4⁺ T cells overexpressing GATA-3 (Fig. 3b). Similarly, following OVA challenge, the number of eosinophils recovered in the BAL fluid from recipients of T cells overexpressing GATA-3 was enhanced 2.3 ± 0.4-fold (Fig. 3c), consistent with the ability of T cells overexpressing GATA-3 to produce higher levels of IL-5, a potent chemoattractant, activator and differentiation factor for eosinophils [16, 29].

We also quantified CD4⁺ T cells recovered in the BAL fluid and the lungs. Compared with animals in all the other groups, large numbers of infiltrating CD4⁺ T cells were recovered in the BAL fluid of animals that received CD4⁺ T cells overexpressing GATA-3 (Figs 3d, 4a and b). Following OVA challenge, the number of CD4⁺ T cells recovered from the lungs was also enhanced in recipients of T cells overexpressing GATA-3 (Fig. 5a). Histological analyses revealed increased inflammation in recipients of T cells overexpressing GATA-3 compared with recipients of T cells transduced with the control vector (Fig. 5b). Cytokine levels in the BAL fluid were also assessed following antigen challenge. Both IL-4 and IL-13 were increased in the BAL fluid of animals receiving CD4⁺ T cells overexpressing GATA-3, while the levels of IFN- γ did not differ significantly in any group (Table 1). These data provide evidence that adoptively transferred, gene-modified CD4⁺ T cells induced a Th2-biased airway eosinophilic inflammatory response that was dependent upon antigen challenge and enhanced by T cell overexpression of GATA-3. Nevertheless, remarkably few EGFP⁺ T cells were recovered in the BAL fluid and lung.

Recruitment of adoptively transferred CD4⁺ enhanced green fluorescent protein⁺ cells to airways

In order to induce airway inflammation, antigen-specific T cells are recruited to the lung and draining lymph nodes.

