

Investigating *Gata3* as a positional candidate gene for allergic asthma in a murine model

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Summary

Gata3 is a positional candidate gene for allergic asthma. We determined allergen-induced GATA-3 mRNA and protein expression in asthma susceptible and resistant mice and generated *Gata3* sequence data. Our data indicate that the *Gata3* gene in isolation is not a causative agent of asthma susceptibility in our model.

Introduction

Allergic asthma is the clinical result of an inappropriate immune response to common environmental allergens. Underlying the clinical manifestation of asthma is chronic airway inflammation that is characterized by a predominant T-helper-2 (Th2) cell bias. These antigen-activated Th2-cells secrete a profile of cytokines that drive subsequent eosinophilia and elevated serum IgE. This aberrant immune response is believed to have a genetic foundation. However, as many genes regulate the complicated signal transduction network involved in allergic inflammation, the task is to discern those genes that play a key role in asthma.

GATA-3 is a transcription factor whose gene may have central relevance in allergic asthma. While GATA-3 is important in vertebrate fetal development and expressed widely in neuronal tissue during embryogenesis, its relevance to allergic asthma relates to its role in T-cell biology. It promotes T-cell development through recognition and activation of T-cell receptor (TCR) genes through GATA binding motifs located in the TCR genes enhancer regions (Ko *et al.*, 1991; Ho *et al.*, 1991; Marine & Winoto, 1991). GATA-3 also determines Th1/Th2 balance; it is expressed at low levels in Th0 cells and inhibits or even inverts the Th1 pathway by way of interleukin (IL)-12 and interferon (IFN)- γ expression inhibition, while favoring Th2 differentiation (Zhang *et al.*, 1997). It induces IL-4 as

a time-restricted instruction switch for Th2 cell differentiation through changes in chromatin conformation (Takemoto *et al.*, 2000; Seki *et al.*, 2004). Inhibition of GATA-3 expression will block generation and effector function of Th2 cells (Ray *et al.*, 2001). Furthermore, GATA-3 was required for development and maintenance of Th2 cells both *in vivo* and *in vitro* in a conditional knockout study (Pai *et al.*, 2004). Thus, GATA-3 is an important determinant of Th1/Th2 balance, making it a molecule of interest in the study of allergic asthma (Zhang *et al.*, 1999; Lee *et al.*, 2000; Nawijn *et al.*, 2001; Justice *et al.*, 2002). Indeed, GATA-3 has been implicated in asthma as Erpenbeck *et al.* (2006) found GATA-3 expression increased in BAL cells from asthmatics undergoing allergen challenge, while Caramori *et al.* (2001) reported higher levels of GATA-3 protein in blood-derived T cells from asthmatics as compared to controls. However, neither of these investigators found GATA-3 to be increased in bronchial biopsies from asthmatics, thus additional studies of this transcription factor are warranted.

Murine studies have significantly advanced our understanding of the role of GATA-3 in asthma. Through the use of a GATA-3 dominant negative mutant model, the function of endogenous wild-type GATA-3 was inhibited; this led to the inhibition of airway eosinophilia, mucus production, Th2 cytokines expression, and IgE synthesis in mice sensitized and challenged with ovalbumin (OVA) (Zhang *et al.*, 1999). Local antisense GATA-3 treatment in a different murine model of asthma resulted in blocked endogenous GATA-3 translation, which led to the inhibition of allergic airway inflammation and airway hyperresponsiveness (Finotto *et al.*, 2001). More recently, Kiwamoto *et al.* (2006) identified increased airway smooth muscle hyperplasia and subepithelial fibrosis in allergen-challenged transgenic mice overexpressing GATA-3.

