

Reciprocal patterns of allergen-induced GATA-3 expression in peripheral blood mononuclear cells from atopics vs. non-atopics

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Summary

Background T helper (Th)2 cytokines are considered to play a central role in the induction and expression of allergic disease. However, the relative importance of individual cytokines is unclear, and overall disease pathogenesis appears to involve the coordinate activities of a range of Th2 cytokines acting in sequence or in parallel. The present study examines an alternative approach to the study of cytokine gene function in atopy, focusing instead upon T cell transcription factors (TFs) which play a role in the regulation of multiple cytokine genes.

Objective To investigate the allergen-induced expression of the TF GATA-3 and c-Maf in peripheral blood mononuclear cells (PBMCs) and in cytokine-driven Th polarization.

Methods PBMC from house dust mite (HDM)-atopic and non-atopics were stimulated *in vitro* with allergen or anti-CD3/IL-2. TF expression was analysed by semiquantitative RT-PCR and major findings were validated by real-time PCR. Cell separations were performed to analyse the contribution of CD45RO⁺ cells. CD4⁺ cord blood cells were Th1 or Th2 polarized *in vitro* by exogenous cytokines and TF expression analysed by Northern blot and real-time PCR.

Results We demonstrate for the first time that during differentiation of CD4⁺ CD45RA⁺ naïve human T cells towards Th2 commitment, and during allergen-specific reactivation of peripheral CD4⁺ CD45RO⁺ Th2 memory cells in established atopics, expression of the Th2-associated TF GATA-3 is rapidly up-regulated, whereas T cells from non-atopics display equally rapid GATA-3 down-regulation under identical conditions of allergen stimulation.

Conclusion These findings identify Th2-associated TFs as key determinants of the atopic phenotype, suggesting their unique potential as therapeutic targets for disease control.

Keywords atopy, Th1/ Th2, transcription factors, GATA-3, c-Maf, allergen

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Introduction

The application of the murine T helper (Th)1/Th2 paradigm [1] to the study of the immunopathogenesis of human allergy [2,3] has provided valuable insight into the role of different cytokines in the disease process. In addition, it offers a plausible theoretical alternative to classical deletional tolerance for control of potentially pathogenic allergen-specific T cells, i.e. via cross-regulation between mutually antagonistic Th cell subsets, a mechanism which is broadly consistent with findings indicative of virtually universal T cell responsiveness to ubiquitous allergens throughout the adult population [4,5]. Furthermore, it provides a framework for the rational design of immunotherapeutic strategies based on immune deviation of pathogenic allergen-specific Th2 responses via selective stimulation of antagonistic Th1 immunity [6,7].

While the central importance of Th2 cytokines in allergic disease is widely accepted, the role of Th1 cytokines is less clear [8]. For example, biopsy studies at the sites of lesional skin in atopic dermatitis indicate the presence of high numbers of interferon (IFN)- γ -producing cells, prompting suggestions that Th1 cells may in some circumstances contribute to disease pathogenesis [9]. Secondly, analysis of short-term peripheral blood mononuclear cell (PBMC) responses to allergens employing highly sensitive assay systems (e.g [10–12]), indicate a heterogeneous Th0-like pattern of cytokine production in many atopics (e.g [10–12]), as opposed to the classical Th2 pattern suggested in earlier cloning studies, suggesting that *in vitro* T cell response patterns may in some circumstances become distorted during the prolonged *in vitro* culture [13]. There is additionally no evidence of delayed type hypersensitivity to allergens in non-atopics, suggesting that their response patterns do not equate directly to classical Th1 immunity [8].

In the present study, we have adopted an alternative approach to these and related issues which minimizes the possibility of *ex vivo* differentiation of responder T cells during analysis, and which does not rely on the simultaneous measurement of a

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wide range of cytokines. We have focused instead upon expression of two transcription factors (TFs) which have previously been demonstrated to play a role in the differentiation of Th2 cells in murine systems, notably the proto-oncogene c-Maf and the zinc finger DNA binding protein GATA-3 [14–17]. We provide evidence for the selective up-regulation of GATA-3 expression during the cytokine-directed differentiation of naïve cord blood cells into Th2 cells, and in PBMC from atopic adults during *in vitro* allergen-specific recall responses. This indicates a potentially important role for this TF in the induction and expression of the allergic phenotype in humans, a suggestion consistent with the recent demonstration of increased GATA expression in bronchial biopsy samples from atopic asthmatics [18,19]. We also demonstrate active allergen-induced down-regulation of GATA-3 expression in PBMC from immunoglobulin (Ig)E antibody-negative non-atopics, similar to that seen in committed murine Th1 cells, suggesting, independent of IFN- γ measurement, that a form of Th1-related immunity underlies the normal 'allergen non-responder' state in non-atopic humans.

Materials and methods

Subjects

Atopic adult volunteers ($n=9$, four males, five females) were selected on the basis of positive serum IgE responses to house dust mite (HDM), together with controls ($n=12$, five males, seven females) who were tested for the presence of HDM-specific IgE in serum and were all negative. The presence of IgE to HDM was defined by the RAST (CAP) system (Pharmacia, Sydney, Australia) and the positive volunteers in this study displayed RAST (CAP) scores ≥ 2 . Total IgE levels (mean $\pm$ SEM) were 130 ± 34 kU/L (range 6–252 kU/L) for the IgE-positive group and 39 ± 8 kU/L (range 11–77 kU/L) for the IgE-negative group. Symptomatology within the HDM-IgE⁺ group was restricted to rhinitis (in particular no subjects with asthma were included). The work was approved by the Princess Margaret Hospital Human Ethics Committee.

