

Reduced T-bet in addition to enhanced STAT6 and GATA3 expressing T cells contribute to human allergen-induced late responses

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Summary

Background T-bet and GATA-3 are transcriptional factors involved in Th1 and Th2 cell differentiation, although their concomitant roles at protein levels in target organs during human allergic disease have not been assessed.

Objectives We investigated the expression of T-bet and GATA-3 in nasal and cutaneous models of Th2 (grass-pollen allergen) and a cutaneous model of Th1 (PPD) responses in man.

Methods Nasal biopsies were obtained at 8 h and skin biopsies at 8 and 48 h after allergen and PPD challenges, respectively, from 10 allergic rhinitis and 6 non-atopic controls. T cells were assessed using immunofluorescence microscopy.

Results There were increases in CD3⁺STAT6⁺ cells ($P = 0.01$ for nose and skin) and CD3⁺GATA3⁺ cells ($P = 0.03$ for skin) in response to allergen compared with diluent in allergics. When compared with non-atopics after allergen challenge the difference between the two groups was also significant for CD3⁺STAT6⁺ ($P = 0.001$ and 0.03) and for CD3⁺GATA3⁺ cells ($P = 0.04$ and 0.001) for nose and skin respectively. Following PPD challenge CD3⁺STAT4⁺ cells and CD3⁺T-bet⁺ cells increased in both groups compared with diluent ($P = 0.02$ and 0.03 for both TFs), whereas only CD3⁺T-bet⁺ cells were significantly greater in non-atopics compared with allergics ($P = 0.04$). The ratio of GATA3⁺:T-bet⁺ T cells in allergen-induced responses was significantly greater in the allergics ($P = 0.008$ and 0.01 nose and skin respectively), whereas the ratio of T-bet:GATA3⁺ T cells was significantly higher in the non-atopics during PPD-induced responses ($P = 0.003$).

Conclusions and Clinical Relevance Dysregulation of Th1 transcription may contribute to heightened expression of STAT6 and GATA3 leading to exaggerated Th2-driven manifestations of allergic disease.

Keywords allergic rhinitis, GATA3, human models of Th1 and Th2, late skin and nasal responses, Stat6, T-bet, tuberculin

Abbreviations AR, allergic rhinitis; BAL, bronchial alveolar lavage; BU, biological units; c-Maf, cellular muscular aponeurotic fibrosarcoma; FITC, fluorescein isothiocyanate; NAC, non-atopic healthy controls; GATA3, GATA-binding protein 3; IQ, interquartile; PBMC, peripheral blood mononuclear cells; PPD, purified protein derivatives; RT, room temperature; SPT, skin prick test; STAT, signal transducer and activator of transcription; T-bet, T-box expressed in T cells; TCR, T cell receptor; TF, transcription factors; TLR, toll like receptor; TRITC, tetramethylrhodamine isothiocyanate.

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Introduction

The subdivision of T cells to Th1/Th2 phenotype based on their distinct cytokine pattern, over two decades ago, broadened our perception of the influence of

T cells on the immunopathogenesis of clinical conditions, such as autoimmune and allergic diseases. In the past decade it has been established that the differentiation of naïve T cells to a Th1 or Th2 phenotype is regulated by specific transcription factors (TF), T-bet and

GATA3 respectively [1, 2]. TFs are proteins that bind to DNA and activate or stifle expression of numerous genes. Once TFs are activated, the signal transducer and activator of transcription (STAT) becomes phosphorylated and binds to DNA in response to different signalling pathways [3, 4].

The differentiation of naïve T cells to Th1 cells not only requires TCR ligation but also signalling by a Th1 promoting cytokine, both of which collectively induce the expression of T-bet. It has been shown that, STAT1 with IFN- γ as a Th1 promoting cytokine induces the expression of T-bet. Similarly, signalling through IL-12/IL-12R activates STAT-4 which in turn up-regulates T-bet expression in newly differentiated Th1 cells [5, 6]. T-bet deficiency in mice resulted in a profound impairment in Th1 cell development with defective IFN- γ production, and overproduction of Th2 cytokines leading to allergic airway inflammation and airway hyperresponsiveness, with bronchial infiltration predominantly consisting of eosinophils and lymphocytes [7, 8]. Although T-bet deficiency has not been described in man, T-bet polymorphisms have been associated with bronchial asthma and increased airway hyperresponsiveness [9].

In contrast with the prolonged Th1 cytokine signalling pathways, the Th2 derived cytokine IL-4 has the ability to up-regulate a number of genes within a few hours of exposure [10]. Signalling through the IL-4R and/or TCR engagement activates STAT6 expression which in turn up-regulates GATA3. Thereafter, GATA3 induces its own expression in a positive feedback loop that results in further IL-4 and other Th2 cytokine (such as IL-13) synthesis [11, 12]. In accordance with the role of STAT6-dependent IL-4 and IL-13 in the pathology of asthma, STAT-6 knockout mice did not develop experimental airway hyperresponsiveness or lung inflammatory responses following OVA inhalation challenge and were protected from all pulmonary effects of IL-13 [13, 14]. In the study by Messi et al., priming of both murine and human naïve CD4⁺ T cells to Th1 or Th2 conditions resulted in IL4/IFN- γ double producing cells *in vitro*, whereas De Fanis et al. highlighted the species-specific features in the regulation of GATA-3. For example, in murine studies GATA-3 has been found to be higher in Th2 compared with Th1 cells. In contrast, its expression was found to be regulated similarly in both human Th1 and Th2 cells with the caveat that there was overexpression of GATA3 in human IL-4 producing cells sorted for the chemoattractant receptor expressed on Th2 cells (CRTH2). These data suggest that GATA3 may not be a unique TF for Th2 cells in humans [15, 16]. These findings using peripheral T cells highlight the need to also study local antigen-specific T cell responses in target organs, as in the present study.

