Research Article

Tissue localization and frequency of antigen-specific effector CD4+ T cells determines the development of allergic airway inflammation

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Summary Previous activation of effector Th2 cells is central to the development of allergic inflammatory responses. We have observed that priming of allergen-specific Th2 cells in C57BL/6 or B10.A mice with allergen delivered via the i.p. or s.c. routes results in very different outcomes following subsequent airway exposure to the same allergen. Systemic allergen immunization (via the i.p. route) resulted in the formation of a lung-resident population of allergen-specific T cells, and mice developed severe allergic airway inflammation in response to inhaled allergen. The localization of cells to the lung did not require the presence of antigen at this site, but reflected a large pool of circulating activated allergen-specific T cells. In contrast, localized immunization (via the s.c. route) resulted in a small T-cell response restricted to the draining lymph node, and mice were not responsive to inhaled allergen. These data indicate that prior sensitization to an allergen alone was not sufficient for the induction of allergic inflammation; rather, responsiveness was largely determined by precursor frequency and tissue localization of the allergen-specific effector Th2 cells.

Key words: allergy, intraperitoneal immunization route, lung, subcutaneous immunization route, Th2 cell.

Introduction

Allergen induced airway inflammation is a major component of the pathology and morbidity of atopic asthma. However, the process by which an individual's airways become sensitized to an allergen is poorly understood. In mouse models of airway inflammation, a defined series of events must occur. The first event involves sensitization against a specific antigen and the development of responsive Th2 cells. This sensitizes the host towards the development of allergic airway inflammation following re-exposure to allergen.[1] The current dogma suggests that only antigen experienced T cells will express the necessary adhesion molecules required to recirculate into those peripheral tissues afflicted by allergies, such as the lung, skin and intestinal mucosa.[2–4] Consequently, only those effector T cells that have previously encountered the allergen will be able to generate a rapid airway inflammatory response following exposure to inhaled antigen.

It is apparent that a degree of allergic airway eosinophilia can be achieved via a lengthy protocol of chronic airway exposure to antigen.[5,6] However, simple immunization of mice using two i.p. injections of antigen in alum adjuvant can sensitize C57BL/6 mice such that a profound airway inflammatory response is induced by just a single exposure to inhaled antigen.[7,8] In the present study, we sought to determine whether the route of allergen sensitization influenced the development of antigen-induced airway inflammation. Surprisingly, immunization of C57BL/6 mice via the s.c. route failed to sensitize the airways towards inflammatory responses, despite the fact that a comparable level of antigen-specific IgG1 and IgE was induced by s.c. and i.p. immunization.

To investigate the role of T-cell activation and localization in these observations, we used a model in which the allergic airway inflammation was mediated by TCR transgenic CD4+ T cells transferred into syngeneic B10.A mice. These studies showed that immunization via the i.p. was an efficient means of activating T-cell responses resulting in an increased number of activated allergen-specific T cells present in the circulation and lung of immunized mice, which correlated directly with the ability of these mice to respond to inhaled antigen. In contrast, s.c. immunization resulted in a small and localized response that was not sufficient for the development of airway responsiveness. This raises the possibility that all individuals may harbour activated Th2 cells specific for environmental allergens but that the atopic condition requires (i) that these cells are already present at the site of allergen contact and/or (ii) the maintenance of sufficiently large numbers of allergen-specific cells within the circulation.

Materials and methods

Mice

The –I line of 5C.C7 TCR transgenic and B10.A mice were bred and maintained at the Animal Facility of the Wellington School of

Medicine (University of Otago, Wellington, New Zealand). The –I line of the 5C.C7 transgenic mice was backcrossed to B10.A mice and maintained by breeding transgenic males to B10.A females. The TCR used to generate the 5C.C7 transgenic strain was derived from the cytochrome *c* specific T-cell clone 5C.C7.[9,10] These cells are specific for pigeon cytochrome *c* peptide 81–104 (PCC) and I-E^k, but proliferate more vigorously when stimulated with peptide fragment 81–103 from the tobacco horn worm moth cytochrome *c* (MCC).[11] All animal experimental procedures used in this study were approved by the Wellington School of Medicine Animal Ethics Committee, and were carried out in accordance with the guidelines of the University of Otago, New Zealand.

Reagents and mAbs

The synthetic MCC peptide KAERADLIAYLKQATK was obtained from Chrion Mimotopes (Melbourne, Victoria, Australia), and is formed from the two N-terminal residues of PCC together with the C-terminal sequence of MCC. Because the N-terminal residues have been shown not to be involved in MHC binding or TCR recognition,[12] this peptide is referred to as MCC for simplicity. Monoclonal anti-CD4 (GK1.5) was grown from hybridoma culture supernatant and conjugated to biotin. Anti-Vα11-FITC, anti-Vβ3-phycoerythrin (PE), anti-Vβ3-biotin, anti-CD44-PE, anti-CD62L-PE and strepdavidin-Cy-chrome C were all obtained from Pharmingen (San Diego, CA, USA) and used at a 1:100 dilution.