Cell Culture

Freshly isolated PBMCs were resuspended at 1×10^6 cells/mL and 1 mL of the cell suspension was cultured for 24 h at 37 °C, 5% CO₂ in round bottom tubes in serum free medium AIM-V [4] (Life Technologies, Mulgrave, Australia) supplemented with 4×10^{-5} 2-Mercaptoethanol with or without the addition of 10 μ g/mL of whole extract of HDM (*Dermatophagoides pteronyssinus*, CSL Limited, Parkville, Australia) or with soluble anti-CD3 (1:100 culture supernatant from OKT3 cell line, ATCC, Manassas, VA, USA) and 20 U/mL recombinant human interleukin (IL)-2. After culture, the cells were centrifuged, the supernatants were collected and stored at –20 °C for future analysis of the cytokine content, and the cell pellet was used immediately for total RNA extraction.

CDNA synthesis

cDNA was synthesized from total RNA using 250 ng oligo dT (Biotech International, Bentley, Australia) and 2.5 U of avian myeloblastosis virus reverse transcriptase (AMV, Promega, Madison, WI, USA) in the presence of 12.5 U ribonuclease inhibitor (Applied Biosystems, Scoresby, Victoria, Australia),

in a final volume of 25 μ L [12]. Oligo dT was annealed to the RNA template by heating to 70 °C for 5 min. The cDNA synthesis was performed at 42 °C for 60 min. cDNA was synthesized from 1 μ g total RNA from polarized cell lines using 1.25 μ M (final concentration) random hexamers (Applied Biosystems) and 31 U of MultiScribe Reverse Transcriptase (Applied Biosystems), in the presence of 12.5 U RNase inhibitor, in a total volume of 25 μ L.

Semi-quantitative RT-PCR

Details of generation and detection of PCR products are as previously described [12,20]. The GATA-3 primer sequence employed, which generated a 454-bp product, was 5' GAC GAG AAA GAG TGC CTC AAG 3' and 5' TCC AGA GTG TGG TTG TGG TG 3'. C-Maf primers generated a 479-bp product: 5' ACC TTC CAC AAT CAA GCC 3' and 5' GTA ACC CAT TCT GGT ATC TTT G 3'. Annealing temperature for both GATA-3 and c-Maf primers was 57 °C and the samples were amplified for 30 cycles. For determination of β -actin levels the samples were amplified for 25 cycles. Primers were supplied by Geneworks (Adelaide, South Australia, Australia).

The cycle numbers for GATA-3, c-Maf and β -actin were determined by extensive PCR analysis to ensure that we had reached detection levels and that the reaction remained in the linear phase. Subset of samples were also routinely tested by analysis on a 1.5% agarose gel and staining with ethidium bromide to ascertain that PCR products of the expected size were obtained.

The PCR products were detected by slot-blot, employing biotinylated probes for B-actin, GATA-3 and c-Maf which were synthesized by PCR, as described previously [12,20], using the same primers as above. The results are expressed as index of TF/ β -actin.

Real-time PCR analyses using TaqMan

After we have completed the analyses of the samples using the standard RT-PCR/slot-blot procedure, PCR analysis by the real-time method (TaqMan, Applied Biosystems, Foster City, CA, USA) became available at our laboratory. We accordingly proceeded to test cDNA from subjects' samples and polarized cell lines from which cDNA was still available, for confirmation of our previous PCR results.

Standards

18S rRNA, GATA-3 and IL-12RB2 DNA standards were generated by PCR amplification of cDNA obtained from phytohaemagglutinin (PHA)-stimulated (24 h) PBMCs, using the same primers as for the real-time reaction (Table 1). PCR products were cloned into the vector pCR 2.1 using the original TA Cloning kit (Invitrogen, Carlsbad, CA, USA), and *E. coli* competent cells (Invitrogen) were transformed with such vectors. The sequence identity of each DNA insert was confirmed by cycle sequencing. Serial 10-fold dilutions from each purified plasmid preparations were used as standards. β -actin standard was a serial 1:2 dilution of PHA-stimulated cDNA. The linear range of the PCR for 18S rRNA, GATA-3 and IL-12RB2 was from one copy to 10^8 copies of plasmid, and for the β -actin, from neat to 1:128 dilution. For β -actin reactions the correlation coefficients were 0.98 and 0.99. For GATA-3 it was between 0.93 and 0.98. For IL-12RB2 it was 0.81 and for 18S rRNA, 0.98.

Table 1. Primers and probe sequences used for real-time PCR

Target	Oligonucleotide	Sequence	Final concentration	Product size
β -actin*	Sense	5' ATT GCC GAC AGG ATG CAG AA 3'	900 nm	150 bp
	Anti-sense	5' GCT GAT CCA CAT CTG CTG GAA 3'	900 nm	
	Probe	5' VIC-CAA GAT CAT TGC TCC TCC TGA GCG CA TAMRA 3'	250 nm	
GATA-3	Sense	5' GGA GGT GGA TGT GCT TTT TAA CA 3'	300 nm	117 bp
	Anti-sense	5' ACC TGG CTC CCG TGG TG 3'	300 nm	
	Probe	5' FAM- ACG GTC AAG GCA ACC ACG TCCC C TAMRA 3'	250 nm	
IL-12RB2†	Sense	5' ACA TTC TTG GAC ATA GTG AGG CC 3'	300 nm	80 bp
	Anti-sense	5' GTA CAT CTG CTC ACG GAA GCC 3'	900 nm	
	Probe	5' FAM-TCC TCC GTG GGA CAT TAG AAT CAA ATT TCA AA TAMRA 3'	250 nm	

*From ref [21]. †Sequence provided by Dr M. Jenmalm.

PCR reaction and quantification

PCR premixes were prepared using Platinum® Quantitative PCR Super Mix-UDG (Life Technologies), MgCl₂ at 5.5 mM (final concentration), and optimized concentration of primers and probe (Table 1). 18S rRNA was used as a commercially available premix at 1:20 final dilution (Applied Biosystems). Standard TaqMan conditions were used, except for 2 min instead of 10 min for Taq polymerase activation.

For each assay the baseline was determined manually, and the threshold cycle (Ct) for each well was compared to the standard curve of the standards serial dilutions. The ratio between the relative amount of target genes and housekeeping (18S rRNA for cell lines and β -actin for subject's samples) was calculated and the resulting figures expressed as 'Taqman units'.

