

The lung cytokine microenvironment influences molecular events in the lymph nodes during Th1 and Th2 respiratory mucosal sensitization to antigen *in vivo*

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SUMMARY

Originally defined by their patterns of cytokine production, Th1 and Th2 cells have been described more recently to express other genes differentially as well, at least *in vitro*. In this study we compared the expression of Th1- and Th2-associated genes directly during *in vivo* sensitization to ovalbumin (OVA) in Th1- and Th2-polarized models of airways inflammation. Th1-polarized airway inflammation was achieved by the intranasal instillation of adenoviral vectors (Ad) encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-12, followed by daily aerosolizations of OVA; instillation of Ad/GM-CSF alone with OVA aerosolization led to Th2-polarized responses. Lymph nodes were obtained at various time-points, RNA extracted, and analysed by real-time quantitative polymerase chain reaction (PCR). Consistent with reports from *in vitro* and human studies, mice undergoing Th1-polarized inflammation showed preferential expression of the transcription factor t-bet, the chemokines IFN- γ inducible protein (IP)-10 and macrophage inflammatory protein 1 alpha (MIP-1-alpha), and the chemokine receptor CCR5. In contrast, the transcription factor GATA-3, the chemokines I-309 and thymus and activation regulated chemokine (TARC), and the chemokine receptors CCR3 and CCR4 were preferentially expressed in the Th2 model. Importantly, we also show that Ad/transgene expression remains compartmentalized to the lung after intranasal instillation. Flow cytometric analysis of lung myeloid dendritic cells indicated that B7.1 was expressed more strongly in the Th1 model than in the Th2 model. These studies provide a direct comparison of gene expression in *in vivo* Th1- and Th2-polarized models, and demonstrate that molecular events in the lymph nodes can be altered fundamentally by cytokine expression at distant mucosal sites.

Keywords allergic airway inflammation chemokines cytokines real-time quantitative PCR Th1/Th2

INTRODUCTION

Molecular signals delivered to T helper cells during their primary activation determine their differentiation, effector and directional activities, and profoundly affect the nature of the ensuing immune-inflammatory response. This has been demonstrated convincingly in *in vitro* systems, where Th cells stimulated in the presence of interleukin (IL)-12 and anti-IL-4 antibodies differentiate into Th1 cells (distinguished by the production of interferon (IFN)- γ), while those stimulated with IL-4 and anti-IFN- γ follow the Th2 differentiation pathway (distinguished by the production of IL-4, -5, and -13) [1,2]. This pattern of cytokine expression

fundamentally affects the downstream nature of the immune-inflammatory response *in vivo*. Th1-polarized responses are associated with enhanced cytotoxic T lymphocyte (CTL) killing, production of distinct immunoglobulin isotypes (IgG2a in mice) and mononuclear and neutrophilic inflammation. In contrast, Th2-polarized responses tend to result in the production of IgE and are characterized typically by eosinophilia in the blood and eosinophilic inflammation in the target organ.

Although Th1 and Th2 cells were defined originally by their profile of cytokine expression, analysis of such *in vitro* polarized Th1 and Th2 cells has also revealed differential expression of a number of other molecules, including chemokines, chemokine receptors and transcription factors [2–5]. However, whether the differential expression of these genes observed *in vitro* holds true during *in vivo* immune responses as well has not been examined carefully in systems where a direct comparison is possible.

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Although Chiu *et al.* compared gene expression of chemokine receptors in CD4⁺ T cells after *in vivo* exposure to Th1- or Th2-polarizing infections, the approach taken in that study does not allow us to be certain that the differential expression they observed after sorting and *in vitro* restimulation was actually present *in vivo* [6].

We have described previously that the transient transgenic expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in mouse airways during exposure to aerosolized ovalbumin (OVA) leads to an inflammatory response reminiscent of asthma, which is characterized by the presence of Th2 cytokines in the bronchoalveolar lavage (BAL) and eosinophils in the BAL and tissue [7]. When we concurrently expressed IL-12 with GM-CSF during OVA exposure, the airway inflammatory response was no longer eosinophilic, but rather dominated by mononuclear cells and neutrophils, along with abundant IFN- γ in the BAL [8]. We used these models of bona fide Th1- and Th2-polarized airways inflammation to examine the expression of a variety of molecules reportedly affiliated with Th1 or Th2 responses *in vivo*, at the primary site of T cell activation, using real-time quantitative polymerase chain reaction (PCR) (TaqManTM). Our data demonstrate that distinct patterns of gene expression are observed readily in the thoracic lymph nodes during Th1- and Th2-polarized inflammatory responses. Moreover, we show that cytokine transgene expression compartmentalized in the airway is able to alter fundamentally molecular events in the thoracic lymph nodes, in spite of the absence of transgene expression in these lymph nodes. Flow cytometric analysis indicated that the divergence of the immune responses may be, in part, attributable to differing patterns of co-stimulatory molecule expression by lung dendritic cells.