Antigen-induced airway inflammation

The model of ovalbumin (OVA)-induced airway inflammation has been described previously.[7] Briefly, it involves two immunizations of C57BL/6 mice with 2 μg OVA in 200 μL of alum adjuvant via the i.p. or s.c. (at the base of the neck) route at day zero and day 14, followed 7 days later by an i.n. challenge with 100 μg OVA in PBS. In the model of MCC-induced airway inflammation, similar protocols are followed as in the OVA model except that MCC is substituted for OVA in the immunization regime and B10.A mice are used as hosts for the adoptively transferred MCC specific transgenic T cells. Specifically, cell suspensions were made from lymph nodes of 5C.C7 mice and the percentage of total lymphocytes that expressed the transgenic Vα11$^+$Vβ3$^+$ TCR was determined by FACS analysis. Cell concentrations were adjusted so that $3–5 \times 10^6$ T cells expressing the transgenic TCR would be present in a total volume of 500 μL in incomplete modified Dulbecco's medium (IMDM), and this volume was injected into the tail vein of sex matched B10.A mice. Two days later, recipient mice were primed i.p. or s.c. with 250 μg MCC in 200 μL alum adjuvant on day two and day 22. Six days after the second i.p. or s.c. immunization (day 28) mice were anaesthetized by injection of a mixture of Ketamine and Xylazine (Phoenix, Auckland, New Zealand), and 100 μg MCC in a 50 μL volume of PBS administered by i.n. inoculation. For both the OVA and MCC allergen models, airway inflammation was determined at day four following i.n. challenge by performing broncho-alveolar lavage (BAL) and differential cell counts were performed as previously described.[7]

Histology of the lungs

Lungs of OVA immunized mice were collected at day 10 after the second i.p. or s.c. immunization. Lungs were immediately placed into 2–3 mL of 10% phosphate-buffered formalin. Fixed tissues were then embedded in paraffin and 2–3 μm sections were cut and mounted onto glass slides. Sections were stained with haematoxylin–eosin (HE) and examined by light microscopy.

Analysis of serum antibody levels

OVA-specific IgG or total serum IgE levels were determined by ELISA. Dilutions of test sera were made on 96-well plates (Nalge Nunc International, Rochester, NY, USA) coated overnight at 4°C with 100 μg/mL OVA (Sigma, St Louis, MO, USA) or with anti-IgE capture antibody (clone 4B3-39). Plates were blocked with PBS plus 5% BSA (Sigma) then incubated with serial dilutions of serum samples. Antibody binding was detected with peroxidase-labelled goat antimouse IgG1 (Sigma) or biotinylated anti-IgE detecting antibody (clone 3-11). Binding of anti-IgE antibody was visualized by further incubation with peroxidase-labelled strepdavidin. All reactions were developed with 1 mmol/L ABTS (Sigma) in citrate phosphate buffer (pH 9.2) and 0.03% hydrogen peroxide. Reactions were stopped at the appropriate time with 2 mmol/L sodium azide and the development of the coloured product was quantified by measuring absorbance at 414 nm using an Anthos Hill (Anthos, Wals, Austria) plate reader. OVA-specific IgG1 titres are expressed in U/mL (reciprocal of 50% antibody titre), whilst total serum IgE is expressed in ng/mL.

Isolation of mononuclear cells from lymphoid and peripheral tissues

Peripheral blood lymphocytes were isolated by collecting 100 μL of tail blood into 1 mL Alsevers solution (20.5 g dextrose, 4.2 g NaCl, 8 g sodium citrate, 1 L H_2O). For the isolation of lymphocytes from the lung, lymph nodes or spleen, mice were injected i.p. with 150 μL heparin (1 IU/mL; Leo Pharmaceutical Products, Ballerup, Denmark), anaesthetized, then perfused via the right heart ventricle with approximately 5 mL PBS. Mice were then killed and lung tissue was incubated for 30 min in complete IMDM containing 2.4 μg/mL collagenase type II (Gibco-BRL, Life Technologies, Gaithersburg, MD, USA) and 0.1% DNase1 (Sigma). Complete IMDM consisted of IMDM (Sigma) plus 5% FCS (Gibco BRL), 2 mmol/L glutamine (Sigma), 1% penicillin-streptomycin (Sigma) and 5×10^{-5} mol/L 2-ME (Sigma). Remaining tissue was dispersed by passage through an 18 gauge needle, and mononuclear cells were purified by gradient centrifugation over Lympholyte-M (Cedarland Laboratories, Hornby, Ontario, Canada) or 70% Percoll (Pharmacia Biotech, Uppsala, Sweden) as per manufacturer's instructions. Lymph nodes and spleens were collected into IMDM then made into cell suspensions by squeezing through two layers of gauze. Red blood cells from blood or spleen were lysed by incubation in 3 mL 0.14 mol/L NH_4Cl, 17 mmol/L Tris-HCl for 5 min or 10–20 min, respectively, at 37°C.