Primers and probes

GATA-3 RT-PCR primers and dual-labelled fluorogenic probe were designed using the program Primer Express (Applied Biosystems). The primer pair was designed to span a exon/intron boundary, and amplification of RNA samples not reverse transcribed was not observed. Amplification of these samples for 18S rRNA (which amplifies genomic DNA) showed amplification, demonstrating the presence of contaminating genomic DNA. Primers and probes for 18S rRNA were purchased directly from Applied Biosystems and used as per the manufacturer's instructions. Analyses of RNA samples not transcribed showed that the Ct values for these sample were approximately ten cycles lower than levels detected in transcribed samples. β -actin was also designed across exon/intron boundaries [21]. IL-12RB2 primers and probe sequences were kindly provided by Dr M. Jenmalm, Linköping, Sweden. Analyses of RNA samples not transcribed showed that Ct values for these samples were around eight cycles lower than transcribed samples. Probes were synthesized by Applied Biosystems (Table 1).

T cell polarization

Enriched CD4⁺ T cells from cryopreserved cord blood were polarized employing a modification of the methods described in [22,23]. Cells were stimulated via culture in 2 μ g/mL PHA plus recombinant IL-2 (20 U/mL) in the presence of recombinant IL-12 (Hoffman La Roche, Nutley, NJ, USA) and 1 μ g/mL anti-IL-4 (clone MP4-25D2, Pharmingen, North Ryde, Australia) for Th1 polarization, or in 10 ng/mL recombinant IL-4

(Pharmingen) and plus two anti-IL-12 antibodies for Th2 polarization (20C2, Hoffman La Roche, and C8.6, Genzyme, Cambridge, MA, USA). After 3 days in culture, cells were collected and expanded for 3 more days in RPMI 10% FCS and 100 U/mL IL-2. After this period, 2×10^4 cells were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, Castle Hill, NSW, Australia) and 400 ng/mL Ionomycin (Sigma) for 24 h in RPMI + 10% FCS. Supernatants were collected and stored at -20 °C for cytokine analysis. The remaining cells were submitted to a second cycle of stimulation. FACS analysis was performed at the end of the second cycle (Day 12) at which stage CD3⁺ CD4⁺ cells contributed 92–93% of the culture.

Northern blot analysis

Total RNA from cells undergoing polarization was obtained using TRIZOL (Life Technologies), following manufacturer's recommendations. 5–10 μ g of total RNA was loaded in a 1.2% Formaldehyde agarose gel and transferred to a Nylon membrane (Amersham, Castle Hill, NSW, Australia), and the RNA was ultra-violet cross-linked. Membranes were incubated with Ultrahyb™ (Ambion, Austin, TX, USA), following manufacturer's instructions. cDNA probes were generated by random priming using High Prime (Boehringer Mannheim, Castle Hill, NSW, Australia) and [α ³²P] CTP.

PBMC fractionation

Freshly isolated PBMC from a subgroup of the HDM-IgE⁺ and HDM-IgE⁻ study subjects were enriched for CD45RO⁺ cells by repeated cycles of depletion via Dynabeads (Dynal, Oslo, Norway) conjugated with the CD45RA-specific antibody 2H4-D1 (Coulter, Miami, FL, USA). CD45RA contamination after two or three cycles of depletion was $\leq 2\%$; the fractionated population was 89–97% CD3⁺ and 83–97% CD45RO⁺. In parallel experiments, CD4-enriched and CD4-depleted post-stimulated PBMC were prepared employing antibody-conjugated Dynabeads used as per manufacturer's instructions. CD8⁺ contamination in the CD4-enriched populations was 0.2–0.3%, while the CD4-depleted populations contained up to 5% CD4⁺ T cells.

ELISA

IL-4, IL-13 and IFN- γ protein (for antigen-stimulated supernatants) was measured in supernatants with commercial kits (CLB, Amsterdam, the Netherlands). IL-5 and IL-10 proteins

were measured using commercially available antibodies pairs (Pharmingen) as described previously [12].

Statistical analysis

Paired responses were analysed using Wilcoxon matched-pairs signed rank test. Comparisons between unpaired groups were analysed with Mann-Whitney *U*-test. Categorical variables were analysed by Fisher's exact test. The statistical package StatView 4.5.1 for Macintosh was used (Abacus Concepts, Berkeley, CA, USA).

Results

GATA-3 expression in adult PBMC

PCR analysis of cDNA obtained from PBMC cultures (Fig. 1a; upper panel) demonstrated up-regulation of GATA-3 mRNA expression following HDM stimulation in eight of the nine

HDM-IgE⁺ individuals tested (stimulated > background; $P=0.038$). In contrast, nine of the 12 HDM-IgE⁻ subjects displayed frank down-regulation of GATA-3 expression under identical conditions of challenge (stimulated < background; $P=0.0076$). This reciprocal pattern is most clearly seen in Fig. 1(b) where respective differences between background and stimulated expression levels in the two groups are expressed as a mean delta value.

It is also of interest to note that in the unstimulated (control) state, mean GATA-3 expression levels were significantly lower in the atopic group than in non-atopics ($P<0.0045$) but as a result of reciprocal changes in expression levels following allergen challenge, this pattern was reversed in the 24 h cultures (atopic > non-atopic; $P<0.019$).

Where PBMCs from the same subjects were stimulated in the presence of soluble anti-CD3 and IL-2 (Fig. 1a; lower panel, and Fig. 1b), differences were again evident, but these were less clear cut than those observed in allergen-stimulated cultures.