The prevalence of allergic diseases, such as allergic rhinitis and asthma, has increased in the past three

decades in children and adults [17]. Cross-sectional investigations demonstrated that asthma and rhinitis co-exist in a majority of patients [18]. One reason for the increase in the prevalence of atopic disease has been attributed to environmental factors, including the reduction of early-life exposure to microorganisms [19]. In agreement with this are findings that suggest early exposure to *Mycobacterium tuberculosis* has been inversely associated with the prevalence of atopic diseases [20, 21].

Allergic rhinitis is characterized by Th2 mediated allergic responses, elevated IL-4, IL-5 and IL-13 production, eosinophilic airway inflammation and increased concentrations of allergen specific IgE [22]. Consistent with the Th2 propensity of allergic rhinitis were the observations of high T cell numbers expressing GATA3, c-Maf and STAT-6 in the nasal mucosa of allergic rhinitis and in bronchial biopsies and sputum from atopic asthmatics compared with non-atopic healthy controls [23, 24]. Interestingly T-bet expressing T cells were absent in the bronchial mucosa of asthmatic patients which was in sharp contrast with the abundance of T cells expressing T-bet in non-asthmatic healthy volunteers [7].

To test whether reciprocal roles of GATA3 and T-bet and their associated signalling pathways are valid in man, we performed an *in vivo* time course experiment that compared the expression of TF using allergen specific nasal and skin provocations and skin challenges with tuberculin. The results were compared with the corresponding diluent challenges. In addition, we sought to establish whether a possible impairment in the Th1 pathway may account in part for the heightened Th2 responses seen in allergic rhinitis, both of which might contribute to the pathology of allergic conditions.

Methods

Participants

Sixteen volunteers (10 allergic rhinitis; F:M: 3 : 7 and 6 non-atopic controls, F:M, 1 : 5) were recruited for the study. The median age of the rhinitic group was 27 years (IQ 24, 29) and the controls, 29 years (IQ 25, 35). Patients all lived within urban areas.

Allergic rhinitis was defined by a history of seasonal symptoms for at least 2 years and a positive skin test > 3 mm weal to grass pollen. Non-atopic healthy controls were defined by the absence of nasal or allergic symptoms and negative skin testing to a panel of 11 common aeroallergens. Skin prick testing was performed with a sterile stainless steel lancet (ALK Abello, Horsholm, Denmark) and standardized reagents [Timothy grass *Phleum Pratense* extract, mixed grass pollen,

Birch pollen, Mixed tree, Cat hair, Dog hair, House dust mite (*Dermatophagoides pteronyssinus*), Mixed weeds, *Aspergillus fumigatus*, *Cladosporium Herbarum*, *Alternaria Alternata*, Soluprick; ALK Abello]; a weal of >3 mm compared with the diluent (negative control) was considered positive. Data for total and allergen specific IgE for the group have been reported previously [25] and were as follows: (Total and specific IgE (mean $\pm$ standard error of the mean) in participants with allergic rhinitis were, respectively, 407 (115) IU/mL and 42.5 (9.3) IU/mL and in normal volunteers were 16.4 (3.1) IU/mL and < 0.34 IU/mL. Exclusion criteria for both groups were: a history of anaphylaxis, current systemic steroid or immunosuppressive medication, other nasal, systemic or other chronic diseases, severe asthma or specific immunotherapy within the last 5 years. The study was approved by the ethics committee of The Royal Brompton and Harefield Hospitals NHS Trust and was performed with the subjects' written informed consent [25].

Study design

Allergen and PPD challenge.

Skin: Skin challenges were carried out with allergen and PPD and their relevant diluents. PPD and allergen challenges were performed in random order and at an interval of 3 weeks in both patients and control groups. Briefly, in one visit, the two PPD injections were given to the extensor surface of one forearm 10 cm apart and a third diluent injection was administered on the other forearm. The procedure was repeated with two allergen and one diluent injection after a minimum of 3 weeks interval. Each volunteer received a total of six skin challenges, two allergen (10 Biological units in 0.02 mL; Alk Abello) and one diluent, two PPD (10 tuberculin units in 0.02 mL; Evan Vaccines, Richmond, VA, USA), and one diluent.

Nasal: One week after completing skin challenges, participants underwent separate nasal challenges with diluent and allergen (Phleum pratense). Nasal challenges were separated by at least 3 weeks and were performed outside of the pollen season. A total allergen dose of 1250–2500 BU (2–4 sprays of a solution of 5000 BU/mL of Aquagen Phleum pratense; ALK Abello), a dose previously shown to induce late nasal responses, was used for the nasal challenge. The order of challenges (allergen or diluent) was randomized [25].