In a previous study, our laboratory conducted a genome-wide linkage analysis in a mouse model of allergic asthma that revealed two quantitative trait loci (QTL) for allergen-induced bronchial hyperresponsiveness located on murine chromosome 2: *Abhr1* and *Abhr2* (Ewart *et al.*, 2000). The murine gene for GATA-3 (*Gata3*) maps within the *Abhr1* region. In humans, *GATA3* gene haplotypes in Finnish families were recently reported to be associated with asthma and serum IgE levels (Pykäläinen *et al.*, 2005). Thus, because of its location and function, *Gata3* was considered an important positional candidate gene for

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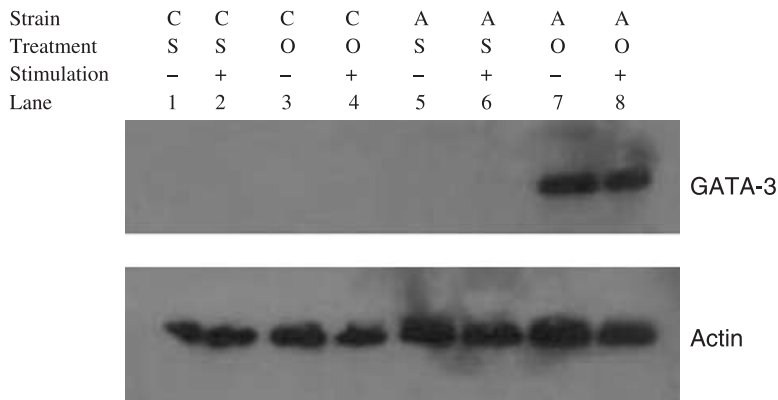


Figure 1. Murine spleen cells from A/J mice sensitized and challenged with OVA produce GATA-3 protein. Spleens were harvested 72 h after *in vivo* challenge with OVA or saline ($n = 8$ mice per group). Spleen cells were disaggregated and incubated *in vitro* with 1 mM Bt2cAMP + 25 ng/mL PMA for 8 h. Nuclear proteins were then isolated from spleen cells after which GATA-3 and actin proteins were detected by Western blot using the following antibodies: GATA-3 (Santa Cruz Biotechnology, Santa Cruz, CA), actin (Sigma-Aldrich, St. Louis, MO) and sheep-antimouse IgG-HRP (Amersham Biosciences, Buckinghamshire, UK). Symbols: C, C3H/HeJ mice; A, A/J mice; S, saline; O, ovalbumin; +, cAMP/PMA stimulation; -, unstimulated.

Abhr1. To further investigate its role in allergen-induced bronchial hyperresponsiveness, *Gata3* was studied at protein, mRNA and DNA levels in the current project. Here we report on the intersecting fields of genetics and immunology as we explore *Gata3* as a positional candidate gene for *Abhr1*.

We first tested the two-part hypothesis that (a) GATA-3 levels are higher in mice with genetic susceptibility to allergen-induced airway hyperresponsiveness (A/J strain) as compared to the less responsive C3H/HeJ strain, and (b) GATA-3 production is induced by allergen stimulation. Initial protein studies were performed on spleen cells harvested from A/J and C3H/HeJ mice at the time of peak airway hyperresponsiveness, 72 h after OVA or saline challenge using our standard *in vivo* exposure protocol as follows (Ewart *et al.*, 2000). On day 0, mice were sensitized intraperitoneally with 10 µg chicken egg OVA (Sigma, St. Louis, MO) in 200 µL phosphate-buffered saline or saline alone. On day 14, mice were anesthetized (ketamine, 45 mg/kg and xylazine, 8 mg/kg, intraperitoneally) and challenged with a tracheopharyngeal instillation of 1.5% OVA (675 µg) in 45 µL saline or saline alone, then recovered from anesthesia. At various time points after challenge mice were euthanized by overdosed pentobarbital and tissues harvested. Spleens were harvested 72 h after *in vivo* challenge and Western blot measurement of nuclear proteins harvested from those spleen cells after an additional 8 h of *in vitro* stimulation with dibutyryl adenosine cyclic AMP (Bt2cAMP) + phorbol 12-myristate 13-acetate (PMA) demonstrated that appreciable GATA-3 production was limited to A/J mice exposed *in vivo* to OVA and was independent of *in vitro* stimulation (Fig. 1, lanes 8 and 9). GATA-3 was not readily detected in cells from *in vivo* OVA-exposed or control C3H/HeJ mice, nor in saline treated A/J mice with or without *in vitro* stimulation. These results are consistent with our hypothesis that GATA-3 production is induced by allergen. It is interesting to note that while GATA-3 levels were higher in A/J mice, as anticipated, this was dependent on allergen exposure *in vivo* and no strain difference was appreciable under *in vitro*-only stimulation conditions. Thus, *in vivo* allergen exposure is a critical

factor in priming immune cells for GATA-3 production, and this was not mimicked by our *in vitro* stimulation conditions.