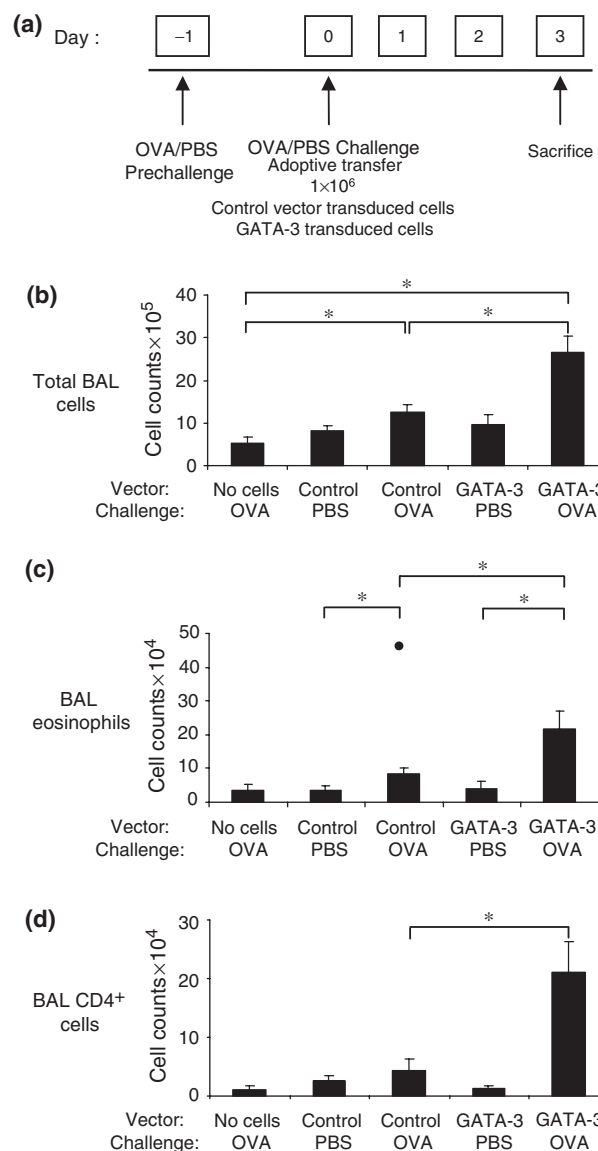


Fig. 3. Adoptively transferred T cells overexpressing GATA-3 induce enhanced airway inflammation and eosinophilia following antigen challenge. (a) Protocol for adoptive transfer of transduced splenocytes and antigen challenge. Bronchoalveolar lavage (BAL) was performed following the killing of challenged recipients. (b) Total BAL fluid cells and (c) BAL fluid eosinophils recovered from challenged recipients are shown (* $P < 0.05$). The dot in (c) represents an animal that was excluded because the number of BAL fluid eosinophils was greater than three standard deviations from the mean. (d) BAL fluid cells were stained with anti-CD4 antibodies and analysed by flow cytometry (* $P < 0.01$). Pooled data from four independent experiments are presented as mean ± SEM ($n = 3-7$ rats per group). PBS, phosphate-buffered saline; OVA, ovalbumin.

We therefore quantified EGFP⁺ cells recovered from BAL fluid, lung homogenates and draining lymph nodes and assessed EGFP expression in lung sections by immunofluorescence. EGFP⁺ cells were detected in the BAL fluid and lung homogenates of recipient animals, although they comprised only a minor fraction of the total CD4⁺ cells

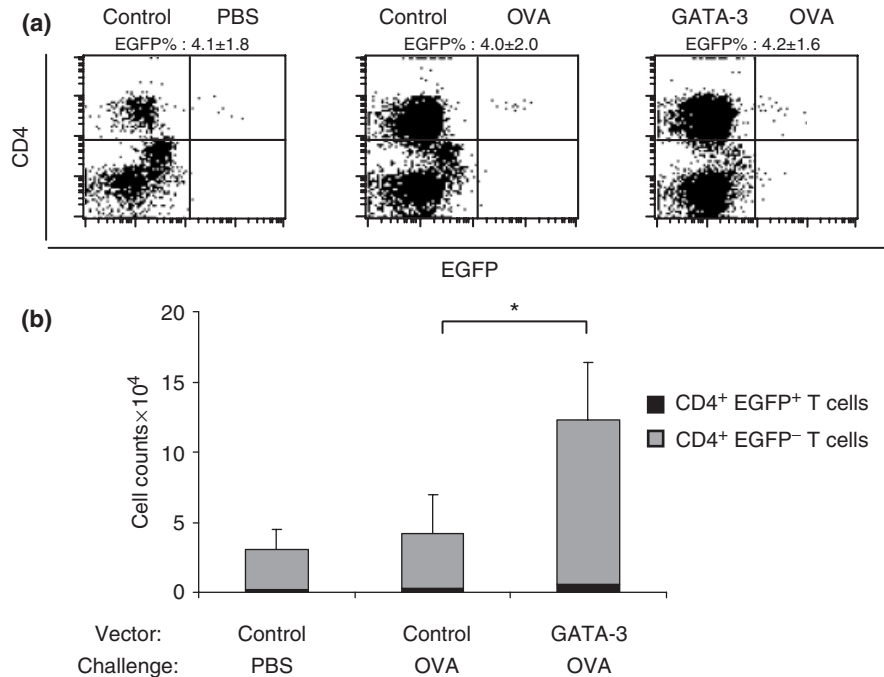


Fig. 4. Adoptively transferred enhanced green fluorescent protein (EGFP⁺) CD4⁺ T cells comprise only a small proportion of total CD4⁺ T cells recruited to the airway. (a) Bronchoalveolar lavage (BAL) fluid cells from T cell recipients were stained with anti-CD4 antibodies and analysed by flow cytometry to detect CD4⁺ and EGFP⁺ cells. (b) Quantification of total CD4⁺EGFP⁺ and CD4⁺EGFP⁻ cells recovered in BAL fluid of transduced T cell recipients (**P* < 0.05). The black bar represents CD4⁺EGFP⁺ T cells and the grey bar represents CD4⁺EGFP⁻ T cells. Pooled data from at least three independent experiments are presented as mean ± SEM (*n* = 3–7 rats per group). PBS, phosphate-buffered saline; OVA, ovalbumin.