Nasal and skin biopsy: Biopsies were taken at 8 h post allergen and diluent challenge for the nose and at 8 and 48 h after allergen and PPD challenges in the skin. Nasal biopsies (2.5 mm) were taken from the inferior turbinate using 10% cocaine as local anaesthetic as

described [22]. Three mm skin biopsies were taken from the extensor aspect of the forearm. Diluent biopsies were taken after 8 h (allergen) and 48 h (PPD). Four micron thick sections were cut from the snap frozen biopsies, fixed in acetone and stored at -80°C . Six micron sections were cut from paraformaldehyde fixed biopsies and used for *in situ* hybridization for the assessment of IL-4 mRNA [26].

Immunofluorescence

Dual immunofluorescence [27] was carried out to co-localize STAT6, GATA3, STAT4 and T-bet to CD3. In brief, the sections were rehydrated in PBS and blocked for endogenous biotin using Avidin-Biotin blocking kit (Vector Lab., Peterborough, UK) and incubated with 20% appropriate sera to block Fc receptor binding. This was followed by addition of rabbit anti-STAT6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-GATA3 (Abcam, Cambridge, UK), rabbit anti-STAT4 (Santa Cruz Biotechnology) and monoclonal anti-T-bet (Santa Cruz Biotechnology) for 1 h at RT. After several washes in PBS, the sections were incubated with affinity purified biotinylated donkey anti-rabbit or donkey anti-goat, followed by further washes and incubation with anti-CD3 (Dako, Cambridge, UK) for 45 min at RT. Normal rabbit, normal goat IgG (Santa Cruz Biotechnology) and mouse IgG1 isotype control (Dako) were used as negative controls. The sections were finally incubated with streptavidin conjugated Alexa Fluor 594 (Invitrogen, Paisley, Scotland, UK) (for the transcription factors) and FITC-labelled rabbit anti-mouse (for CD3⁺ T cells) for 30 min. The slides were mounted using anti-fade fluorescence mountant 'ProLong' (Invitrogen).

In situ hybridization

Riboprobes, both antisense and sense, were prepared from cDNA for IL-4 (gifts from Q Hamid, McGill University, Montreal, Canada) [26].

Blocking peptide

Blocking peptides sc-621P and sc-486P were used in absorption studies to selectively block the binding abilities of STAT6 and STAT4 antibodies, respectively, (Santa Cruz Biotechnology) and to confirm the specificities of the staining [28].

Quantification

The sections were analysed independent of the clinical protocol using a Nikon Eclipse (E400) fluorescent microscope (Tokyo, Japan) at 400 $\times$ magnification under appropriate absorption/emission wavelengths (590/

617 nm for Alexa Fluor 594 or TRITC and 488/519 nm for FITC labelled antibody or Alexa Fluor 488). The images were captured using Nikon Digital Still Camera DXM1200 and analysed with Lucia 4.82 software (system for Image processing and analysis; Prague, Czech Republic) by two blinded independent observers. Immuno-stained cells were counted one grid (nose) and two grids (skin) immediately beneath the entire epithelium/epidermis and results were expressed as the number of positive cells/mm². Inter-observer variations for the counts were less than 10%. Average number of fields captured was 15 for skin and 8 for nasal biopsies equivalent to 1.5 mm² (0.4–2.4 mm²) and 0.8 mm² (0.2–1.8 mm²), respectively, of sub-epithelial tissue per biopsy [28].

Statistical analysis

Data were expressed as median and interquartile (IQ) range. Between-group comparisons were performed using the Mann–Whitney *U*-test. Within-group comparisons were performed using the Wilcoxon matched-pairs signed-ranks test. Correlations were performed using Spearman rank correlation coefficient with statistics software package (Minitab Inc., State College, PA, USA). All tests were two-tailed and *P*-values < 0.05 were considered significant.

Results

Clinical response

Allergic rhinitis (but not non-atopics) demonstrated both early and late nasal and skin responses to allergen challenge, but not to diluent challenge (*P* = 0.001 and 0.01, respectively, for both). Significant increases in nasal symptoms were recorded during early (0–60 min) and late nasal responses (1–8 hr) after allergen compared with diluent challenge (*P* = 0.001 and 0.03 respectively). The cutaneous late-phase responses at 8 and 48 h of allergen challenge were significantly greater than the diluent response (*P* = 0.001 for both). The size of skin late response to allergen at 8 h was also significantly greater than at 48 h (*P* = 0.002). In contrast, the surface area of late skin responses following intradermal PPD challenge was similar in both groups i.e. 3941 and 3502 mm² rhinitics and non-atopics (*P* = 0.001 and 0.04, respectively, when compared with diluent challenge).