FACS analysis

FACS analysis of lymphocytes was carried out in 96-well round bottom plates using $10^5–10^{-6}$ cells per well, incubated with the indicated mAb diluted in 100 μL FACS buffer (PBS with 2% FCS and 0.01% sodium azide) for 10–15 min on ice. All samples were subjected to prior incubation with 2.4G2 (10 μg/mL) to inhibit *Fc*γRII-mediated uptake of mAbs. Flow cytometric analysis was performed on a FACSort (Becton Dickinson, Franklin Lakes, NJ, USA) using the CellQuest software. For all samples, between 50 000 and 100 000 live cells were collected for analysis.

Dendritic and T-cell co-culture

Mononuclear cells were isolated from the lung as described above. Peritoneal cells were collected by flushing the peritoneal cavity with 10 mL PBS and resuspending the pellet in complete IMDM. For all samples, 0.6 mL of 0.1 mol/L EDTA (pH 7.2) was added for every

7 mL of cell suspension, and samples were passed through a stainless steel sieve, layered over cold FCS containing 0.1 mol/L EDTA (FCS-EDTA) and then centrifuged. Pellets were resuspended in 5 mL cold Nycodenz (Progen, Heidelberg, Germany) and layered on top of a further 5 mL of cold Nycodenz. A final layer of 1–2 mL FCS-EDTA was added to the top of the sample and samples were spun for 10–15 min at 400 *g*. All cells except the pellet were collected and stained with anti-CD11c (N418) mAb-biotin for 30 min on ice in PBS containing 2 mmol/L EDTA and 2% BSA. CD11c$^+$ cells were purified using Strepdavidin MicroBeads (MACs Reagents; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched DC were cultured at the indicated concentrations in cIMDM in a 96-well plate (Nunc) with lymph node cells (2×10^5/well) from 5C.C7 TCR transgenic mice. Assays were incubated for 72 h at 37°C with [^{3}H] thymidine added (1 μCi/well) for the last 12 h. Total [^{3}H] thymidine incorporation was measured as an indicator of cell proliferation.

Results

I.p. but not s.c. immunization results in sensitization of the airways

C57BL/6 mice were immunized twice with identical doses of OVA antigen in alum adjuvant via the s.c. or i.p. route on days zero and 14. Ten days later, mice received an i.n. challenge with OVA, and the resulting airway eosinophilia and antibody response was analysed. Immunization via the i.p. route sensitized animals such that a single i.n. exposure to soluble OVA induced a profound airway eosinophilia (Fig. 1a). Surprisingly, s.c. immunization with an identical quantity of OVA in the same volume of alum adjuvant failed to result in the mice becoming responsive to inhaled OVA (Fig. 1a). Even when mice received three s.c. OVA/alum immunizations rather than the usual two, no sensitization of the lung was observed (Fig. 1a). However, both i.p. and s.c. immunized mice produced OVA-specific IgG1 (Fig. 1b) and had increased levels of total IgE (Fig. 1c), indicating that comparable B-cell responses were induced within the lymphoid tissues of both groups of mice.

To analyse the contribution of allergen-specific T cells to the airway inflammatory response, we used a model of airway eosinophilia whereby the inflammatory response is driven by TCR transgenic T cells.[13] In this model, $3–5 \times 10^6$ 5C.C7 T cells expressing the transgenic Vα11$^+$Vβ3$^+$ TCR were injected i.v. into normal syngeneic B10.A mice, and 2 days later, recipient mice were immunized with 250 μg MCC peptide in alum adjuvant (days two and 22). On day 28, recipient mice received an i.n. challenge with 100 μg MCC in PBS, and cellular infiltration into the airways was determined by BAL 4 days later. As reported for OVA immunization and airway challenge, mice immunized via the i.p. but not the s.c. route developed an acute airway inflammatory response (characterized by eosinophil and lymphocyte accumulation) following i.n. challenge (Fig. 3a). Importantly, no airway eosinophilia was observed in B10.A mice that were immunized i.p. and challenged i.n. with MCC, but which had not received 5C.C7 T cells, indicating that the response was driven solely by the TCR transgenic T cells with no contribution by endogenous T cells (Fig. 3a). To investigate the T-cell response, we analysed the proportion of Vα11Vβ3$^+$ T cells present in the lungs following i.n. challenge. These
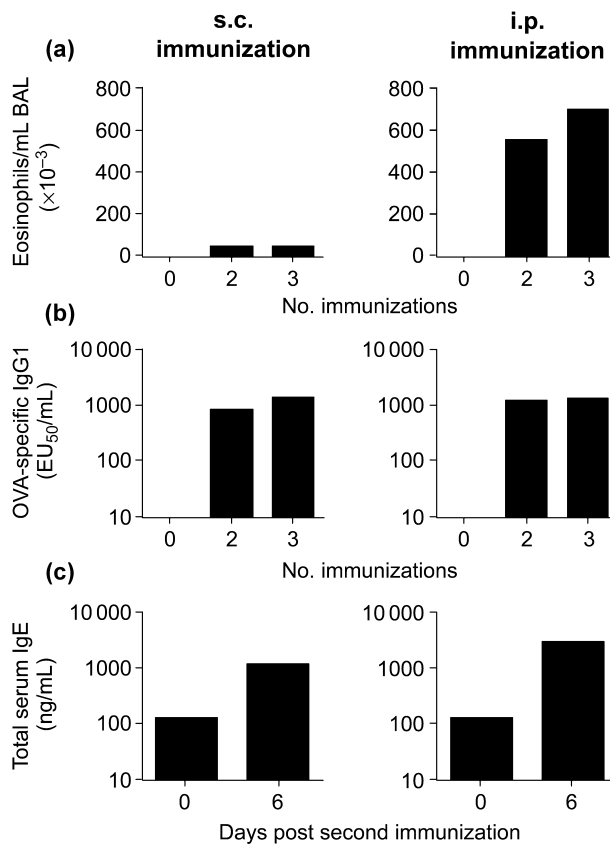