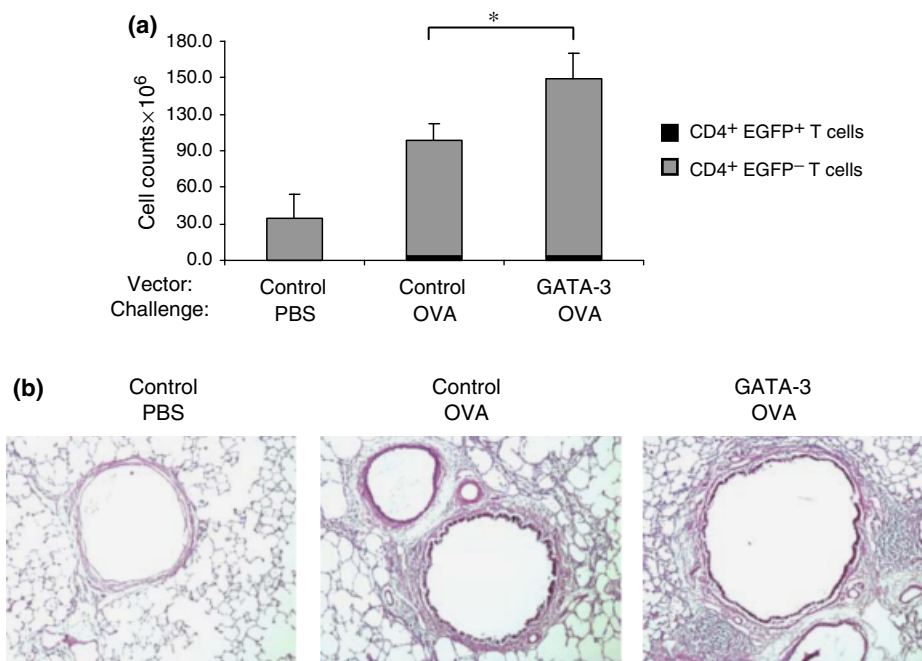


Fig. 5. Adoptively transferred T cells overexpressing GATA-3 induce enhanced antigen non-specific CD4⁺ T cell recruitment to the lung following antigen challenge. (a) Lung cell suspensions were stained with allophycocyanin (APC)-conjugated anti-rat CD4 antibody and analysed by flow cytometry to quantify the total CD4⁺EGFP⁺ and CD4⁺EGFP⁻ cells recovered (**P* < 0.05). (b) Lungs from challenged recipients were fixed in 10% formalin and paraffin-embedded. Tissue sections were then stained with haematoxylin and eosin (H&E) and examined by light microscopy. Representative sections are shown. Pooled data from at least three independent experiments are presented as mean ± SEM (*n* = 3–4 rats per group). EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; OVA, ovalbumin.

Table 1. Cytokine production in bronchoalveolar lavage of challenged recipients of transduced cells

	No cells	Control PBS	Control OVA	GATA-3 PBS	GATA-3 OVA
IL-4	0	3.43 ± 1.57	3.27 ± 1.38	4.88 ± 2.64	5.92 ± 1.89*
IL-13	40.83 ± 12.01	48.11 ± 12.75	28.21 ± 11.22	32.08 ± 9.83	72.25 ± 14.79*
IFN- γ	93.17 ± 2.12	107.16 ± 0.00	92.67 ± 6.72	99.16 ± 2.12	86.17 ± 0.71

One experiment representative of three independent experiments is shown.

* $P < 0.05$ compared with control OVA.

OVA, ovalbumin; PBS, phosphate-buffered saline.