Th2 transcription factors in the nose and skin after allergen and PPD challenge

STAT6. When compared with diluent, CD3⁺STAT6 expressing cells were increased at 8 h post allergen

challenge both in the nose (~24-fold, *P* = 0.01) and skin (~7-fold, *P* = 0.01) in rhinitics, but not controls (Table 1; Figs 1a and b). CD3⁺STAT6⁺ cells were also increased at 8 h post allergen challenge in the nose (*P* = 0.001) and in the skin (*P* = 0.03) in rhinitics compared with non-atopics. At 48 h post cutaneous allergen provocation CD3⁺STAT6⁺ cells were more numerous in the rhinitics compared with non-atopics (*P* = 0.005; Table 1).

GATA3. In rhinitics (but not in non-atopics) CD3⁺GATA3⁺ cells after allergen provocation were increased (~1.5-fold) in the nasal mucosa compared with diluent challenge, although these increases did not reach significance (*P* = 0.07, Table 1; Fig. 1c). CD3⁺GATA3⁺ cells in the nasal mucosa were greater in rhinitics compared with non-atopics (*P* = 0.04). In response to allergen, cutaneous CD3⁺GATA3 expressing cells in rhinitics were increased at 8 and 48 h (by 4- and 2.5-fold respectively, *P* = 0.03 and 0.01 respectively) compared with the diluent challenge (Fig. 1d; Table 1). When compared with non-atopics, GATA3⁺ T cells at 8 h (*P* = 0.001) and 48 h post allergen challenge (*P* = 0.005) were greater, by 20- and 4.5-fold, respectively, in the rhinitics (Table 1).

There was a linear association between IL-4 mRNA expressing cells using *in situ* hybridization and cutaneous CD3⁺STAT6⁺ cells in atopics at 8 h post allergen provocation (*r* = 0.75, *P* = 0.02; Fig. 1e).

Th1 transcription factors in the nose and skin after allergen and PPD challenge

STAT4. CD3⁺STAT4⁺ cells in the nasal mucosa and in the skin of rhinitics and controls did not change at 8 or 48 h post allergen challenge (Table 1). In contrast, in response to tuberculin challenge, CD3⁺STAT4⁺ cells at 48 h (but not 8 h) were increased in the skin by 2-fold in rhinitics (*P* = 0.02), and by 15-fold in non-atopics (*P* = 0.03) when compared with diluent (Fig. 2a; Table 1).

T-bet. In response to 48 h of PPD (but not allergen) challenge, CD3⁺T-bet⁺ cells were increased in both rhinitics (by ~5-fold, *P* = 0.02) and non-atopics (by 17-fold, *P* = 0.03) compared with diluent challenge (Fig. 2b). When compared with non-atopics, CD3⁺T-bet⁺ cells at 48 h post PPD challenge were significantly less in the rhinitics (*P* = 0.04).

Kinetics of TF expression. Kinetics of nasal and cutaneous CD3⁺ T cells, Th2 expressing TF cells (CD3⁺STAT6⁺ and CD3⁺GATA3⁺) in response to allergen challenge and Th1 expressing TF cells (CD3⁺STAT4⁺ and CD3⁺T-bet⁺) following tuberculin provocation mirrored late