Figure 1 I.p. and s.**c.** immunization with ovalbumin (OVA)/alum had different consequences for lung inflammatory responses following airway exposure to OVA. Mice were immunized twice (days 0 and 14) or three times (days 0, 14 and 28) i.p. or s.c. with 2 μg OVA in 200 μL alum adjuvant. Seven days later, mice were anaesthetized and given 100 μg OVA in 50 μL of PBS by i.n. inoculation. (a) At day three after the i.n. challenge, mice were killed and a broncho-alveolar lavage (BAL) was performed using 1 mL PBS flushed three times into the lung. Differential cell counts on Diff Quik (Dade-Behring, Deerfield, IL, USA stained cytospins were performed to determine the number of infiltrating eosinophils. Blood was collected (b) at the time the mice were killed following the i.n. challenge or (c) 6 days after the second immunization, and OVA-specific serum IgG1 or total IgE was measured, respectively. Data represent pooled values for four to five mice and are representative of three separate experiments.

cells represented 24% of total CD4$^+$ T cells in mice immunized via the i.p. route and receiving an i.n. challenge, whereas the proportion of Vα11Vβ3$^+$ T cells in mice immunized via the s.c. route remained at the level seen in control, non-immunized mice (Fig. 3b).

These data indicate that only the i.p. route of immunization can sensitize mice towards the development of acute airway inflammatory responses following allergen inhalation, and that this response occurs in parallel with the recruitment of allergen-specific T cells to the lung.

Table 1 Proportion of CD4$^+$ T cells expressing the Vα11$^+$Vβ3$^+$ TCR present in various tissues following i.p. or s.c. immunization with MCC/alum

	Tissue	S.c. immunization		I.p. immunization	
		Alum	MCC + alum	Alum	MCC + alum
S.c. draining tissues	Skin	1.2	5.5†	0	3.1†
	Axillary lymph node	1.6	2.7†	1.8	2.8†
I.p. draining tissues	Peritoneum	0.5	1.4	0.2	21.7†
	Blood	2	0.9	2.3	3.9
	Spleen	1.9	1.3	1.5	7.3†
	Lung	1.4	1.2	1.3	9.5†
	Mediastinal lymph node	0.7	1.7	ND	6.5

†Values significantly greater than those obtained following alum immunization alone using a Student's *t*-test ($P \leq 0.05$).
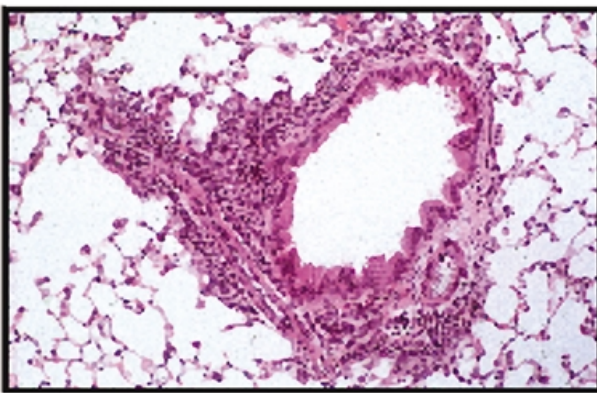ND, not done.

(a)
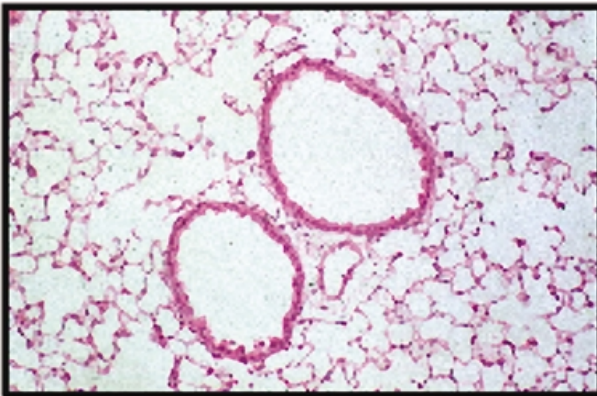
(b)

Figure 2 (a) I.p. and (b) s.c. immunization with ovalbumin (OVA)/alum had different consequences for lung inflammatory responses following airway exposure to OVA. Mice were immunized twice (days 0 and 14) i.p. or s.c. with OVA/alum, then 10 days after the last immunization, lungs were fixed, embedded in paraffin, and sections were stained with haematoxylin–eosin (HE) as detailed in the Materials and methods. Histological sections are shown at 40× magnification.