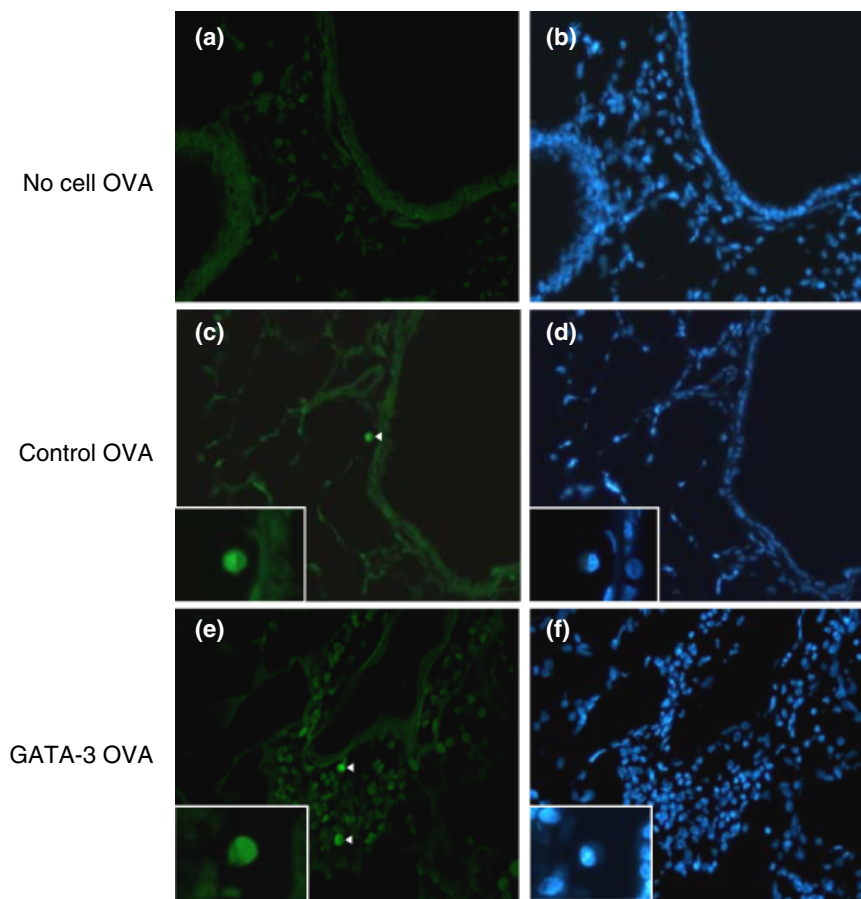


Fig. 6. Adoptively transferred $CD4^{+}EGFP^{+}$ T cells traffic to the lung. Lungs from antigen-challenged recipients of transduced cells were fixed in 10% formalin and paraffin-embedded. Prepared tissue sections were then permeabilized with 0.1% Triton X-100 and stained with goat polyclonal anti-GFP antibody to detect $CD4^{+}EGFP^{+}$ cells in the lung (a, c, e). Nuclei were counterstained using Hoechst 33342 (b, d, f). Data are representative of at least two independent experiments. The arrows indicate $EGFP^{+}$ cells. EGFP, enhanced green fluorescent protein; OVA, ovalbumin.

present (Figs 4a, b and 5a). Consistent with the flow cytometry data, $EGFP^{+}$ cells were also identified in lung sections from recipient animals following OVA challenge and they comprised only a minor fraction of the inflammatory cells present (Fig. 6).

We quantified the total number of $CD4^{+}EGFP^{+}$ cells isolated from the BAL fluid and lung. Of the one million $CD4^{+}EGFP^{+}$ cells administered by i.p. injection, we recovered $4.1 \pm 2.1 \times 10^4$ and $3.3 \pm 1.6 \times 10^5$, respectively, in saline and OVA-challenged recipients of cells trans-

duced with the control virus and $4.0 \pm 0.6 \times 10^5$ from OVA-challenged recipients of cells overexpressing GATA-3 (Fig. 7). These data demonstrate that the OVA challenge enhanced recruitment of antigen-specific $EGFP^{+}$ cells to the lung. However, the number of $EGFP^{+}$ cells recovered in the lung did not differ between OVA-challenged groups, suggesting that the number of antigen-specific cells in the lung can, under some circumstances, be dissociated from the levels of cytokines they produce as well as the inflammation they induce.

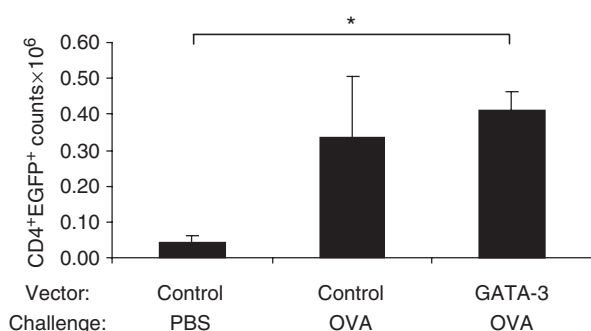


Fig. 7. Quantification of adoptively transferred CD4⁺ enhanced green fluorescent protein (EGFP⁺) T cells recovered from the bronchoalveolar lavage (BAL) fluid and the lung. Total numbers of CD4⁺EGFP⁺ cells recovered from the BAL fluid and lung were quantified (**P* < 0.05). Pooled data from at least three independent experiments are presented as mean ± SEM (*n* = 3–7 rats per group). PBS, phosphate-buffered saline; OVA, ovalbumin.