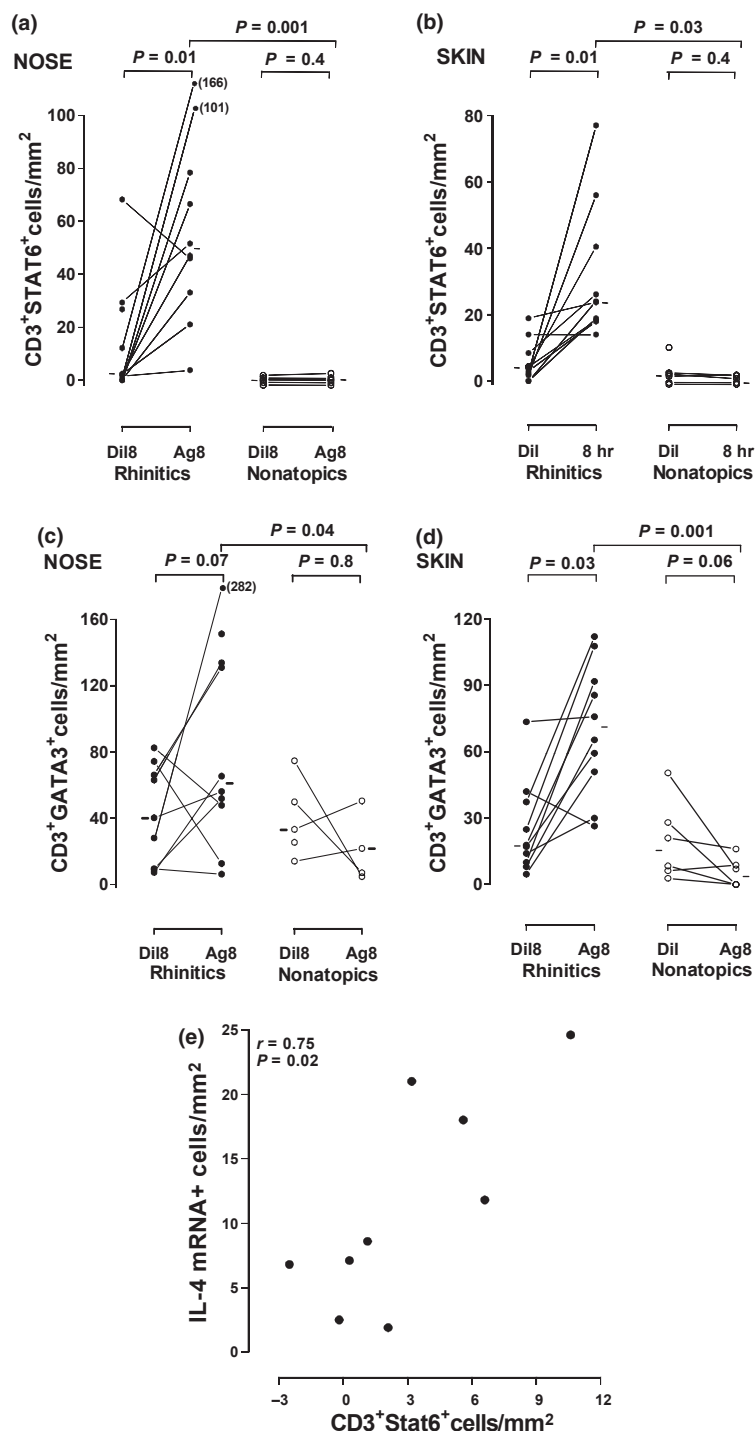


Fig. 1. CD3⁺STAT6⁺ cells/mm² (a and b) and CD3⁺GATA3⁺ cells/mm² (c and d) in the nasal mucosa and in the skin in allergic rhinitis (•) and non-atopics (O) at 8 h after diluent and allergen (Ag) challenge respectively. The association between CD3⁺STAT6 expressing cells (dual IF) and IL-4 mRNA⁺ cells (*in situ* hybridization) in the skin section of rhinitics after allergen challenge are shown (e). Unpaired data are shown without connecting line. Bars (-) depict medians. P-values are shown.

nasal and skin responses to allergen and PPD, peaking at 8 and 48 h respectively (Table 1).

The ratio of CD3⁺GATA3⁺:CD3⁺T-bet⁺ cells at 8 h post allergen challenge was higher in the nasal mucosa ($P = 0.008$) and in the skin ($P = 0.01$) in

rhinitics compared with non-atopics (Fig. 3a). Conversely, the ratio of CD3⁺T-bet⁺:CD3⁺GATA3⁺ cells was greater at 48 h of PPD challenge in the non-atopics compared with rhinitics ($P = 0.003$ respectively; Fig. 3b).

Table 1. Median numbers (IQ range) of total CD3⁺ and STAT6, GATA3, STAT4 and T-bet expressing T cells/mm² in the nasal mucosa in response to 8 h after allergen challenge and in skin biopsies following diluent, 8 and 48 h after allergen or PPD provocation

		Nose			Skin					
		Dil8	Ag8	Dil8	Ag8	Ag48	Dil48	PPD8	PPD48	
AR	CD3 ⁺ cells	172 (135, 201)	238** (160, 302)	37.3 (17, 65)	138 (79, 258)	59.5 (43, 96)	45 (28, 59)	59.5 (28, 95)	469** (277, 754)	
N = 10										
NAC	CD3 ⁺ cells	351 [#] (330, 400)	255* (139, 280)	56 (35, 69)	80.5 (56, 112)	31.5 [#] (17, 44)	33.8 (15, 80.5)	80.5 (63, 133)	670* (559, 738)	
N = 6										
AR	CD3 ⁺ STAT6 ⁺	2.2 (0, 27)	49.3*** (30, 84)	3.46 (1.3, 9.8)	23.8*** (18, 44)	12 (4.1, 26)	3.2 (1.8, 9)	2.9 (1.3, 8.4)	2.1 (0.4, 5.7)	
N = 10										
NAC	CD3 ⁺ STAT6 ⁺	0 (0, 1.0)	0.35 ^{###} (0, 1.2)	1.7 (0, 4.3)	0.7 ^{##} (0, 1.7)	0.77 ^{###} (0, 1.7)	0 [#] (0, 1.4)	1.9 (0.75, 5.1)	1.4 (0, 4.7)	
N = 6										
AR	CD3 ⁺ GATA3 ⁺	40.3 (9.3, 70)	60.7 (39, 138)	17.5 (9.5, 38)	70.5** (45, 95)	44.8* (21, 64)	17.7 (8.7, 22)	14.2 (5.4, 33)	63.8** (27, 90)	
N = 10										
NAC	CD3 ⁺ GATA3 ⁺	33.3 (9.3, 62)	21.7 [#] (5.8, 38)	14.7 (5.3, 33)	3.5 ^{##} (0, 10)	9.8 ^{##} (4.5, 18)	3.8 (2.1, 14)	5.8 (0, 32)	17.0 [#] (5.2, 29)	
N = 6										
AR	CD3 ⁺ STAT4 ⁺	5.9 (1.5, 10)	5.3 (4.4, 12.5)	9.3 (1.5, 18)	9.8 (8.0, 29)	10.1 (7.5, 19)	7.6 (5.7, 13)	10.3 (4.8, 14)	16.4** (13, 90)	
N = 10										
NAC	CD3 ⁺ STAT4 ⁺	11.6 (3.2, 36)	13.4 (5.9, 24)	4.3 (1.0, 7.2)	3.95 (3.0, 12)	3.1 [#] (1.3, 4.7)	3.5 (1.1, 10)	10.1 (4.6, 17.5)	54.5** (42, 72)	
N = 6										
AR	CD3 ⁺ T-bet ⁺	3.74 (1.1, 9.7)	0.87 (0, 8.9)	4.2 (0, 21)	8.4 (1.0, 31)	10.7 (5.0, 17)	10.9 (3.5, 38)	9.6 (3.6, 26)	53.2** (23, 110)	
N = 10										
NAC	CD3 ⁺ T-bet ⁺	25.2 (2.7, 34)	7.4 (4.6, 25)	5.4 (3.5, 14)	7 (1.9, 11)	3.1 (0, 8.5)	7.7 (3.6, 23)	20.1 (11, 34)	135.4 ^{###} (102, 232)	
N = 6										