I.p. but not s.c. immunization results in the accumulation of activated allergen-specific T cells in the lung and circulation before i.n. challenge

We observed that C57BL/6 mice immunized with OVA via the i.p. route had a mononuclear infiltrate surrounding the airways even before being subjected to i.n. challenge (Fig. 2). In contrast, the airways of mice immunized via the s.c. route were normal, without signs of lymphoid infiltrate (Fig. 2). It was plausible that this infiltrate might contain allergen-specific T cells that would respond to inhaled allergen and contribute to the allergic inflammatory response. We therefore investigated the effect of i.p. or s.c. immunization with MCC/alum on the accumulation of T cells expressing Vα11$^+$Vβ3$^+$ TCR in various tissues before the administration of i.n. MCC.

As expected, i.p. immunization with MCC/alum resulted in a marked accumulation of Vα11$^+$Vβ3$^+$ T cells at the site of peptide deposit (peritoneum) and in the draining lymphoid tissues (spleen and mediastinal lymph node) (Table 1). More interestingly, i.p. immunization appeared to increase the proportion of Vα11$^+$Vβ3$^+$ T cells found within all tissues investigated, including non-draining lymph nodes and lung (Table 1). In contrast, s.c. immunization consistently increased the percentage of CD4$^+$ T cells expressing the Vα11$^+$Vβ3$^+$ TCR in the draining axillary and brachial lymph nodes, but did not result in the significant accumulation of Vα11$^+$Vβ3$^+$ T cells within any other tissue investigated (Table 1). Similar trends to those shown in Table 1 (day three after the second immunization) were also seen at a later time point (day seven), indicating that these observations were not simply a result of differences in the kinetics of T-cell expansion or circulation following i.p. versus s.c. immunization. The total numbers of Vα11$^+$Vβ3$^+$ CD4$^+$ T cells detected within all investigated tissues of mice immunized i.p. with MCC/alum increased approximately 20-fold over those levels found in mice immunized with alum alone (5.5×10^6 *vs* 2.5×10^5). In comparison, the numbers of antigen-specific T cells in mice immunized s.c. increased only 1.2-fold over background (4.2×10^5 *vs* 3.3×10^5).

We have previously shown that the proportion of Vα11$^+$Vβ3$^+$ T cells in the blood increases following the second i.p. immunization with MCC, and that these cells are quickly depleted following i.n. challenge,[13] presumably reflecting their migration into the lung. We therefore monitored the proportion of Vα11$^+$Vβ3$^+$ T cells in the blood of i.p. and s.c. immunized mice before and following i.n. challenge. As
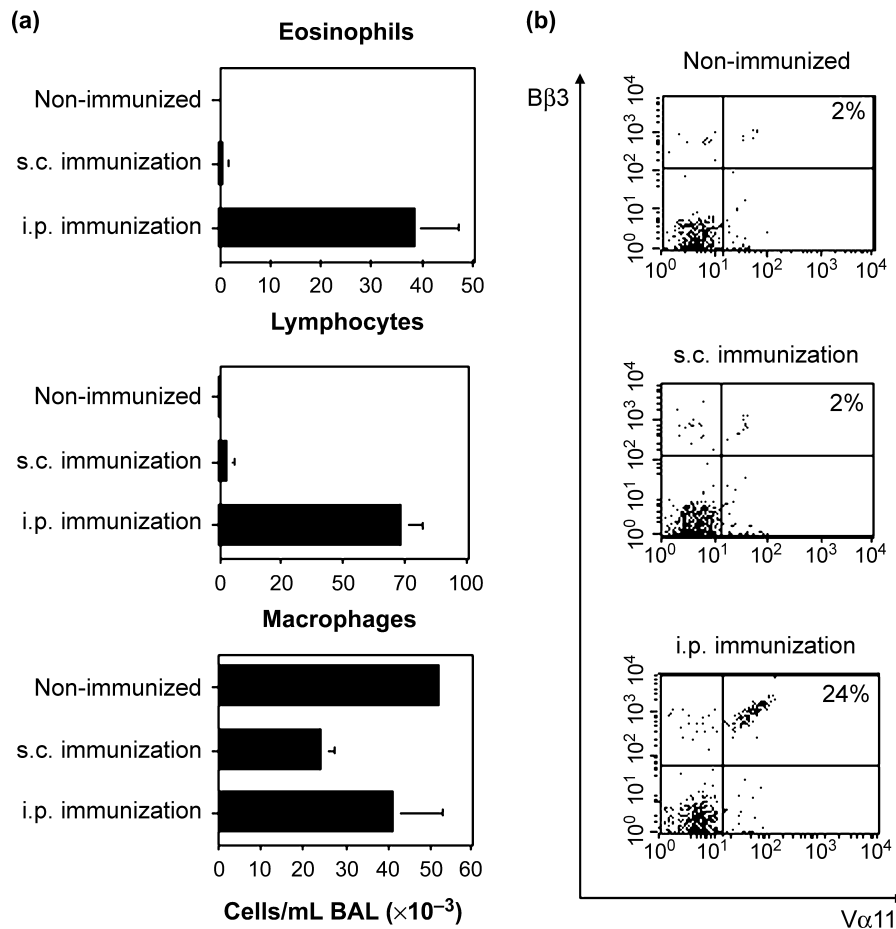
Figure 3 I.p. immunization, but not s.c. immunization, with MCC/alum induced airway inflammation following MCC i.n. challenge. Vα11+Vβ3+ CD4+ T cells ($3-5 \times 10^6$) were injected i.v. into B10.A mice on day zero. At day two and 22 recipient mice were immunized either s.c. or i.p. with 250 μg MCC in 200 μL alum adjuvant. On day 28, mice were anaesthetized and given 100 μg MCC in 50 μL of PBS by i.n. inoculation. (a) Four days later, mice were killed and broncho-alveolar lavage (BAL) and differential cell counts were performed as detailed in Figure 1. Data represent mean ± SD of five to six mice per group and are representative of two separate experiments. (b) Lung cells from the same mice were stained with anti-CD4-biotin, SA-Cy-chrome C, anti-Vα11-FITC and anti-Vβ3-PE, then analysed by FACS. Dot plots show cells from one mouse, are gated for CD4 expression and are representative of seven to ten animals per group from two separate experiments. Numbers indicate the percentage of gated CD4+ T cells expressing Vα11 and Vβ3.