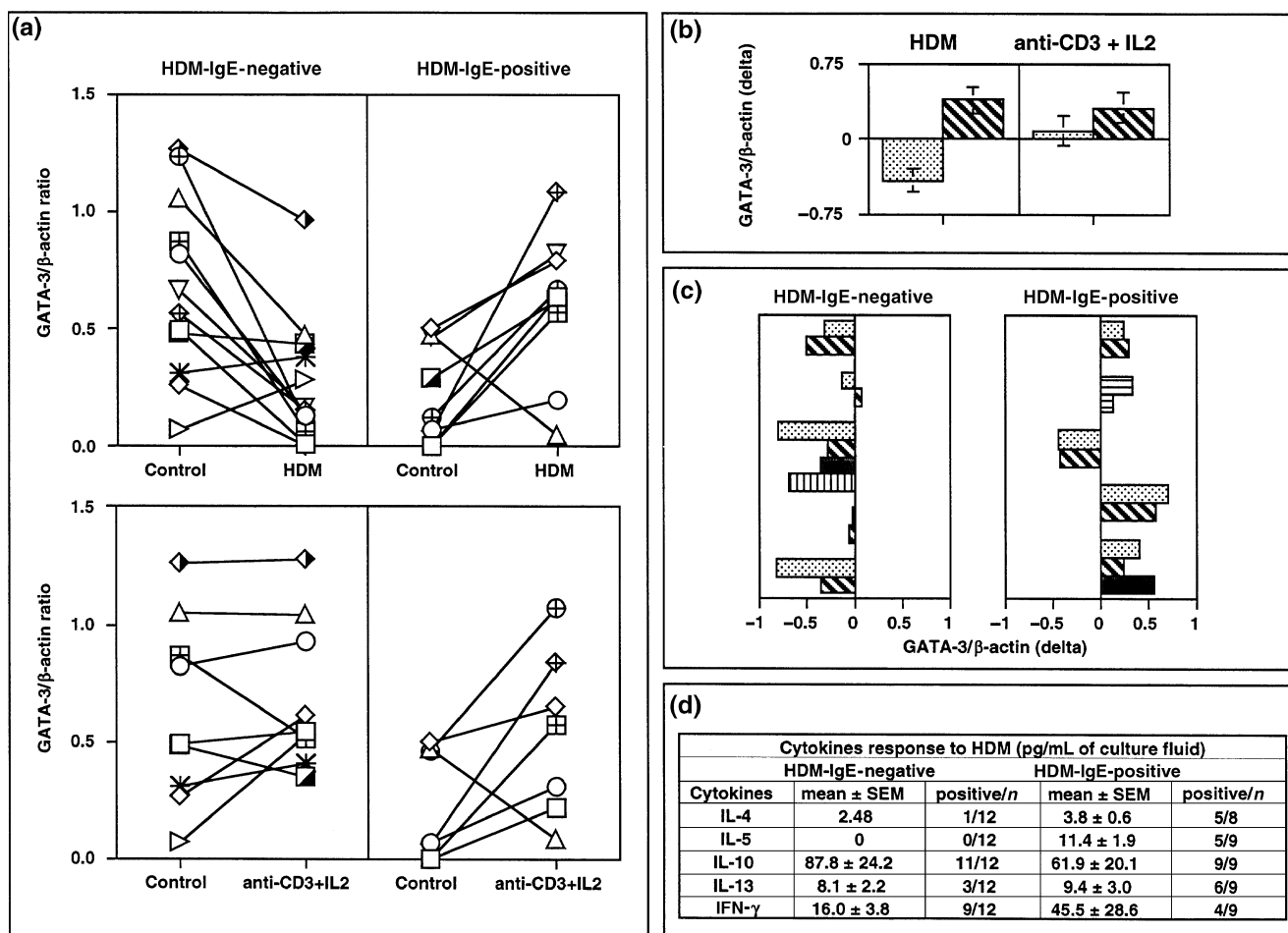


Fig. 1. Analysis of GATA-3 expression and cytokine production in PBMC following allergen-specific or polyclonal T cell stimulation. (a) PBMC from HDM-IgE-negative ($n=12$) and positive ($n=9$) individuals were cultured for 24 h in medium alone (control) or medium supplemented with allergen (HDM, 10 µg/mL) or with soluble anti-CD3 and recombinant IL-2. GATA-3 expression was analysed by RT-PCR and normalised against β-actin levels. (b) Differences (Δ) between the stimulated and control levels for each subject in (a) are represented as mean ± SEM for HDM-IgE negative (dotted) and positive (hatched) groups. Positive Δ values indicate up-regulation of GATA-3 expression and negative values indicate down-regulation. (c) Repeated testing of five HDM-IgE negative and five HDM-IgE positive individuals; data are expressed as the difference between allergen-stimulated and control levels of GATA-3 as per (b). (first culture, dotted; second culture, hatched; third culture, filled; fourth culture, striped). (d) Cytokine production in response to HDM stimulation in cultures employed in (a) was assayed by ELISA. Shown in the table are mean ± SEM of values above background controls. Positive represents the number of individuals who showed detectable production of a given cytokine in response to HDM stimulation. The lower limits of detection for individual cytokines were: IL-4, 5 pg/mL; IL-5, 6 pg/mL; IL-10, 6 pg/mL; IL-13, 3 pg/mL; IFN-γ, 5 pg/mL.

However, it is noteworthy that six out of seven atopic subjects displayed up-regulation of GATA-3 ($P < 0.04$ by Fisher's exact test), whereas expression levels remained unchanged or declined in seven out of nine non-atopic subjects. Parallel stimulation of cultures from these subjects with Con A or PHA (data not shown) resulted in heterogeneous patterns of GATA-3 expression which were similar in both groups.

In ten individual subjects we repeated the HDM stimulation on up to four occasions (Fig. 1c). In the atopic subjects the same pattern of change was observed in all five cases, including in the only subject that down-regulated GATA-3 after HDM stimulation. Response patterns were also consistent in the cultures from non-atopic subjects.

Analysis by real time PCR

Analyses of cDNAs from five of the HDM-IgE⁺ and four of the HDM-IgE⁻ subjects from Fig. 1(a) by real-time PCR showed a similar pattern to the results obtained with slot-blot, i.e. increased expression of GATA-3 after HDM stimulation in the HDM-IgE⁺ group and decrease in the HDM-IgE⁻ group (Table 2). It is not feasible to make direct correlations between values obtained through the slot-blot experiment and the ones using real-time PCR, due to the more extensive linear range of the Taqman method. Nevertheless, the trend observed with the two methods are very similar. It is noteworthy that the 'outlier' HDM-IgE⁺ subject who showed down-regulation to HDM in the slot-blot (positive subject 5 in Table 2) gave qualitatively similar results using the TaqMan. Replicate samples (cultured one year apart) from one subject (negative subjects 1 and 1a in Table 2) also gave very similar results.