To refine our understanding of conditions needed for GATA-3 production in A/J mice, additional durations of *in vitro* stimulation were selected, flanking the initial 8 h *in vitro* stimulation. Western blot studies conducted on spleen cells harvested 72 h after OVA challenge and stimulated *in vitro* for 4, 12 and 24 h demonstrated a time-dependent increase in GATA-3 production from 4 to 24 h in A/J mice (data not shown). Thus, GATA-3 production by spleen cells from OVA-exposed A/J mice is sustained. As was the case after 8 h of Bt2cAMP/PMA exposure, substantial GATA-3 production was limited to the A/J strain and was independent of *in vitro* stimulation. Additionally, the level of production was sustained and increased in a time-dependent manner relative to the duration the cells were in culture. Even after 24 h of *in vitro* stimulation, GATA-3 was not appreciably induced by *in vivo* OVA treatment in C3H/HeJ mice.

To further explore the role of GATA-3 in allergen-induced airway hyperresponsiveness, we next examined the time course of GATA-3 expression leading up to the peak of airway hyperresponsiveness at 72 h. GATA-3 message was measured by real-time reverse transcription-PCR in lungs, tracheobronchial lymph nodes and spleens harvested from A/J and C3H/HeJ mice ($n = 6$ /group) 6, 12, 24, 48 and 72 h after challenge with OVA or saline. As anticipated, A/J mice, with their propensity to develop asthma-like characteristics following allergen exposure, demonstrated significantly increased GATA-3 expression in both spleens and tracheobronchial lymph nodes as compared to saline-treated controls at the 6 h time point (Fig. 2). In contrast, GATA-3 expression was not induced by allergen exposure in C3H/HeJ mice. This is consistent with the C3H/HeJ strain's resistance to airway hyperresponsiveness and other asthma-related features. Similarly, peripheral blood T cells from asthmatics have been reported to express fivefold more GATA-3 than control subjects (Caramori *et al.*, 2001), a result that is consistent with increased GATA-3 from lymphoid organs in A/J as compared to C3H/HeJ mice.