expected, the proportion of Vα11+Vβ3+ T cells was increased in the blood of i.p. immunized mice, but these were rapidly lost following i.n. challenge (Fig. 4). Interestingly, the proportion of Vα11+Vβ3+ T cells present in the blood of s.c. immunized mice was barely detectable, being no higher than that found in non-immunized mice, and the level of these cells did not alter following i.n. challenge (Fig. 4).

To ensure that s.c. immunization did actually result in T-cell activation, we analysed the expression of CD62L and CD44 by Vα11+Vβ3+ CD4+ T cells in the spleen of i.p. immunized mice, or the axillary lymph node of s.c. immunized mice, because these represent the major draining lymphoid organs following challenge via either route. As expected, Vα11+Vβ3+ CD4+ T cells from naive 5C.C7 mice were CD62L^{hi}CD44^{med} (Fig. 5; dashed line), consistent with a naive phenotype. In contrast, Vα11+Vβ3+ CD4+ T cells from the spleens of mice immunized i.p., or the axillary lymph node of mice immunized s.c., were CD62L^{med-lo}CD44^{hi} (Fig. 5; solid line), consistent with an activated phenotype. Note though that Vα11+Vβ3+ CD4+ T cells from s.c. immunized mice did show a less dramatic downregulation of CD62L compared to that seen following i.p. immunization.

These data indicate that both i.p. and s.c. immunization with MCC/alum can induce the activation of Vα11+Vβ3+ CD4+ T cells within draining lymphoid tissues, but that only i.p. immunization results in a greater number of activated T cells in the circulation, non-draining lymph nodes and lung.

I.p. immunization does not cause antigen deposition in the lung

It was not clear whether the accumulation of allergen-specific T cells within the lung of i.p. immunized mice before i.n. challenge reflected non-specific trafficking of activated peptide-specific T cells from the blood through peripheral tissues, or a process of selective recruitment. We reasoned that the major factor likely to drive the specific recruitment of T cells to the lung would be the presence of antigen at this site. We therefore conducted an experiment designed to determine whether i.p. immunization can result in the deposition of antigen within the lung. For this purpose, we immunized mice i.p. with 1 mg MCC peptide, isolated CD11c+ dendritic cells (DC) from the peritoneal cavity and the lung 24 h later, then co-cultured the DC with naive 5C.C7 TCR transgenic T cells. A similar degree of enrichment was obtained from both sites (between 70 and 90% CD11c+ cells); however, these were likely to represent different subpopulations of DC. MCC peptide presentation by CD11c+ DC isolated from the peritoneal cavity or lung following i.p. immunization was then determined by their ability to stimulate the proliferation of naive Vα11+Vβ3+ T cells. As expected, CD11c+ DC isolated from the peritoneal cavity were able to induce Vα11+Vβ3+ T-cell proliferation (Fig. 6a). In contrast, CD11c+ DC isolated from the lungs of i.p. immunized mice did not induce Vα11+Vβ3+ T-cell proliferation, even when very high numbers of DC were used (Fig. 6b).
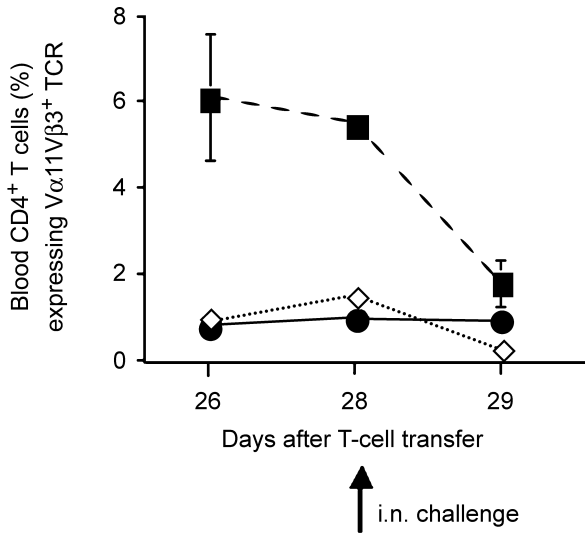
Figure 4 I.p. immunization, but not s.c. immunization, with MCC/alum induced accumulation of Vα11+Vβ3+ CD4+ T cells in the blood before i.n. challenge. B10.A mice were injected with Vα11+Vβ3+ CD4+ T cells then subjected to MCC immunization and i.n. challenge as detailed in Figure 3. Blood was collected from the tail into Alsever's solution at the indicated time points before and after the i.n. challenge and lymphocytes were stained with anti-CD4-biotin, SA-Cy-chrome C, anti-Vα11-FITC and anti-Vβ3-PE then analysed by FACS. Data show mean ± SD of five to six mice per group and are representative of three separate experiments. ●, s.c. immunization; ■, i.p. immunization; ◇, non-immunized.