Table 2. Percentage of CD4⁺ T lymphocytes recovered from recipients of transduced cells

	Spleen	Draining lymph nodes
Control PBS	31 ± 1.8	24.65 ± 1.8
Control OVA	29.16 ± 2.9	14.48 ± 7.2
GATA-3 OVA	31.99 ± 3.7	28.83 ± 2.1*

Data from two independent experiments is shown, *n* = 6.

**P* < 0.05 compared with control OVA.

OVA, ovalbumin; PBS, phosphate-buffered saline.

In vivo expansion of lymph nodes in recipients of T cells overexpressing GATA-3

Finally, inflammatory responses in the lung draining (mediastinal) lymph nodes and spleens of recipient animals were assessed. Adoptively transferred CD4⁺ T cells did not induce inflammation in the spleen, providing evidence that these cells did not induce inflammation at sites distinct from that of antigen challenge (Table 2 and data not shown). However, increased numbers of CD4⁺ T cells were recovered from the draining lymph nodes in T cell recipients challenged with OVA (Fig. 8a and data not shown). As in the lung, CD4⁺ T cell numbers were further enhanced in recipients of T cells overexpressing GATA-3. In addition to the increase in CD4⁺ T cells in the lymph nodes, increased numbers of total cells were also recovered from the draining lymph nodes of animals receiving T cells overexpressing GATA-3 following OVA challenge (Fig. 8b). Nevertheless, unlike in the lung, EGFP⁺ cells were not detected in the lymph nodes. These data suggest that T cells overexpressing GATA-3 may indirectly induce the proliferation or recruitment of cells in the lymph node.

Discussion

Models of adoptive transfer have helped to define the role of T cells and their cytokines in orchestrating allergic

asthma. In experimental asthma, adoptively transferred, antigen-specific CD4⁺ T cells induce airway inflammation in part by activating other effector cells that participate in airway inflammatory responses [7, 9, 30]. We have previously shown that small numbers of T cells, harvested from antigen-sensitized donor rats and retrovirally transduced *in vitro*, drive robust inflammatory responses 48 h following adoptive transfer and antigen challenge [8]. By adoptively transferring CD4⁺ T cells expressing EGFP, we have now shown that the majority of CD4⁺ T cells that infiltrated the lung within days of antigen challenge were not the adoptively transferred T cells themselves. Moreover, these CD4⁺ T cell-dependent allergen-driven inflammatory responses in the lung and draining lymph nodes were augmented in recipients of CD4⁺ T cells overexpressing the Th2-specific GATA-3 transcription factor.

By stimulating splenocytes from OVA-sensitized BN rat donors with antigen *in vitro*, we are able to obtain a population of T cells that can be efficiently retrovirally transduced in numbers sufficient for adoptive transfer experiments [8, 25]. Following culture with antigen, the T cells that originally encountered antigen *in vivo* during sensitization become reactivated and undergo proliferation, allowing them to be transduced with recombinant retroviruses [25, 26]. As we and others have shown previously, a subset of the transduced cells are OVA-specific [8, 25, 26]. Consistent with this, upon sequential *in vitro* exposure to OVA and recombinant retroviruses, CD4⁺ T cells are selectively transduced (Fig. 1 and [8]). For the studies described here, we used retroviruses derived from a control retroviral vector encoding EGFP only as well as retroviruses encoding the rat GATA-3 transcription factor. Cells transduced with retroviruses encoding GATA-3 expressed higher levels of both GATA-3 mRNA and protein as well as the Th2 cytokines, IL-4 and IL-5. We hypothesized that cells transduced under these conditions and subsequently adoptively transferred to naïve recipients would induce airway inflammation and eosinophilia following OVA challenge. Because the effector role of Th2 cells in asthma is dependent upon Th2 cytokine release, we expected that animals receiving T cells overexpressing GATA-3 would have an augmented response to OVA challenge. Following adoptive transfer and OVA challenge, we found that recipients of retrovirally transduced T cells exhibited increased airway inflammation. The inflammatory response was OVA dependent, eosinophilic in nature and was greatly enhanced by T cells overexpressing GATA-3. We found larger numbers of CD4⁺ T cells in recipients of T cells overexpressing GATA-3. However, the vast majority of these CD4⁺ T cells did not express EGFP. These data suggest that the GATA-3⁺ EGFP⁺ cells, upon OVA challenge, recruited large numbers of endogenous CD4⁺ T cells to the lung. This increased recruitment was also reflected in the number of CD4⁺ T cells recovered in