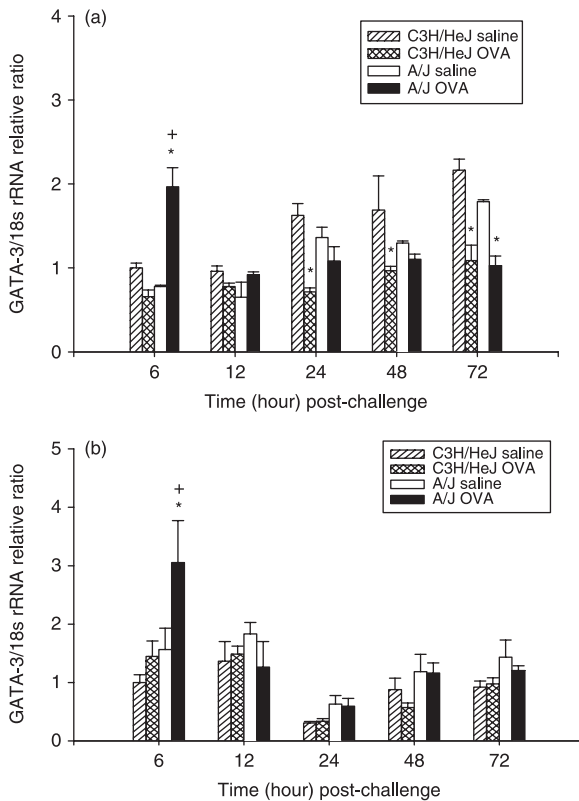


Figure 2. GATA-3 expression in tracheobronchial lymph nodes (a) and spleens (b) following OVA or saline sensitization and challenge in A/J and C3H/HeJ mice ($n = 6/\text{group}$). GATA-3 expression was determined by real-time RT-PCR fluorogenic 5' nuclease assays of tissues harvested 6, 12, 24, 48 and 72 h after OVA or saline challenge. Samples were tested in duplicate and the standard curve analysis method was applied (Applied Biosystems, 2001) using 18 s rRNA as an internal control. The real-time RT-PCR product crossed the junction of exons 3 and 4; oligo sequences were forward primer 5'-cctaccgggttcggatga-3', reverse primer 5'-gtgtgtgagggacggaagac-3', and probe tcgagggcccaaggcagatcc. Results were analysed by two-factor ANOVA with post hoc comparisons performed by Student–Newman–Keul tests. * Significant difference due to treatment and + significant difference due to strain ($P \leq 0.05$).

The early surge in GATA-3 expression in A/J mice was expected based on its role as a transcription factor driving the Th2 cytokine response. These results support the importance of GATA-3 in regulation of cytokine expression in A/J mice. It was somewhat surprising, however, that GATA-3 expression in A/J mice was not sustained in either lymphoid organ, and indeed was statistically decreased in lymph nodes from the OVA group at 72 h. This low level of GATA-3 message in A/J mice at 72 h is in contrast to our protein data and may be due to regulatory mechanisms that generate disparity in message–protein expression. Thus, the regulatory mechanisms that influence GATA-3 levels should be further explored. GATA-3 expression in lymph nodes was also decreased in OVA-treated C3H/HeJ mice as compared to saline controls at the later time points. While the GATA-3 expression in the control groups increased slightly across the time points, the strain and treatment groups within each

time point were assayed simultaneously (on the same 96-well plate). Therefore, the data within each time point were not subject to day-to-day assay variation, making the OVA-induced decrease in GATA-3 in the C3H/HeJ mice at 24, 48 and 72 h an interesting observation suggesting strain-specific Th cell polarization subsequent to OVA exposure. Indeed, C3H/HeJ mice demonstrate a Th1-biased immune response by 24 h after OVA challenge (data not shown).

No change in GATA-3 expression was detected in lungs from either strain following allergen challenge (data not shown). This may be surprising if one anticipates that the lung, as a target organ of asthma, would be the site of early induction of the allergic immune response. However, we believe that the early response to allergen is most likely to be targeted to immune cells, thus we specifically examined the tracheobronchial lymph nodes. Our results indicate that the initial response to allergen, in terms of GATA-3, may be primarily generated by the local lymph nodes. This is consistent with the report by Caramori *et al.* (2001) in which they found no difference in GATA-3 protein expression in bronchial biopsies from asthmatics as compared to normal controls, yet GATA-3 was increased in T cells from asthmatics. Similarly, Erpenbeck *et al.* (2006) found increased GATA-3 expression in BAL cells but not bronchial biopsies from asthmatics subsequent to segmental allergen challenge. It is also conceivable that subpopulations of cells within the lung may be responsive in terms of GATA-3 production but their responses may be masked by high background levels of this protein. Specifically, high levels of GATA-3 protein and mRNA have been found in human bronchial epithelial cells (Caramori *et al.*, 2001). Future studies using techniques such as laser capture microdissection could be used to determine specifically which cell types in asthmatic murine lungs produce GATA-3.

Our results of strain-specific GATA-3 profiles at both the RNA and the protein levels support the hypothesis that the *Gata3* gene is important in our model of allergic asthma; therefore, we continued our investigation at the DNA level. The murine *Gata3* gene contains approximately 23 kb of coding region, 13 kb of 5' promoter region and 8 kb of 3' flanking region (George *et al.*, 1994). It is composed of six exons with sizes ranging from 125 to 828 bp and five introns with sizes from 418 to 6.5 kb (George *et al.*, 1994). The functional domains of murine GATA-3 protein are two zinc fingers (Cys-X2-Cys-X17-Cys-X2-Cys): (i) an amino zinc finger (N-finger) and (ii) a carboxyl zinc finger (C-finger), located in exon 4 and exon 5, respectively (Ko *et al.*, 1991; George *et al.*, 1994).