These data indicate that MCC is not detectable on CD11c+ DC present in the lungs of mice following i.p. immunization, and that the presence of peptide-specific T cells at this location is therefore likely to represent non-specific trafficking of activated cells into peripheral tissues.

Discussion

Our data indicate that immunization via the i.p. or s.c. route has very different consequences for lung inflammatory responses following airway exposure to antigen. Both i.p. and s.c. immunization were sufficient to induce CD4+ T-cell activation and T-cell-dependent B-cell antibody production. However, only immunization via the i.p. route could sensitize C57BL/6 mice to subsequent lung inflammatory responses. These findings can in part be explained by the observation that i.p. immunization with antigen or peptide in alum led to the accumulation of specific T cells into the lung before i.n. challenge. In contrast, s.c. immunization resulted in the activation of antigen-specific T cells in the draining axillary lymph nodes, but did not promote accumulation of these cells in lung tissue or predispose the host towards the development of an airway inflammatory response. This was true even when an increased amount of antigen was used in the s.c. immunization procedure. T-cell recruitment to the lung of i.p. immunized mice does not appear to be driven by antigen deposits at this site, and may instead reflect the increased number of activated T cells present in the circulation of these mice.

However, the presence of activated T cells in the lung of i.p. immunized mice before i.n. challenge is unlikely to be the
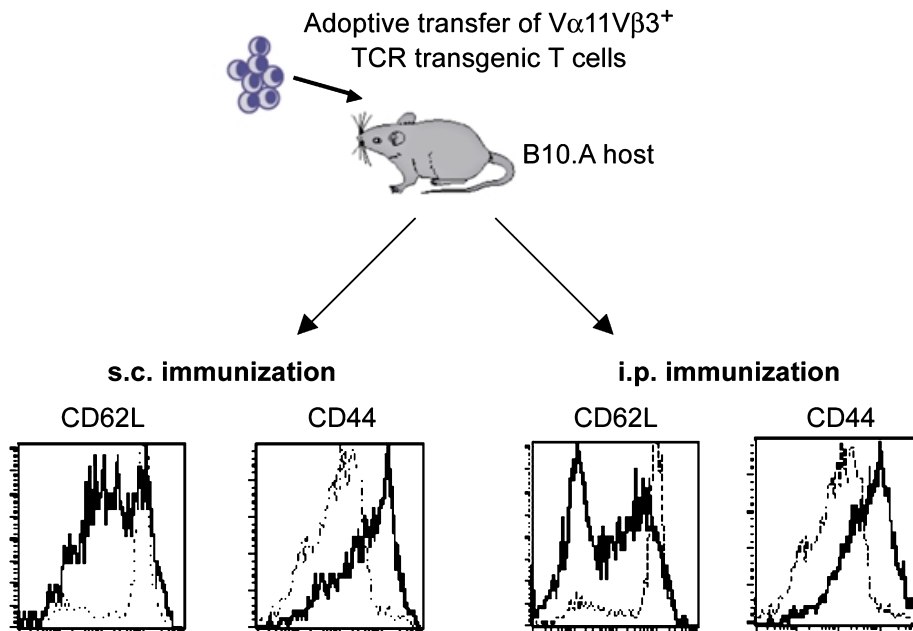