P* = 0.05, *P* = 0.01, ****P* = 0.001 when diluent (Dil)- compared with allergen (Ag) or PPD challenge.[#]*P* = 0.05, ^{##}*P* = 0.01, ^{###}*P* = 0.001 when allergic rhinitis (AR) compared with non-atopic controls (NAC).

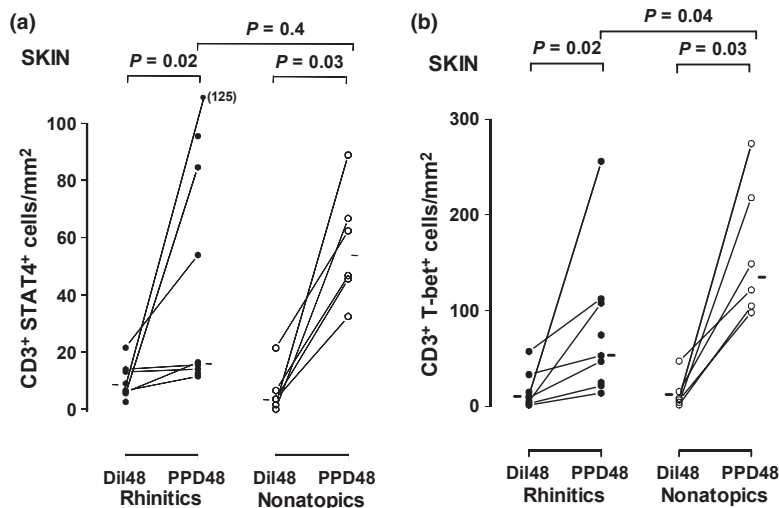


Fig. 2. CD3⁺STAT4⁺ cells/mm² (a) and CD3⁺T-bet⁺ (b) in the skin of rhinitics (•) and nonatopics (O) at 48 h of diluent and PPD challenge. Bars (–) depict medians. *P*-values are shown.

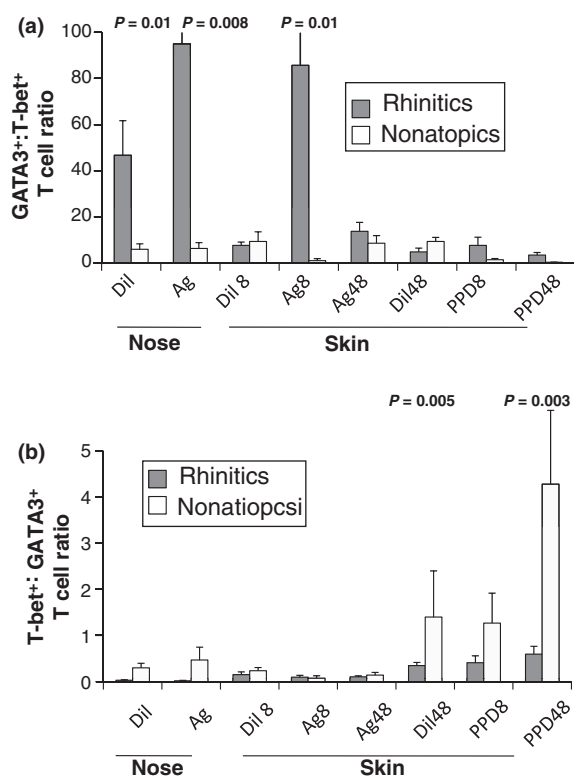


Fig. 3. Ratio of CD3⁺GATA3⁺:CD3⁺T-bet⁺ T cells (a) and CD3⁺T-bet⁺:CD3⁺GATA3⁺ T cells (b) in the nasal mucosa in response to diluent and allergen challenge at 8 h and in the skin in response to diluent, allergen and PPD challenge at 8 and 48 h. *P*-values show differences between rhinitics vs. non-atopics.

Examples of CD3⁺STAT6⁺, CD3⁺GATA3⁺ in the nasal sections from rhinitics at 8 h post allergen challenge (Figs 4a and b) and CD3⁺STAT4⁺ and CD3⁺T-bet⁺ cells

(Figs 4c and d) in the skin sections from non-atopics at 48 h after PPD provocation are shown.