MATERIALS AND METHODS

Subjects

Six to 8-week-old female Balb/c mice were obtained from Charles River (Ottawa, ON, Canada) and maintained under specific pathogen-free conditions, with a 12-h light/dark cycle and food and water *ad libitum*. All experiments described herein were approved by the McMaster Animal Research Ethics Board.

Administration of adenoviral constructs

As described previously [7,8], airway expression of cytokines was achieved by the intranasal delivery of a replication-deficient human type 5 adenovirus with the cytokine gene inserted into the E1 region of the viral genome. The viral dose was administered

intranasally to isoflurane-anaesthetized animals in 30 : 1 PBS on day -1. For adenoviral vectors (Ad)/GM-CSF, the dose was 3×10^7 plaque-forming units (pfu); for Ad/IL-12, the dose was 1×10^7 pfu. As a control, an empty replication-deficient adenovirus (RDA) was administered. In order to assess transgene expression, an Ad vector expressing a mouse-exogenous gene (OVA) was delivered at a dose of 3×10^7 pfu.

Sensitization to OVA

One day after administration of Ad/G-CSF alone or Ad/GM-CSF and Ad/IL-12, mice were sensitized to OVA by exposing them to a 1% OVA aerosol (1% wt/vol in 0.9% saline; Sigma-Aldrich, Oakville, ON, Canada) for 20 min daily for 10 days. The aerosol was generated using compressed medical air at 7 l/min through a Bennet/Twin nebulizer, into a plexiglas chamber.

Preparation of cDNA samples

At various time-points during OVA aerosolization mice were anaesthetized by isoflurane and killed by exsanguination. Thoracic lymph nodes and lung tissue were collected, pooled and stored in RNALater (Ambion, Inc., Austin, TX, USA). Total RNA was extracted using TriPure (Roche, Indianapolis, IN, USA) using a Polytron Aggregate homogenizer (Kinematica, Lucerne, Switzerland). Genomic DNA was removed from these samples using the Qiagen RNeasy kit (Qiagen Inc., Mississauga, ON, Canada). RNA was reverse transcribed to cDNA using the Qiagen OMNIscrip kit (Qiagen) using random hexamers (GIBCO, Rockville, MD, USA) and oligo-dT (GIBCO) as primers.

Real-time quantitative PCR analysis

PCR primers and FAM-labelled probes for GATA-3, IFN- γ inducible protein (IP)-10, I-309, t-bet and OVA (Table 1) were designed using the PrimerExpress version 1.5 software package (Applied Biosystems, Foster City, CA, USA). Primer and FAM-labelled probe sets for IFN- γ , IL-4, CCR3, CCR4, CCR5 and thymus and activation regulated chemokine (TARC) were obtained as predeveloped assay reagents (PDARs) from Applied Biosystems. GAPDH primers and VIC-labelled probes were obtained from Applied Biosystems. PCR was carried out in the ABI Prism 6700 Sequence Detection System, operated by Sequence Detector version 1.7 software (Applied Biosystems), using TaqMan Universal PCR Master Mix (Applied Biosystems) for all PCR reagents; 1 μ g of cDNA was added to each well, and all measurements were conducted in triplicate wells. Gene expression was quantified relative to the expression of the housekeeping gene

Table 1. Sequences of custom primers and probes for real-time quantitative PCR

Gene	Forward primer	Reverse primer	Probe
GATA-3	CTA CCG GGT TCG GAT GTA AGT C	GTT CAC ACA CTC CCT GCC TTC T	AGG CCC AAG GCA CGA TCC AGC
I-309	ACA AAA CGT GGG TTC AAA ATC A	GGG AAG GTG GCT CAT CTT CA	CTG AAG AAG GTG AAC CCC TGC TAACCG
IP-10	GGA TGG CTG TCC TAG CTC TGT AC	TGG GCA TGG CAC ATG GT	AGG GCGT TCG CAC CTC CAC ATA GCT
t-bet	ACC AGA ACG CAG AGA TCA CTC A	CAA AGT TCT CCC GGA ATC CTT	CTG AAA ATC GAC AAC AAC CCC TTT GCC
OVA	CCA TGC AGC ACA TGC AGA A	GGA ATG GAT GGT CG CCC TAA	AGA GAC GCT TGC AGC ATC CAC TCC A

GAPDH, and normalized to that measured in naive control mice (where applicable).