Figure 5 Both i.p. and s.c. immunization with MCC/alum induced the expression of activation markers on Vα11+Vβ3+ CD4+ T cells. B10.A mice were injected with Vα11+Vβ3+ CD4+ T cells then subjected to MCC immunization as detailed in Figure 3. Three days following the second immunization, mice were killed and cell suspensions were made from spleen (i.p. immunized mice) or axillary lymph nodes (s.c. immunized mice). Cell suspensions were stained with anti-Vβ3-biotin, SA-Cy-chrome C, anti-Vα11-FITC and anti-CD44-PE or anti-CD62L-PE. Dot plots show cells gated for Vα11 and Vβ3 expression. Dashed lines indicate naive Vα11+ Vβ3+ T cells from blood of 5C.C7 mice; solid lines indicate Vα11+ Vβ3+ T cells from MCC/alum immunized mice. Dot plots show cells from one mouse and are representative of three to four individual animals.
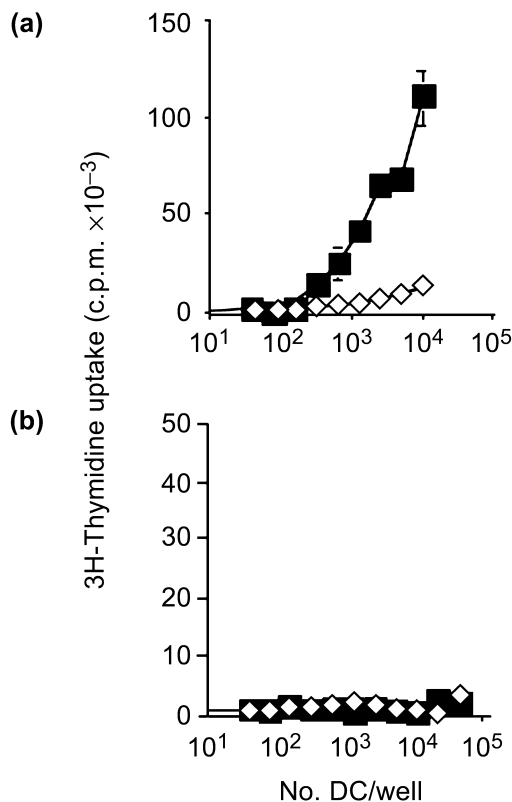
(a)

(b)

Figure 6 MCC peptide is not presented by lung dendritic cells (DC) following i.p. immunization. 5–6 B10.A mice were immunized i.p. with 1 mg MCC in alum (■) or with alum alone (◇). Twenty-four hours later, DC were isolated from the (a) peritoneal cavity or (b) lung and pooled as detailed in the Materials and methods. DC were then incubated at the indicated concentrations with 2×10^5 lymph node cells from naive 5C.C7 mice and cultured for 72 h with [^{3}H] thymidine added (1 μCi/well) for the last 12 h. Total [^{3}H] thymidine incorporation was measured as an indicator of cell proliferation. Values represent mean ± SE of triplicate cultures and are representative of two separate experiments.

only explanation for the observed increase in airway inflammatory responses. I.p. immunization also resulted in the production of a larger pool of activated Vα11⁺Vβ3⁺ CD4⁺ T cells in the blood compared to s.c. immunization. These cells are likely to represent the pool of antigen-specific T cells available for recruitment into the lung immediately following i.n. challenge, resulting in the generation of an increased airway inflammatory response.

Another possible explanation for the differences seen in lung inflammatory responses in i.p. and s.c. immunized mice is that functionally different T cells are generated in these two groups. A number of recent studies have indicated that Th1 and Th2 cells express distinct sets of adhesion molecules[14] and chemokine receptors,[15–17] resulting in differences in the recruitment patterns of these cells into inflamed peripheral tissues. In the present study, the levels of OVA-specific IgG1 total IgE were similar in mice immunized with OVA/alum via the i.p. or s.c. route, indicating that both routes of immunization can induce the development of a Th2 response. Thus,

these cells probably express a similar set of surface receptors, although we have not formally tested this.

Of special note, large differences were found in the total number of activated Vα11⁺Vβ3⁺ CD4⁺ T cells generated following i.p. or s.c. immunization with MCC. Therefore, the similarities in IgG1 and IgE levels seen in i.p. or s.c. immunized mice may reflect that B cells, not T cells, are limiting in the generation of an antibody response. This view is supported by a previous finding in our laboratory that anti-CTLA-4 mAb treatment of mice infected with *Nippostrongylus brasiliensis* significantly increases the pool of IL-4- and IL-5-producing T cells in the draining lymph nodes,[18] but does not alter the antibody response (K. McCoy and G. Le Gros, unpubl. obs., 1998).

Many investigators have reported that allergen sensitization of BALB/c mice via the s.c. route is sufficient for the induction of allergic airway inflammation.[19,20] BALB/c mice are well accepted to generate a more robust Th2 response, and to show increased allergic airway inflammatory responses compared to C57BL/6 mice.[19] Thus we feel that the differences between these reports and our findings are likely to reflect the genetic background of the strains used, perhaps resulting from different frequencies of atopy inducing Th2 cells generated in these various strains. Alternatively, the precise site of s.c. immunization, or the number and frequency of i.n. challenges administered, may play a role.

In summary, in our model, i.p. immunization with antigen or peptide in alum leads to the generation of a larger number of lung resident and circulating activated CD4⁺ T cells in the peripheral blood compared to s.c. immunization. Following i.n. challenge, the cells resident within the lung of i.p. immunized mice could respond immediately, and the ensuing inflammatory response would result in further accumulation of specific T cells from the blood and hence accentuate the response. Although s.c. immunized mice contained previously activated allergen-specific T cells, these cells were not available in high enough numbers, or within the appropriate location, required for the induction of an acute airway inflammatory response. These findings indicate that the mere presence of responsive Th2 cells may not be sufficient for atopic asthma. Instead, the development of atopy may be largely determined by the frequency and tissue localization of allergen-specific Th2 cells.

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