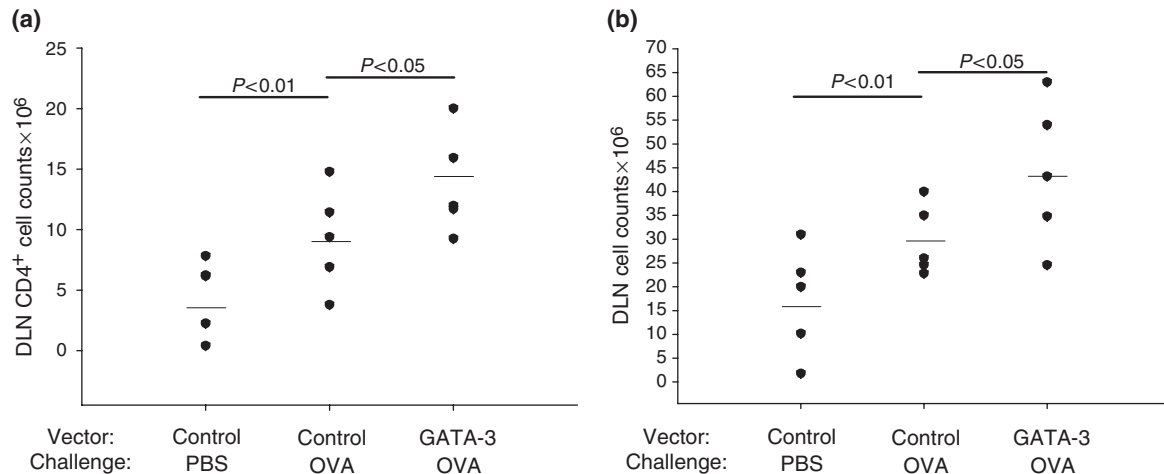


Fig. 8. Antigen-induced expansion of lymphoid cell populations in recipients of T cells overexpressing GATA-3. (a) Total DLN cells were recovered 72 h following challenge and were stained with anti-CD4 antibodies to quantify the lymphoid CD4⁺ T cell population by flow cytometry. (b) DLNs from antigen-challenged recipients were recovered and the total cells were counted. Pooled data from two independent experiments are presented as mean ± SEM ($n = 5$ rats per group). DLN, draining lymph node; PBS, phosphate-buffered saline; OVA, ovalbumin.

the draining lymph nodes of recipients post-OVA challenge.

Our *in vitro* data indicate that IL-4 production was increased in both EGFP⁺ and EGFP⁻ cells 14 days following transduction of CD4⁺ T cells with retroviruses encoding GATA-3. The increased IL-4 expression in EGFP⁺ GATA-3⁺ cells is likely due to the direct binding of retrovirally encoded GATA-3 to cis-acting elements in the IL-4 gene locus. Because GATA-3 autoregulates its own expression, there may also be a contribution from host cell GATA-3 induced by the virally encoded protein [18]. Subsequently, increased IL-4 production by the transduced cells likely promoted the Th2-biased differentiation and IL-4 production of non-transduced EGFP⁻ cells in the same culture.

IL-4 production by antigen-specific T cells has also been shown to promote differentiation of naïve T cells to Th2-type effector cells with specificity towards the same or other antigens *in vivo* in adoptive transfer models of experimental asthma [9, 31, 32]. The ability of adoptively transferred, antigen-specific T cells to promote differentiation of naïve T cells into antigen-specific effector cells is due to the colocalization of both naïve and effector T cells with dendritic cells in the lymph node and the subsequent IL-4-dependent differentiation of the naïve T cells [31, 32]. Several other studies have investigated the localization of adoptively transferred T cells *in vivo* [9, 10, 31–34]. Data from Bradley et al. [33] indicate that memory T cells migrate and localize to the site of antigen challenge, consistent with our ability to identify EGFP⁺ T cells in the lung. Data from other groups have also demonstrated preferential trafficking of effector CD4⁺ T cells expressing low levels of CD62L to the lung [34, 35]. These