GATA-3 expression vs. cytokine production

Production of cytokines in response to HDM stimulation was assessed by ELISA in the cultures employed in Fig. 1(a), and is shown in Fig. 1(d). Employing the different Th2 cytokines the frequency of positive responses detected in the atopic group was, respectively, IL-4 (5/8, one individual not tested), IL-5 (5/9) and IL-13 (6/9). In contrast, in PBMC cultures from HDM-IgE⁻ individuals, no IL-5 production in response to HDM was detected. One subject showed production of IL-4 (confirmed in a separate culture) and three individuals in this group showed production of IL-13 in response to HDM. In relation to

production of IL-10 and IFN- γ , no differences between the groups were observed. Production of cytokines in response to anti-CD3 and IL-2 showed that all subjects in both groups produced IL-4, IL-5, IL-10, IL-13 and IFN- γ in equivalent levels (not shown).

Cellular source of GATA-3 expression

GATA-3 expression was assessed in allergen-stimulated and mitogen-stimulated cultures of PBMC from atopics and non-atopics before and after depletion of CD45RA⁺ cells; CD45RO-enriched cultures comprised 89–97% CD3⁺ T cells. Representative results are shown in Fig. 2, which demonstrate that the qualitative differences in allergen-specific patterns of GATA-3 expression between atopics and non-atopics remain after removal of naïve T cells, and in the case of the atopic subgroup, the degree of GATA-3 up-regulation was consistently higher than that observed with unfractionated PBMC. In other experiments (not shown), positive selection of CD4⁺ cells from post-stimulated cultures in both groups, followed by RNA extraction of both CD4-enriched and CD4-depleted populations and subsequent RT-PCR analysis of GATA-3 expression, indicated that GATA-3 expression was restricted to the CD4-enriched population. Collectively, these results suggest that the principal cells responding to specific allergen via GATA-3 modulation express the CD4⁺ Th memory phenotype.

C-Maf expression in PBMC

In other experiments we analysed the parallel expression of c-Maf in HDM-stimulated cultures. Although the HDM-IgE⁺ group displayed more frequent up-regulation of c-Maf expression in response to allergen stimulation, there were no statistically significant differences between these subjects and the non-atopic group (Fig. 3).

Analysis of GATA-3, c-Maf and IL-12 receptors (IL-12R) in polarized cell lines

T cell lines were derived from cord blood propagated under standardized conditions for Th1 or Th2 polarization. Cytokine profiles were analysed after the first and second round of polarization, and a representative example is shown in Fig. 4(a). Cells polarized in the presence of IL-4 produced more IL-4, IL-5, IL-10 and IL-13 than cells polarized in the

Table 2. HDM-specific GATA-3 mRNA levels using slot-blot and real-time PCR (TaqMan)

HDM-IgE	subject	Slot-blot			TaqMan		
		control	HDM	HDM*	control	HDM	HDM*
Negative	1	1.05	0.47	−0.58	105.74	2.01	−103.73
	1a	nt	nt		103.44	21.01	−82.43
	2	0.26	0	−0.26	22.21	0	−22.21
	3	0.87	0.06	−0.81	37.04	0	−37.04
	4	0.51	0.53	0.02	26.25	27.67	−1.42
Positive	1	0	0.57	0.57	0	31.29	31.29
	2	0.46	0.82	0.35	0	368.8	368.8
	3	0.06	1.08	1.02	0	80.73	80.73
	4	0.29	0.62	0.33	21.33	591.58	570.25
	5	0.47	0.05	−0.42	17.19	0	−17.19

*delta (the difference between GATA-3 stimulated and unstimulated levels); nt: not tested. The results are expressed as GATA-3/ β -actin ratio.

presence of IL-12, however, the Th1-like line still produced detectable levels of IL-4, IL-10 and IL-13 at the end of the culture period.

Production of IL-5, in contrast, was close to the limit of detection after the second cycle of IL-12 polarization. IFN- γ production was observed at high levels in the IL-12 polarized line, and was produced in much lower levels in the IL-4 polarized line.

Northern analysis showed that GATA-3 expression was readily detected from Day 2 until Day 12 in the IL-4 polarized cell line (Fig. 4b). GATA-3 expression was high by Day 3 in cells

cultured in the presence of IL-4. Low levels of GATA-3 were observed sporadically in the IL-12 line, peaking at Day 9, but expression was again low on Day 12. GATA-3 was also expressed at high levels on Days 6 and 12, three days after removal of IL-4 from the culture. Reanalysis of these samples by real-time PCR (Fig. 5a) showed that GATA-3 levels were highest at Day 3, but were detected at significant levels at Days 6 and 12. Interestingly, despite the fact that levels of GATA-3 did not appear to increase after the second round of stimulation, production of IL-4 and IL-5 increased (Fig. 4a). This suggests that maintenance of permissive levels of GATA-3 (as opposed to a new burst of high level production) may be sufficient to support ongoing Th2 cytokine gene expression, once Th2-polarization is complete. We also analysed GATA-3 expression by real-time PCR from Day 0 to Day 3 in two other polarizations (data not shown). Levels of GATA-3 in non-polarized (naïve) cells were low but detectable, but increased over 70 times in the IL-4 line, without any significant change in cells cultured with IL-12.