Flow cytometric analysis

At various time-points during OVA exposure, mice were anaesthetized with isoflurane, killed by exsanguination and lungs removed. Lung tissue was perfused by injecting 10 ml Hanks's balanced salt solution (HBSS) into the right atrium of the heart. After cutting into ~3 mm pieces, pooled lung tissue from several mice was incubated in collagenase III (Worthington Biochemical, Freehold, NJ, USA) in HBSS (GIBCO BRL, Grand Island, NY, USA) (150 U/ml) for 1 h, and then ground through a tissue screen into HBSS. This cell suspension was layered on top of a 30/60% Percoll density gradient, and centrifuged at 2500 r.p.m. for 25 min at room temperature. Cells at the 30/60% interface were collected, washed twice and resuspended in PBS; 10^6 cells were stained for flow cytometry in polystyrene tubes. After blocking with Fc block ($1 \mu\text{g}/10^6$ cells) (PharMingen), cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c ($1 \mu\text{g}/10^6$ cells) (PharMingen), phycoerythrin (PE)-conjugated anti-CD11b, and either biotin-conjugated anti-I-A^d, anti-B7.1 or anti-B7.2 ($1 \mu\text{g}/10^6$ cells) (PharMingen); streptavidin-PerCP (20 $\mu\text{l}/10^6$ cells) (Becton Dickinson, San Jose, CA, USA) was added to label fluorescently the biotin-conjugated antibodies. Cells were stored in 1% paraformaldehyde overnight, run on a FacSCAN flow cytometer (Becton Dickinson) running CellQuest acquisition software and data were analysed using WinMDI (Scripps Institute, La Jolla, CA, USA).

Data analysis

Real-time quantitative PCR data are expressed as mean $\pm$ standard deviation (s.d.) of triplicate wells; one representative experiment is shown. Statistical analysis was performed using SigmaStat version 2.03. Differences were considered statistically significant when $P < 0.05$ by ANOVA.

RESULTS

Detection of transgene expression in the lungs and thoracic lymph nodes of intranasally infected mice

In order to determine the tissue distribution of transgene expression after intranasal instillation of the Ad vectors, we administered an Ad vector expressing a completely exogenous sequence (OVA) to mice in a fashion identical to that by which the cytokine vectors are administered. Mice were sacrificed at various time-points after intranasal Ad/OVA administration, and expression of the transgene detected in lung or lymph node tissue by TaqManTM (Fig. 1). As expected, transgene expression was completely undetectable in the lungs or lymph nodes of uninfected mice. Expression of the transgene was detected readily in the lung at the earliest time-point examined (day 3) to day 9, peaking on day 6 after infection. However, even after 40 cycles of PCR amplification, absolutely no transgene expression was detectable in the lymph nodes at any time-point after intranasal infection.

Transcription factor expression

The transcription factor t-bet is an important activator of the IFN- γ gene [9–11], whereas GATA-3 is known to initiate IL-5 and IL-13 transcription [12–14]; thus, differential expression of these factors is likely to be important in the acquisition of the Th1 or Th2 phenotypes, respectively. We examined t-bet and GATA-3

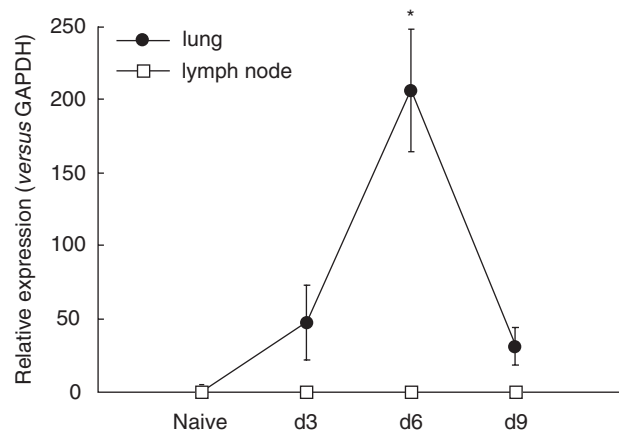


Fig. 1. Expression of a mouse-exogenous transgene in lung and lymph node was measured by real-time quantitative PCR (TaqManTM) at various time-points after intranasal administration of 3×10^7 pfu of an adenoviral vector carrying the transgene. Transgene expression was quantified relative to the presence of a housekeeping gene, GAPDH. Black circles (●) show transgene expression in the lung, while white squares (□) indicate transgene expression in the lymph nodes. Points are mean $\pm$ s.e.m. of individual mice, $n = 4$ –5. *Significant difference ($P < 0.05$) from naive mice by ANOVA.

expression in the lymph nodes during the Th1 and Th2 models. Figure 2a demonstrates that t-bet expression is significantly up-regulated only in the Th1 model. In contrast, GATA-3 expression is up-regulated only in the Th2 model on day 4 (Fig. 2b); although this up-regulation is transient and modest in extent (~twofold over naive), this observation has been made consistently between repeated TaqMan assays and between multiple experiments in this study, as well as in other studies [15].