Absorption studies. Using peptides sc-621P (STAT6; Fig. 4e) and sc-486P (STAT4) and the corresponding antibodies abolished STAT6 and STAT4 staining in both nasal and skin sections from the atopic group. No peptide was available for either GATA3 or T-bet antibodies for the absorption study. IgG isotype controls did not show any staining (Fig. 4f).

Discussion

In this study, we have demonstrated significant increases in CD3⁺STAT6⁺ and CD3⁺GATA3⁺ cells following allergen challenge in the nasal mucosa and skin in allergic rhinitis compared with non-atopic normal controls. Furthermore, cutaneous CD3⁺STAT6⁺ cells were closely associated with the numbers of IL-4⁺ cells after allergen challenge. In contrast, increases in cutaneous CD3⁺T-bet⁺ cells in response to tuberculin were significantly greater in non-atopic controls compared with subjects with allergic rhinitis. The kinetics of IL-4 mRNA expressing cells closely mirrored the findings of STAT6 (Th2) profiles.

We hypothesized that in a predominantly Th2 condition, such as allergic rhinitis, impaired Th1 responses may contribute to the enhanced expression of Th2 cytokines and their related transcriptional machineries. Reduced numbers of cutaneous T-bet expressing CD3⁺ cells as well as a decreased ratio of T-bet:GATA3 expressing T cells in response to tuberculin supports the notion that T cells from atopic individuals are predisposed to differentiate towards a Th2 phenotype upon specific antigenic stimulation. Our findings are in accord

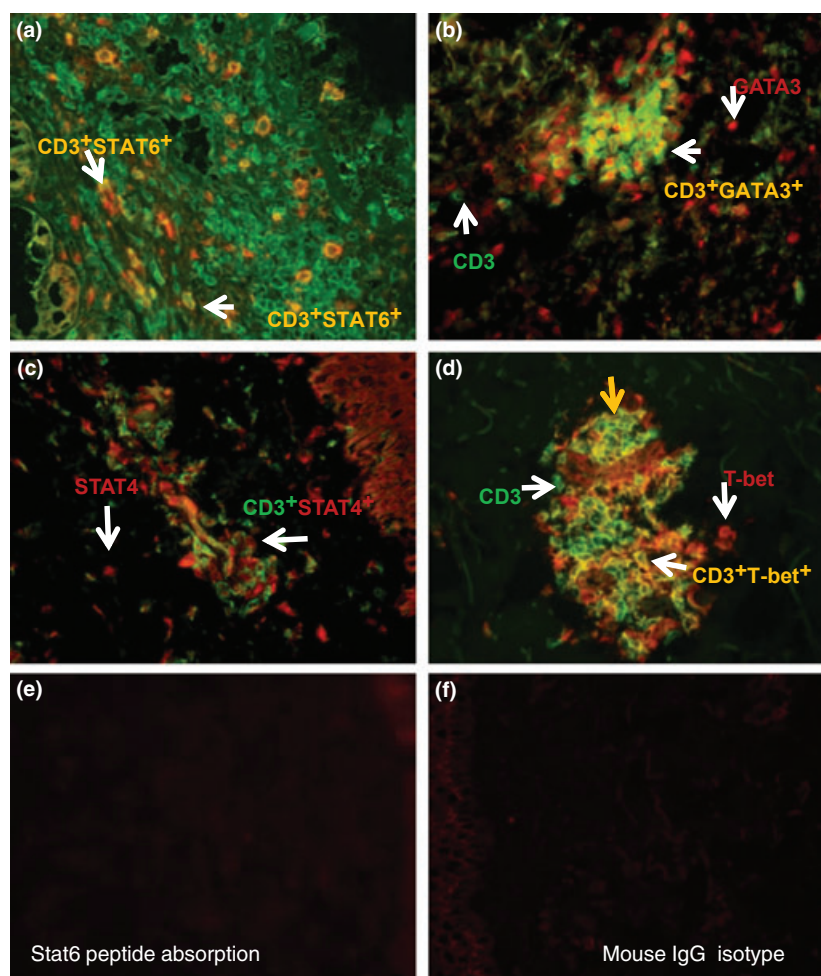


Fig. 4. Examples of CD3⁺STAT6⁺ cells (a) and CD3⁺GATA3⁺ cells (b) in the nose and skin after 8 h of allergen challenge in rhinitics. CD3⁺STAT4⁺ (c) and CD3⁺T-bet⁺ cells (d) in the skin from non-atopic subjects after 48 h of PPD challenge are shown (400× magnification). Absorption of STAT6 antibody with STAT6 peptide abolished STAT6 staining (e) and staining using mouse IgG isotype control (f) are shown.

with studies carried out in children in whom an inverse relationship was found between positive tuberculin responses and atopy, suggesting that the exposure to *Mycobacterium tuberculosis* may modify the immune profile and inhibit the development of atopic disorders in a genetically predisposed individual [20, 21]. Moreover, bacterial lipoproteins such as those found in PPD (*M. tuberculosis*) trigger through TLR pathways leading to a prominent antigen specific Th1 cytokine switch, i.e. IFN- γ secretion from antigen specific T cells, whereas allergens induce Th2 responses with IL-4 (as well as IL-5, IL-9 and IL-13) as prominent cytokines [29, 30]. Our hypothesis was further strengthened by the observation that in contrast with allergen challenge which increases the cutaneous CD3⁺ T cells influx in rhinitics but not in controls (~4-fold vs. 1.5-fold respectively at 8 h i.e. peak of late responses), intradermal PPD provocation resulted in 10-fold increase in the recruitment of CD3⁺ cells in allergic rhinitics vs. a 20-fold increase in controls.