In an effort to determine whether sequence variation in *Gata3* was present between A/J and C3H/HeJ mice, which could underlie expression differences in these strains, we sequenced over 12 kb of genomic DNA in and around the *Gata3* gene including ~9 kb upstream of the ATG initiation site, all coding regions and splice sites, and ~1.5 kb of 3' UTR in both mouse strains (sequence reported as GenBank accession numbers DQ400108–DQ400115 for A/J sequence and DQ400116–DQ400123 for C3H/

Table 1. *Gata3* comparative sequence analysis

Nucleotide position	Strains from which reported sequence derives		
	This report: A/J ^a , C3H/HeJ ^b	Celera: A/J, 129 × 1/SvJ, 129S1/SvImJ, DBA/2J	George <i>et al.</i> BALB/c
–1562 to –1558	GGGCA	GGGCA	CAGG
–1000 to –999	GG	GG	G
–991	A	A	T
–979 to –977	CCC	CCC	CC
–961 to –960	CC	CC	C
–940 to –939	CC	CC	C
–931 to –930	GA	GTA	GA
–907 to –904	GGGG	GGGGG	GGGG
–693 to –962	TG	TG	TCG
54–56	CCC	CCCC	CCC
364	C	C	Y ^c
389–392	CCCC	CCCC	CCC
470–475	CCCTCC	CCCTCC	N ^d CNCC
479	T	T	A
485	C	C	N
547–548	CC	CC	CCC
689–706	(T)18	(T)18	(T)19
962–964	GGG	GGG	GG
20 673	A	C	N/A ^e

^a GenBank no. DQ400108–DQ400115; ^b GenBank no. DQ400116–DQ400123; ^c Y represents C or T; ^d N represents A, G, C, or T; ^e N/A no sequence information available.

HeJ sequence). Furthermore, we repeated the sequencing of all exons. We identified a microsatellite in intron 5 that was polymorphic between the strains; (GT)₂₆ in A/J (GenBank DQ400114) and (GT)₂₈ in C3H/HeJ (GenBank DQ400122) mice. However, this microsatellite is not likely to be functionally important for regulation of *Gata3* gene expression. No other polymorphisms were found in the examined sequence between A/J and C3H/HeJ mice. However, we did identify a number of sequence differences from those reported previously (Table 1). Based on the fact that we obtained duplicate sequence in two strains, repeated sequencing of all exons and identified high sequence similarity with database sequence, we have high confidence in the accuracy of our results.

The lack of detected functional sequence variation between A/J and C3H/HeJ strains does not directly support our hypothesis of *Gata3* as a positional candidate gene for allergen-induced airway hyperresponsiveness in our model. However, while we performed an extensive evaluation of the genomic sequence related to this gene, it was beyond the scope of this project to identify and examine the full extent of the *Gata3* regulatory regions as they are both expansive and currently undefined. Specifically, a 625 kb yeast artificial chromosome (YAC) bearing the murine *Gata3* gene as well as approximately 450 kb 5′- and 150 kb 3′ of flanking sequence contained insufficient sequence to fully support the tissue-specific expression of GATA-3 in thymus and specific neural crest-derived cells based on a LacZ reporter gene study (Lakshmanan *et al.*, 1998). Therefore, there are additional regulatory sequences that exist either further upstream or downstream of this 625 kb *Gata3* region. Thus, we can conclude that *Gata3*

gene and flanking DNA sequence do not contain the polymorphism underlying *Abhr1*; however, distant regulatory regions may contain the causative genetic variant. Together our protein, expression and sequence data indicate that while *Gata3* may drive Th2 cell differentiation, the gene and its minimal promoter in isolation are not the causative agents of *Abhr1*-related asthma susceptibility.

To elucidate the complex genetic background leading to the asthma phenotype, it is necessary to explore a diverse number of genes that may be considered as 'positional' candidate genes. Through refined mapping techniques and supported by mechanistic evidence, certain genes assume a prominent position as likely candidates for further studies. *Gata3* was one such gene. Future studies should involve clear definition of the *Gata3* regulatory regions and comparative sequence analysis of its regulatory regions that lie within *Abhr1*.

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