Cytokine and chemokine expression in the thoracic lymph nodes during Th1 and Th2 polarization

Our previous work [7,8] demonstrates that mucosal exposure to GM-CSF/IL-12/OVA elicits a bona fide Th1 response, while GM-CSF/OVA elicits Th2 immunity. These conclusions were based on findings in the lung and spleen. Here, we have investigated cytokine expression in the thoracic lymph nodes during sensitization to OVA in these Th1 and Th2 models to determine whether alteration of the lung microenvironment fundamentally alters Th polarization events in the lymph nodes. Lymph node expression of IFN- γ is increased dramatically in the Th1 model on days 4 and 7, returning to near-naive levels by day 11 (Fig. 3a). However, in the Th2 model IFN- γ mRNA is never detected above that seen in naive animals. In contrast, IL-4 gene expression is significantly up-regulated at all time-points examined in the Th2 model, and modestly but significantly up-regulated only on day 11 of the Th1 model (Fig. 3b).

We also examined the expression of supposedly Th1- and Th2-affiliated chemokines in the lymph nodes during sensitization to OVA *in vivo*. IP-10 was significantly up-regulated in the Th1 model, peaking on day 4, but was not up-regulated at all in the Th2 model (Fig. 4a). Expression of macrophage inflammatory protein 1 alpha (MIP-1-alpha) was enhanced in both models on day 4 to a similar degree, but remained heightened in the Th1 model on day 7, whereas it had returned to naive levels in the Th2 model (Fig. 4b). mRNA for I-309 was moderately but significantly

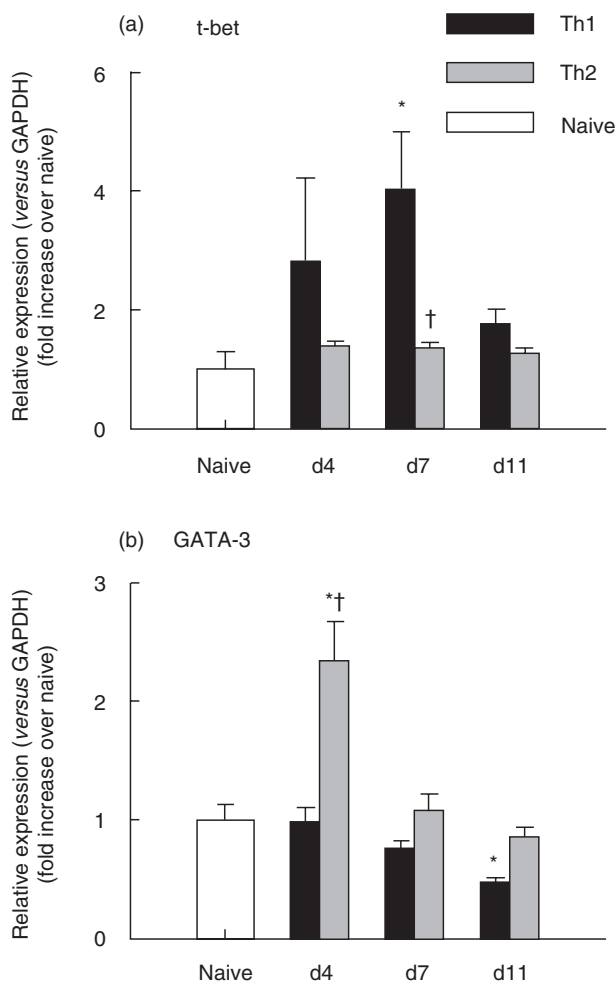


Fig. 2. Expression of the transcription factors t-bet and GATA-3 were measured by real-time quantitative PCR (TaqMan™) in the lymph nodes at various time-points during respiratory sensitization to OVA. Cytokine expression was quantified relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from eight to 10 mice for analysis. Bars are mean $\pm$ s.d. of triplicate measurements. *Significant difference ($P < 0.05$) from naive mice; †significant difference ($P < 0.05$) from the Th1 model at the same time-point.

increased in the Th2 model on day 4, but returned to naive levels by day 7, and was never up-regulated in the Th1 model (Fig. 4c). TARC expression was substantially up-regulated in the Th2 model on days 4 and 7, returning to naive levels by day 11, whereas TARC was only detected above naive levels on day 4 of the Th1 model at a considerably lower level than that seen in the Th2 model (Fig. 4d).