All participants in the study were matched in terms of age and gender, had received BCG vaccination and none had a history of previous tuberculosis or other systemic disease. Although we cannot absolutely exclude possible latent tuberculosis during childhood, the vigorous response of T-bet post PPD (data presented in Figs 2b and 3) most likely represents immunological memory in response to previous BCG vaccination and unrelated to recent TB infection.

The linear association between STAT6⁺ T cells and IL-4⁺ cells in the rhinitics found in the present study supports previous observations on the requirement of IL-4 for the induction of STAT6. In contrast, no relationship was found between IL-4 expressing cells and CD3⁺GATA3⁺ cells. One possible explanation for such disparity is that IL-4 upregulation is an early event e.g. could increase as early as 30 min and peaks 6–8 h following TCR ligation, an event similar to STAT6 activation in T lymphocytes [10]. In contrast, the expression of

GATA3 involves sequential activation steps [2, 10] involving the ligation of IL-4/IL-4R, followed by STAT6 upregulation in the cytoplasm resulting in the induction of GATA3 in the nuclei. The elevated numbers of GATA3⁺CD3⁺ cells, but not STAT6⁺CD3⁺ cells at 48 h post allergen challenge support the notion that distinct kinetics operate for STAT6 and GATA3 expression and the latter follows STAT6 induction in a time dependent fashion. The dynamic responses of GATA3 expression were further supported by the finding of significant numbers of T cells expressing GATA3 after 48 h of challenge with PPD, a Th1 stimulus, which is consistent with the notion that T cells from atopics are inherently prone to Th2 differentiation and may have a biased pattern of TF expression. It has been reported that a certain level of GATA-3 expression is critical for CD4⁺ T cells to maintain a non-polarized phenotype which can bind directly to the Fox P3 and inhibit its transcription [31]. It has also been reported that GATA-3 and T-bet functionally interact with each other in a competitive manner which finally influences the outcome of T cell polarization [32]. Furthermore, the regulatory mechanism of GATA3 in suppressing Th1 cells was revealed in a recent report where after PPD challenge induced delayed hypersensitivity, IFN- γ production in lymph node cells of sensitized mice was significantly lower in GATA-3-transgenic mice [33]. These observations collectively suggest that over expression of Th2 cells can influence both Tregs and Th1 type cell responses.

Furthermore, the increase in the numbers of GATA3⁺ T cells following PPD challenge in our study may also suggest that there is a propensity for a Th2 development and responses in atopic populations regardless of the type of stimuli i.e. whether allergen or PPD. Interestingly the time-dependency of the increase in GATA3 was specific for the type of stimuli. i.e. the GATA3 response peaked at 8 h for allergen and 48 h for PPD. It is possible that GATA3 may be expressed by not only Th2, but also other Th populations.

In contrast with the skin, where allergen challenge induced higher numbers of both STAT6 and GATA3, a significant increase in the numbers of STAT6⁺ T cells, but not GATA3 expressing T cells was observed following nasal allergen challenge. This observation may be explained by the likelihood that the microenvironment may influence the outcome of the response. In the

clean/virgin environment of skin, allergen provocation results in the recruitment of a greater population of Th2 cells, whereas in the 'dirty' environment of the nose the expression of GATA3 is more universally expressed by many T cell phenotypes. An alternative explanation [34] may be that GATA3 expression is a pre-requisite for all T cells to survive and differentiate.

Moreover, no changes were observed in terms of STAT6⁺ T cells following PPD challenge in either AR or normal controls. Our data, revealed that in addition to enhanced local Th2 responses, the impaired ability to mount potent Th1 responses to a Th1 stimulus i.e. *Mycobacterium tuberculosis* might contribute to the severity of immune responses seen in allergic diseases. In one study a single intradermal injection of heat-killed *Mycobacterium vaccae* in atopic asthmatics was associated with a reduction in bronchial late responses to inhaled allergen, as well as decreases in serum IgE and IL-5 production [35]. These data also support the notion that the inclusion of immune adjuvants (CpG, MPL^R) with the ability to boost local antigen-specific Th1 responses during the course of specific immunotherapy may be advantageous as recently confirmed in ragweed seasonal allergic rhinitis [36].

In summary, we have shown that in allergic rhinitis, but not in non-atopic controls, allergen-driven increases in IL-4-expressing T cells and associated Th2-dependent clinical late responses at 8 h in the skin and nose are intimately associated with increased expression of the transcription factors STAT6 and GATA3, but not T-bet. The reverse was apparent during tuberculin driven Th1-dependent 48 h late cutaneous responses in both allergic and non-atopic controls. The relative impairment of T-bet in allergic individuals may contribute to the observed exaggerated Th2 responses, thereby predisposing to allergic inflammation and disease expression.

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