We analysed the expression of IL-12RB2, reported to be expressed selectively in Th1 cells. Expression of IL-12RB2 was clearly observed by Day 2 in cells polarized in the presence of IL-12, and its expression peaked at Day 9, but declined to undetectable levels at Day 12 (Fig. 4b). Analysis of IL-12RB2 expression by real-time PCR showed a similar pattern (Fig. 5b); expression of IL-12RB2 was observed in cells polarized in the presence of IL-4 at Day 3, declining after that, similar to previous observations [22]. Analysis of two other polarizations (data not shown) from Day 0 showed that levels of IL-12RB2 increased dramatically after 3 days in the presence of IL-12, but declined after the removal of IL-12.

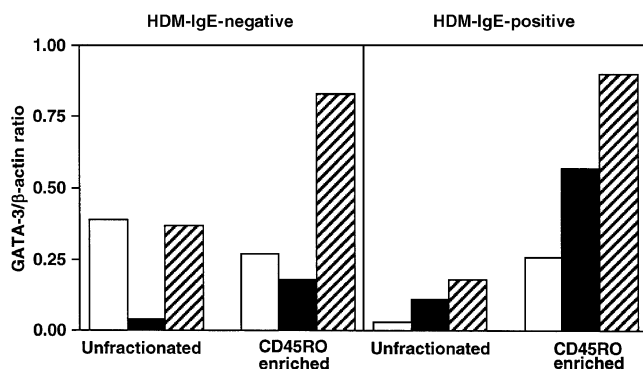


Fig. 2. GATA-3 expression in CD45RO enriched cell populations. Aliquots of unfractionated mononuclear cells were enriched for CD45RA cells. Unfractionated and CD45RO-enriched cells were cultured for 24 h in medium alone (open bars), medium supplemented with HDM (10 μ g/mL, solid bars) or with PHA (1 μ g/mL, hatched bars). GATA-3 expression was analysed by RT-PCR and normalised against β -actin levels. Examples shown are representative of three independent experiments for the HDM-IgE-positive group and two for the HDM-IgE-negative group.

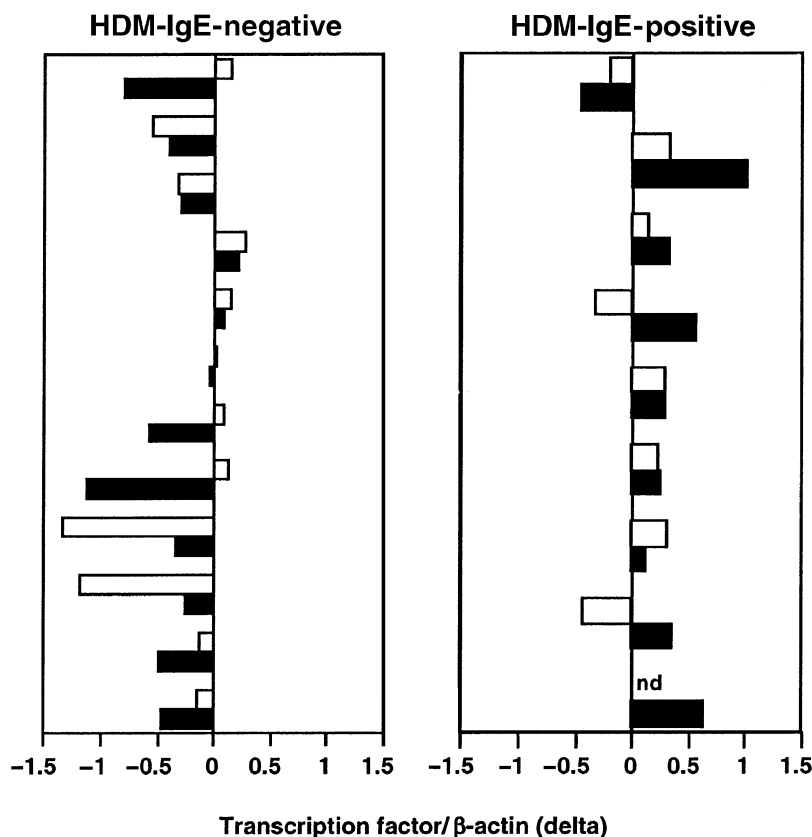


Fig. 3. Comparison of c-Maf and GATA-3 expression following allergen stimulation. PBMC from HDM-IgE-negative ($n = 12$) and positive ($n = 9$) individuals were stimulated *in vitro* for 24 h with allergen (HDM, 10 μ g/mL). c-Maf and GATA-3 expression were analysed by RT-PCR and normalised against β -actin levels and the difference between stimulated and control levels for each subject tested were expressed as Δ values as per Fig. 1(b). Solid bars: GATA-3; open bars: c-Maf).