Chemokine receptor expression

Different patterns of chemokine receptor expression have been associated with Th1- and Th2-polarized cells. Here we examined the expression of the Th1-affiliated CCR5 and the Th2-affiliated CCR3 and CCR4 in the lymph nodes during *in vivo* immune responses. As seen in Fig. 5a, CCR5 expression was up-regulated

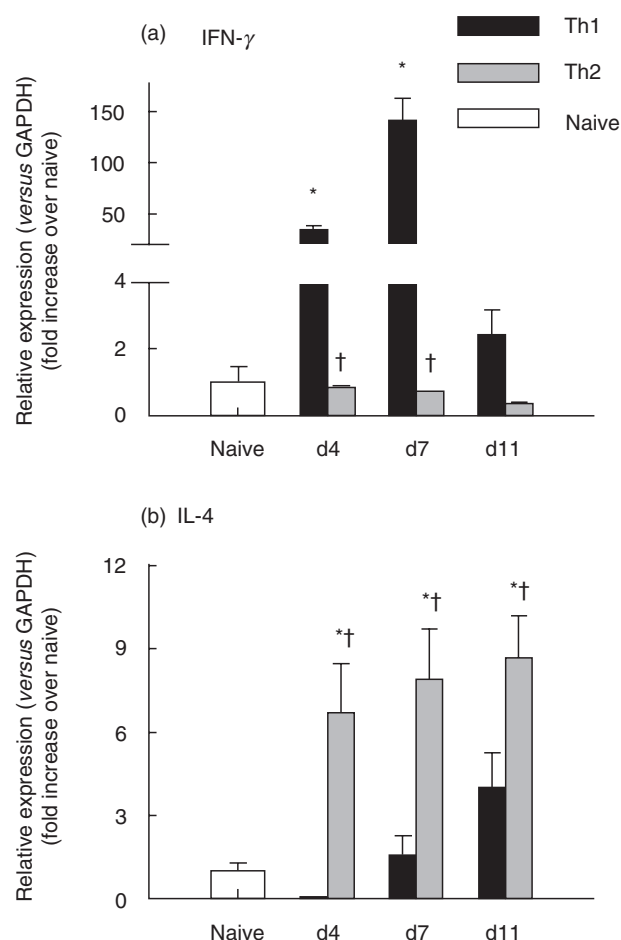


Fig. 3. Expression of the prototypical Th1 and Th2 cytokines IFN- γ and IL-4 were measured by real-time quantitative PCR (TaqMan™) in the lymph nodes at various time-points during respiratory sensitization to OVA. Cytokine expression was quantified relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from eight to 10 mice for analysis. Bars are mean $\pm$ s.d. of triplicate measurements. *Significant difference ($P < 0.05$) from naive mice; †significant difference ($P < 0.05$) from the Th1 model at the same time-point.

only during the Th1 protocol, and not expressed above naive levels in the Th2 model. In contrast, mRNA for CCR3 and CCR4 were significantly up-regulated only during the Th2 protocol and not during the Th1 model (Fig. 5b, c).

Flow cytometric analysis of lung dendritic cells

Assessment of co-stimulatory molecule expression on lung dendritic cells was performed at various time-points during the Th1 and Th2 models. B7.1 and B7.2 expression was analysed on CD11b⁺ CD11c⁺ lung-derived mononuclear cells; 89.9% of these cells were positive for MHC class II expression, indicating that they were dendritic cells of the myeloid phenotype. In naive mice, 18.9% and 5.4% of CD11b⁺ CD11c⁺ lung mononuclear cells expressed B7.1 and B7.2, respectively (Table 2). By day 4 of the Th1 model, 44.4% of CD11b⁺ CD11c⁺ lung mononuclear cells expressed B7.1, and this remained at a similar level on day 7

Table 2. Flow cytometric analysis of co-stimulatory molecule expression

Naive	Co-stimulatory molecule expression on lung-derived CD11b ⁺ CD11c ⁺ cells (%)					
	B7.1 ⁺			B7.2 ⁺		
	18.9			5.4		
	Day 4	Day 7	Day 11	Day 4	Day 7	Day 11
Th1 (GM-CSF/IL-12/OVA)	44.4	39.8	20.8	31.5	28.0	29.4
Th2 (GM-CSF/OVA)	29.1	11.4	10.9	21.9	32.7	33.8

Mice were sensitized to OVA by daily aerosol exposure in the context of airway expression of GM-CSF and IL-12 (Th1) or GM-CSF alone (Th2). Lungs were pooled from five to 10 mice for each time-point, and mononuclear cells were isolated, stained and analysed by flow cytometry at the indicated time-points. Data shown are from one representative experiment.