cells are unable to proliferate and yet maintain their ability to execute effector functions, such as cytokine production [34]. The CD4⁺ T cells that we adoptively transferred expressed CD25 and OX40 and were CD62L^{low} and thus likely consisted of a mixture of effector and memory-effector T cells. These cells would be predicted to traffic primarily to non-lymphoid tissues, including the lung, following adoptive transfer. Indeed, small numbers of EGFP⁺ T cells were detected in the lungs, both by flow cytometry and immunofluorescence. These EGFP⁺ cells were still detectable in the lung after three OVA challenges over a 9-day time course. Moreover, in these experiments CD4⁺ T cells overexpressing GATA-3 retained the ability to enhance recruitment of host CD4⁺ T cells to the lung following three OVA challenges (data not shown). It is likely that the transduced CD4⁺ T cells that we adoptively transferred migrated to the lungs and, following antigen challenge, produced abundant Th2 cytokines, which led to the recruitment of host cells that participated in the inflammatory response. We believe it is unlikely that the host CD4⁺ T cells recruited to the lung in this model are antigen specific due to the short time frame of our experiments. Animals were killed 3 days following adoptive transfer and challenge and it is unlikely that this would be sufficient time for IL-4 produced by the transduced cells to promote differentiation of naïve host cells. Consistent with this, few of the CD4⁺ T cells harvested in the BAL fluid expressed OX40, a T cell marker of activation. To determine whether NKT cells were recruited to the lung, we used RT-PCR to monitor the expression of the NKT cell-invariant T cell receptor [36, 37]. Although the expression of this receptor was detected in the lungs of rats, it did not change following OVA challenge. These

data demonstrate that, although NKT cells are present, their levels do not increase following OVA challenge in this experimental system.

In an elegant study by Huh et al. [38], primed T cells located in the lung were shown to induce maturation of resident mucosal dendritic cells, which subsequently migrated to the regional lymph nodes, where they would be expected to have potent antigen presentation capacity. These data raise the possibility that, in our study, following allergen challenge, the adoptively transferred CD4⁺EGFP⁺ T cells present in the lung induced rapid maturation of mucosal dendritic cells, which subsequently migrated to and induced robust T cell activation in the draining lymph node. Moreover, these data suggest that T cells overexpressing GATA-3 and Th2 cytokines more efficiently induced maturation of mucosal dendritic cells and thus their ability to activate host T cells that then participated in Th2-biased allergen-induced inflammatory responses.

There are several implications for the enhanced recruitment of host T cells in the allergic inflammatory response. Firstly, recruitment of antigen non-specific CD4⁺ T cells to the lung provides a mechanism by which Th2 responses to neo-allergens may occur. As discussed above, it has been shown that the lymph node is the site of collateral priming and phenotype spread. Nevertheless, because dendritic cell activity localized to the lung is required to maintain allergic airway responses [39], we propose that the early recruitment of antigen non-specific CD4⁺ T cells to the lung may also contribute to phenotype spread. In addition, antigen non-specific T cells recruited to the lung may also contribute to airway remodelling. We have recently shown that small numbers of adoptively transferred antigen-specific CD4⁺ T cells drive airway smooth muscle remodelling. CD4⁺EGFP⁺ cells can also be localized in apparent contact with airway smooth muscle bundles following adoptive transfer and antigen challenge. These data suggest that the increase in airway smooth muscle mass may depend in part on direct T cell:smooth muscle cell contact. Our data presented here provide the possibility that antigen non-specific host T cells recruited into the lung may also participate in this process.

In conclusion, we have presented evidence that adoptively transferred CD4⁺EGFP⁺ T cells mobilized to the lung where they induced antigen-dependent eosinophilic inflammation. In recipients of T cells overexpressing GATA-3 influx of both eosinophils and CD4⁺ T cells was augmented, likely due to increased production of Th2 cytokines resulting in enhanced recruitment of endogenous T cells. This process underscores the importance of the phenotype of T lymphocytes recruited at the initial stages of allergen challenge and suggests that highly polarized T cells producing abundant Th2 cytokines may accelerate the development and severity of allergy by inducing the recruitment of a larger number of T lymphocytes that

may then participate in airway inflammation and asthma pathogenesis.

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