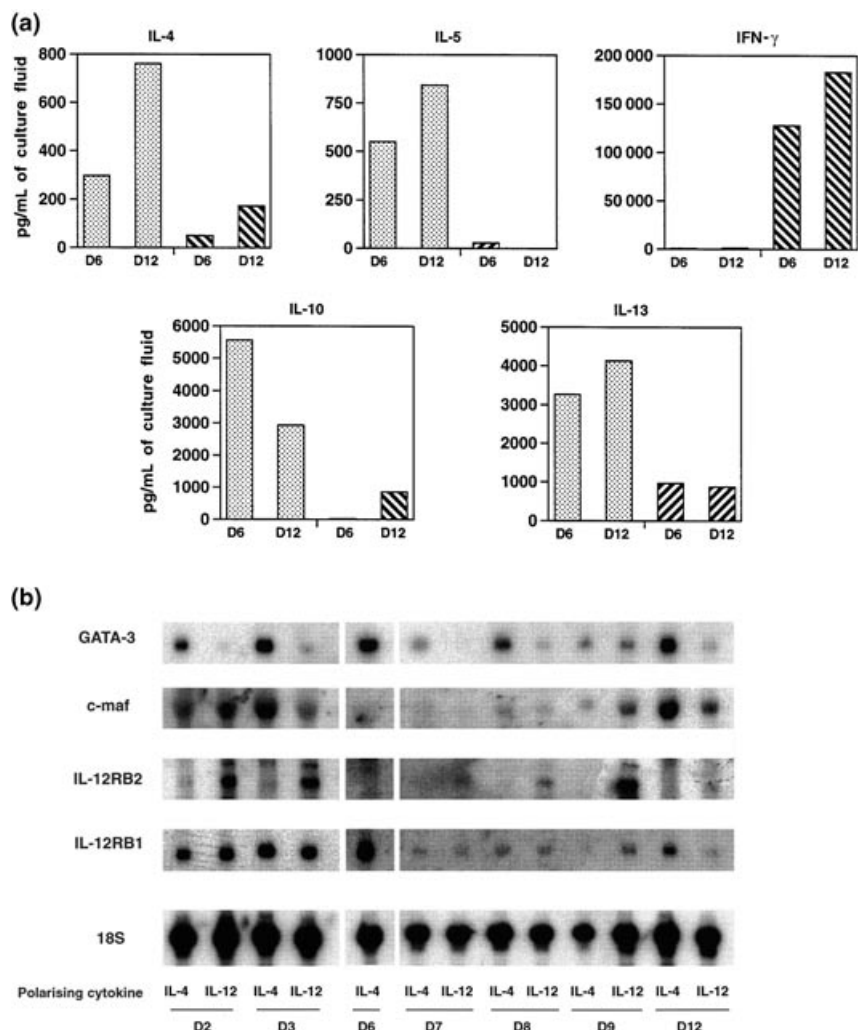


Fig. 4. Expression of GATA-3, C-Maf, IL-12RB1 and IL-12RB2 in T cells undergoing Th1 or Th2 polarization *in vitro*. Cord blood mononuclear cells were polarized *in vitro* by stimulation in the presence of IL-4 (dotted) or IL-12 (hatched) as described in the text. (a) After the first cycle of polarization (Day 6: D6) and again after the second cycle of polarization (Day 12: D12) the cells were restimulated for 24 h with PMA (10 ng/mL) and Ionomycin (400 ng/mL). Cytokine production in culture supernatants was assayed by ELISA. Representative example of three independent polarizations. (b) Total RNA was extracted from cells collected at the specified time-points and analysed by Northern blot.

We also analysed the expression of the transcription factor c-Maf. In contrast to GATA-3, c-Maf expression was detected in similar levels in cells polarized in IL-4 and IL-12 at Day 2. At Day 3, the IL-4 line expressed approximately two times more c-Maf than the IL-12 line, after correction for RNA load. Following 3 days expansion in IL-2, c-Maf was not detected in the IL-4 line, as opposed to GATA-3. c-Maf expression increased slowly until Day 12. By Day 12, c-Maf expression was again around two times higher in the IL-4 line compared to the IL-12 line, after correction for RNA load (not shown).

Discussion

Our results in the present study indicate that a defining feature of the allergen-specific Th2 cytokine response in PBMC from atopics is up-regulation of GATA-3 expression, which occurs concomitantly with production of the cytokines IL-4, IL-5 and IL-13. In contrast, IL-10 secretion was detected in both atopic and non-atopic responses and was thus independent of GATA-3 expression, in agreement with earlier findings indicating production of this cytokine by cloned T memory cells expressing both the Th1 and Th2 phenotypes [24].

The results presented here are in agreement with two recent reports describing increased GATA-3 expression in bronchial biopsies and bronchoalveolar lavage cells from atopic asthmatics [18] and in upper airway mucosa after *in vivo* and *in vitro* allergen challenge [19]. We expand those observations to show that selective increase in GATA-3 gene expression in response to exposure to specific allergen can also be observed in circulating memory T cells, allowing studies of expression of TF in blood samples.

We also observed that unstimulated levels of GATA-3 in non-atopics were higher than in atopics, in contrast to the observation [19] in nasal biopsies. Allergen stimulation in our PBMC samples from non-atopics led to a decrease in GATA-3 expression, whereas in nasal biopsy samples from non-atopics allergen exposure did not exert significant effects [19]. A possible reason for the disparity in GATA-3 expression between non-atopics in these two studies may be the different origins of the cell populations studied. For example, local microenvironmental factors operative in mucosal tissues may modulate the expression of GATA-3 by T cells subsequent to their migration from the peripheral circulation. Nevertheless, in relation to allergen-induced GATA-3 expression in atopics, the nasal biopsy and PBMC samples responded in a similar manner.

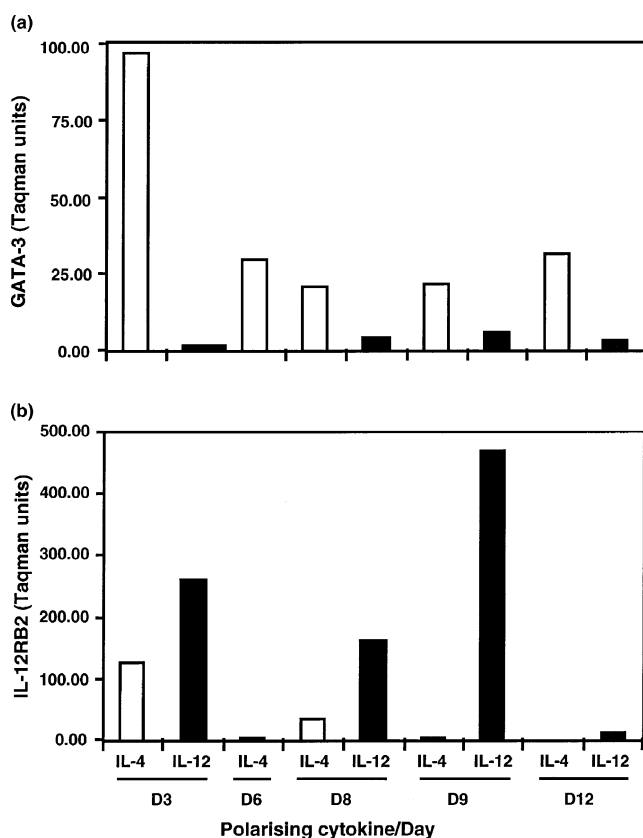


Fig. 5. Expression of GATA-3 (a) and IL-12RB2 (b) analysed by real-time PCR in T cells polarized under Th1 or Th2 conditions. Cord blood mononuclear cells were polarized *in vitro* by stimulation in the presence of IL-4 (open) or IL-12 (filled) as described in the text. Total RNA was extracted from cells collected at the specified time-points, transcribed to cDNA and analysed by real-time PCR (TaqMan) as described in the text. Values for the target genes and the housekeeping gene (18S rRNA) were obtained by comparison with the respective standard curves, and finally expressed as a ratio of target genes to 18S rRNA (TaqMan units).