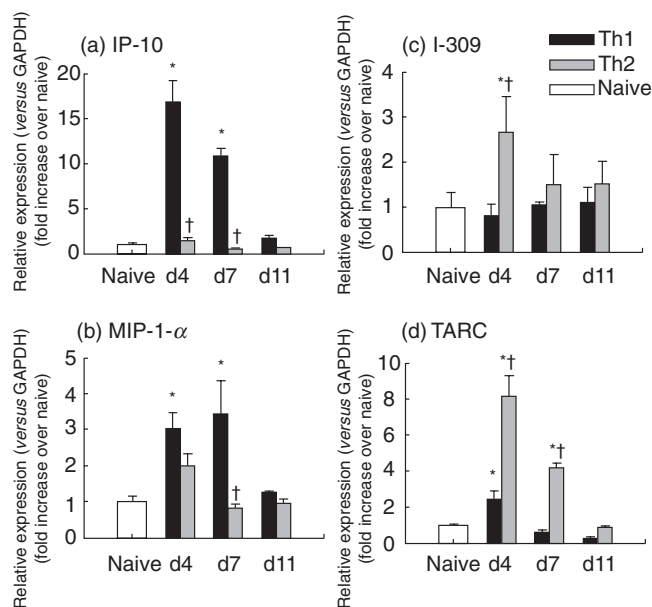


Fig. 4. Expression of the chemokines IP-10, MIP-1- α , I-309 and TARC were measured by real-time quantitative PCR (TaqManTM) in the lymph nodes at various time-points during respiratory sensitization to OVA. Chemokine expression was quantified relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from eight to 10 mice for analysis. Bars are mean $\pm$ s.d. of triplicate measurements. *Significant difference ($P < 0.05$) from naive mice; †significant difference ($P < 0.05$) from the Th1 model at the same time-point.

(39.8%), returning to baseline levels by day 11 (20.8%). Fewer CD11b⁺ CD11c⁺ lung mononuclear cells expressed B7.1 in the Th2 model, peaking at 29.1% on day 4, and returning to below naive levels on days 7 and 11. B7.2 expression was also up-regulated in the Th1 model, peaking by day 4 (31.5%), and remaining relatively stable to days 7 (28.0%) and 11 (29.4%). In the Th2 model, 21.9% of lung CD11b⁺ CD11c⁺ lung mononuclear cells expressed B7.2 by day 4, increasing to 32.7% on day 7, and remaining high at day 11 (33.8%).

DISCUSSION

Although Th1 and Th2 cells were defined originally solely by their cytokine production profile [1], recent work has identified a variety of other genes that are expressed differentially between the two Th subtypes [2–5]. In the present series of experiments we have used models of Th1- and Th2-polarized airway inflammation to examine whether the expression of Th1- and Th2-associated genes can be observed *in vivo* in the thoracic lymph nodes during the time of T cell activation and differentiation. Use of these models in parallel gave us the opportunity to make direct comparisons between equivalent time-points during *in vivo* Th1- and Th2-polarized sensitization to the same antigen, something that has not been possible using other approaches.

For this study, we elected to analyse mRNA derived from whole lymph nodes rather than to use purified populations of cells; this approach has both apparent advantages and limitations. The first major advantage of this approach is that we do not introduce any potential for experimental artefact due to *ex vivo* stimulation of cells during any sorting procedure, nor during an *in vitro* culture step. Because the lymph nodes were removed and placed immediately in RNALater, with no other manipulation, we can be confident that TaqManTM measurements represented precisely the status of gene expression *in vivo* at each time-point. This cannot be said of any previously published literature that we are aware of comparing gene expression during Th1 and Th2 polarization, which have all used *ex vivo* sorting or *in vitro* culture steps. However, the concomitant limitation is that we are unable to ascribe observed changes in gene expression to a particular cell type; thus we have avoided describing our data in terms of Th1 or Th2 cells but rather speak about Th1- or Th2-polarized responses. The second advantage is that, given the absence of reliable commercially available antibodies against a number of these proteins, real-time quantitative PCR is a very powerful, sensitive and specific technique for measuring gene expression. The corollary is that by examining mRNA expression, we cannot be sure that the changes we observed are reflected by a change in protein expression or biological activity. However, we believe that it is reasonable to presume that changes in gene expression are reflected at least to some degree in protein expression; for example, the changes we observed in IFN- γ and IL-4 mRNA expression (Fig. 3) correspond very well with our previously published enzyme-linked immunosorbent assay (ELISA) data in BAL, as well as with divergent cellular responses in the lung [7,8].

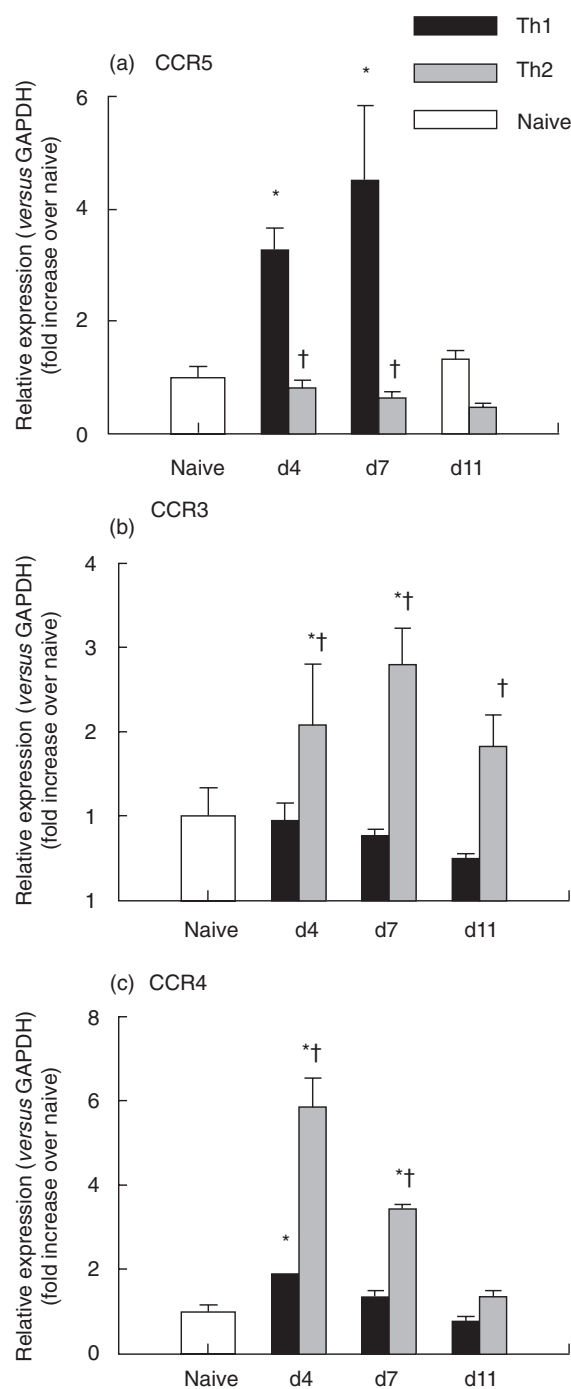


Fig. 5. Expression of the chemokine receptors CCR5, CCR3 and CCR4 were measured by real-time quantitative PCR (TaqMan™) in the lymph nodes at various time-points during respiratory sensitization to OVA. Chemokine receptor expression was quantified relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from eight to 10 mice for analysis. Bars are mean $\pm$ s.d. of triplicate measurements. *Significant difference ($P < 0.05$) from naive mice; †significant difference ($P < 0.05$) from the Th1 model at the same time-point.

Based on our previous work, we contend that airway expression of GM-CSF during exposure to aerosolized OVA results in a Th2-polarized immune response [7], whereas concurrent overexpression of IL-12 and GM-CSF during OVA exposure directs the response to the Th1 end of the spectrum [8]. TaqMan analysis of IL-4 and IFN- γ gene expression in the thoracic lymph nodes (Fig. 3) further corroborates that these are bona fide models of Th1- and Th2-polarized immune responses. Whereas our previous work documented that these cytokines were differentially present in the BAL fluid, the data presented here indicate that the effector programme is acquired in the thoracic lymph nodes during the time of T cell activation and differentiation, confirming that the differences seen in the BAL were not simply attributable to differential recruitment of T cells, but rather reflect the emergence of distinct populations in the lymph nodes. This assertion is corroborated further by the presence of T1/ST2⁺ T helper cells observed in the Th2 model [15], but not in the Th1 model (B. U. Gajewska unpublished data).

Transcription factors, cytokines, chemokines and chemokine receptors were all expressed differentially *in vivo* in the lymph nodes during Th1- or Th2-polarized immunological sensitization to OVA, in a manner consistent with that previously described in *in vitro* studies of CD4⁺ cells [3]. Such a divergence in gene expression in the lymph nodes probably has important functional consequences for the subsequent development of immune-inflammatory responses in the target organ. To summarize, in the present study Th1-polarized responses were associated with increased expression of t-bet, IFN- γ , IP-10, MIP-1-alpha and CCR5, whereas GATA-3, IL-4, I-309, TARC, CCR3 and CCR4 were expressed more prevalently during Th2-polarized responses.

The transcription factors GATA-3 and t-bet have been described as 'master switches' in the development of Th1 and Th2 cells, as they transactivate expression of key Th1 and Th2 genes, including the prototypic cytokines IFN- γ (t-bet) and IL-5 and IL-13 (GATA-3). As reviewed in [11], expression of t-bet or GATA-3 determines not only Th differentiation, but can actually override the influence of exogenous polarizing stimuli or previous polarization. Hence, the differential expression of t-bet or GATA-3 we observed in the lymph nodes in Th1- and Th2-polarized responses probably represents a critical step in the development of these responses. If differences in the levels of t-bet and GATA-3 are indeed responsible for Th1 or Th2 lineage commitment [10,13,16–20], these may be attractive therapeutic targets in Th1- or Th2-mediated immunopathological processes such as autoimmunity and allergy [9,21].