We additionally demonstrate GATA-3 expression during *in vitro* differentiation of naïve human cord blood T cells towards the Th2 cytokine phenotype, establishing for the first time that GATA-3 expression is associated with Th2 differentiation in human cells. This process may be analogous to that which occurs in genetically susceptible individuals during early infancy, a period in which we have demonstrated that Th2 polarization of aeroallergen-specific T cell responses commonly occurs [25,26]. Collectively, these results suggest that GATA-3 may play an important role in both the afferent and efferent phases of allergic disease.

The mechanism of action of GATA-3 in Th2 responses remains to be determined. GATA-3 has been shown to directly transactivate the murine IL-5 gene [15,27]. However, its role in relation to IL-4 gene regulation is less clear, and transfection studies suggest that GATA-3 is not sufficient to fully induce IL-4 production [28]. One recent suggestion is that GATA-3 may function as a chromatin remodelling factor, making the IL-4/IL-5 locus available [29].

We additionally examined c-Maf expression in these responses, in view of its critical role in regulation of IL-4 expression in murine models [16,17]. While qualitative changes in c-Maf expression (i.e. up-regulation vs. down-regulation) in response to specific allergen did mirror those in GATA-3 in 14 of the 20 subjects tested (Fig. 3), the magnitude of the changes in

c-Maf did not reach statistical significance. This is consistent with recent findings with cell lines which suggest that c-Maf may not play a critical role in the activation of the human IL-4 promoter [30]. Similarly, during *in vitro* T cell polarization, c-Maf gene expression levels were only slightly increased in the Th2 line compared to the Th1 line, in contrast to GATA-3 expression. These findings may indicate the existence of a hierarchy of TFs involved in differentiation and expression of the Th2 phenotype in humans, with GATA-3 playing a more direct determinant role than in the mouse, and TFs such as c-Maf and others exerting additive effects.

Our experiments involving generation of Th1-polarized lines from naïve cord blood cells cultured in the presence of IL-12, resulted in up-regulation of IL-12RB2 expression and IFN- γ secretion, as reported previously in murine and human systems [22,31], and this was inversely associated with GATA-3 expression. Interestingly, maintenance of IL-12RB2 expression appears to depend on the continuous presence of IL-12, while GATA-3 expression is maintained even after the removal of IL-4.

Thus, allergen-induced down-regulation of GATA-3 in the non-atopics appears consistent with underlying Th1-like responsiveness, as predicted by the initial T cell cloning studies in this area. This finding provides supporting evidence for the existence of Th1-like allergen-specific immunity, which is independent of direct measurement of *in vitro* IFN- γ production; moreover, this method facilitates assessment of the cytokine 'polarity' of the T cell response at a very early time-point in culture, minimizing the potentially distorting effects of *in vitro* culture on *in vivo* T cell functions. The puzzle of why this Th1-like responsiveness in non-atopics is not associated with *in vivo* manifestations of delayed type hypersensitivity (DTH) remains to be solved. It is likely that some form of negative feedback within the overall response of the non-atopics, such as concomitant IL-10 production [8,12], may be involved, but no direct evidence on this issue is currently available.

The reciprocal nature of the GATA-3 response in atopics and non-atopics appear to correspond to patterns of *in vitro* murine T cell differentiation systems, where cells committed to the Th2 pathway show an increase in the levels of GATA-3 while Th1 conditions lead to a decrease of GATA-3 levels [14,15,32,33]. However, in those studies, and also in our own observations using real-time PCR, levels of GATA-3 are low in unstimulated naïve cells, while we observed higher GATA-3 levels in unstimulated cells from non-atopics compared to atopics. We speculate that, at least in PBMC, GATA-3 expression is under tighter control in atopics compared to non-atopics, resulting in lower expression in the absence of stimulation, but additional *ex vivo* studies are necessary to clarify this observation.

It is also of interest to speculate on the mechanism(s) underlying GATA-3 down-regulation in the non-atopic subjects. A direct role of IL-12 in GATA-3 repression has been proposed [32]. However, a recent study with IL-12RB2 chain transgenic mice failed to observe a decrease in GATA-3 and c-Maf expression following culture with IL-12 [33]. It may be possible that interaction with other TFs, such as the recently described repressor of GATA (ROG) [34] and T bet [35] play an active role in GATA-3 repression. Experiments in our laboratory are in progress to analyse the expression of these TFs in response to allergen stimulation.

In conclusion, this is the first study to demonstrate the utility of monitoring antigen-induced dynamic changes in TF expression in peripheral blood T cells for qualitative assessment of immune responses in a specific human disease, and as such opens up new avenues for future research in this and related areas of disease immunopathogenesis. In relation to allergy, it is becoming clear that the disease involves aberrant expression of a range of Th2-associated cytokines, and it appears increasingly unlikely that therapies targeted at single cytokines will have broad efficacy. The possibility of targeting upstream events in the disease process, as exemplified by the potential role of GATA-3 in regulating a range of Th2 cytokine genes, provides alternatives for future drug development. While specific pharmacological tools to test this possibility in humans are yet to be developed, it is pertinent to note the recent report on an experimental model of atopic asthma, in which expression of a dominant-negative mutant of GATA-3 in mice in a T cell specific fashion led to concomitant down-regulation of IL-4, IL-5 and IL-13, and parallel attenuation of asthma-like manifestations and IgE production [36]. This provides important proof-of-principle for the approach, particularly when viewed in the light of our findings presented here.

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