Disparate production of cytokines is the defining feature of Th subsets. The present analysis demonstrates differential mRNA expression of the prototypical Th1 and Th2 cytokines, IFN- γ and IL-4, respectively, as expected. We have shown previously that protein expression is also different in these models, examining BAL fluid and splenocyte culture supernatants, and observing predominantly IFN- γ in the Th1 model, and IL-4, IL-5 and IL-13 in the Th2 model [7,8]. These observations are not incidental, but rather reflect the primary role of Th cells in determining the nature of immune responses through their production of cytokines, which orchestrate adaptive immune responses through numerous pathways: regulating the development of leucocytes in the bone marrow; influencing the differentiation and effector function of other T cells and natural killer (NK) cells; signalling for isotype switching in B cells; and regulating the expression of other genes [22–26].

It is well established that cells of the myeloid lineage have different patterns of chemokine responsiveness due to disparate chemokine receptor expression; for example, neutrophils tend to express chemokine receptors which bind to the CXC family of ligands, whereas eosinophils express a pattern of receptors which bind CC chemokines. Similarly, chemokine receptors are expressed differentially on Th1 and Th2 cells [4,5]; here we show that this pattern holds true *in vivo* during Th1- and Th2-polarized inflammation. Such biased expression of chemokines and chemokine receptors allows for the preferential attraction and retention of particular Th subsets at sites of inflammation, thereby modifying the nature of the inflammatory process. This has been exemplified in studies showing that overexpression of Th1-associated chemokines can alter the inflammatory response in an otherwise Th2-driving milieu [27], or where chemokine or chemokine receptor knock-out animals generate aberrant inflammatory processes in the target organ [28–34].

These experiments also demonstrate that molecular events taking place in the lymph nodes can be affected substantially by cytokine expression occurring at distant sites. As shown in Fig. 1, intranasal instillation of an adenoviral vector encoding a completely exogenous protein resulted in robust expression of the transgene in lung tissue, but there was no detectable transgene present in the draining thoracic lymph nodes, even after 40 cycles of PCR amplification. This suggests that the impact of GM-CSF and IL-12 overexpression in the airways on the molecular events taking place in the thoracic lymph nodes is not due to the expression of these cytokines locally in the lymph nodes themselves. Table 2 shows that the expression of these cytokines in the airway mucosa conditions the antigen-presenting cells (APC) residing there, associated with different patterns of expression of the co-stimulatory molecules B7.1 and B7.2; we do not presume that the observed differences in B7 expression are solely responsible for the divergent immune responses that emerge, but are rather a 'proof-of-principle' that the local cytokine milieu can substantially alter the phenotype of APCs. Upon migration to the draining lymph nodes, we speculate that these differently conditioned APCs can then present antigen to naive T cells along with an appropriate complement of signals (including B7 molecules, other co-stimulatory molecules and cytokines) which elicit Th1 or Th2 differentiation [35–37]. Our data indicate that in the absence of other modulating factors, GM-CSF alone conditions airway APCs to present antigen in the lymph nodes in a Th2-privileging fashion; if IL-12 is additionally present in the lung, these APCs promote Th1 differentiation instead. Our analysis of molecular events in the lymph nodes demonstrates clearly that the impact of GM-CSF and/or IL-12 expression in the airway microenvironment is not merely the result of local effects on leucocyte recruitment to the airways, but rather that it alters fundamentally the very character of the adaptive immune response as it develops in the thoracic lymph nodes.

This finding has important implications for our understanding of the initiation of airways disease and approaches to its treatment. Even if cytokine expression is localized wholly within the lung, this can still have a profound impact on immunodifferentiation events taking place at a distant site, such as the lymph nodes. Therefore, exposure to agents which stimulate airway epithelial cells or alveolar macrophages to produce cytokines could facilitate immunological sensitization through the effects on lung APCs, as we have proposed previously [38]. For example, agents which can induce GM-CSF in the airway, such as various allergens

[39,40] and pollutants [41,42], could thereby facilitate Th2-polarized allergic sensitization. Additionally, these data suggest that we may be able to target immunotherapeutic strategies locally to the target organ, and affect not only local events in that organ, but fundamentally affect the systemic response to an antigen without administering the therapy systemically.

To conclude, our study demonstrates that gene expression in the thoracic lymph nodes during immunological sensitization is fundamentally different in *in vivo* Th1- and Th2-polarized models of airway inflammation. Using these models, we were able to directly compare gene expression between Th1 and Th2 models *in vivo* during the primary immune response. Our data support previous findings showing that IP-10, MIP-1- α , CCR5 and t-bet, in addition to IFN- γ , are produced preferentially during Th1-skewed immune responses, whereas Th2-polarized immune responses are characterized by the expression of I-309, TARC, CCR3, CCR4 and GATA-3, in addition to IL-4 and other Th2 cytokines. Importantly, such divergent profiles of gene expression in the lymph nodes were driven by cytokine expression at a distant site, without evidence of local transgene expression within the lymph nodes themselves. These data enhance our understanding of molecular events during Th1- and Th2-polarization *in vivo*, and suggest that cytokine expression distant from the sites of T cell activation can have a major impact on the nature of the immune responses that develop subsequently.

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