Regulation of Th2 Cytokine Genes by p38 MAPK-Mediated Phosphorylation of GATA-3

Kittipong Maneechotesuwan, Yao Xin, Kazuhiro Ito, Elen Jazrawi, Kang-Yun Lee, Omar S. Usmani, Peter J. Barnes and Ian M. Adcock

*J Immunol* 2007; 178:2491-2498; doi: 10.4049/jimmunol.178.4.2491

http://www.jimmunol.org/content/178/4/2491

References

This article cites 44 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/178/4/2491.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulation of Th2 Cytokine Genes by p38 MAPK-Mediated Phosphorylation of GATA-3

Kittipong Maneechotesuwan, Yao Xin, Kazuhiro Ito, Elen Jazrawi, Kang-Yun Lee, Omar S. Usmani, Peter J. Barnes, and Ian M. Adcock

GATA-3 plays a critical role in allergic diseases by regulating the release of cytokines from Th2 lymphocytes. However, the molecular mechanisms involved in the regulation of GATA-3 in human T lymphocytes are not yet understood. Using small interfering RNA to knock down GATA-3, we have demonstrated its critical role in regulating IL-4, IL-5, and IL-13 release from a human T cell line. Specific stimulation of T lymphocytes by costimulation of CD3 and CD28 to mimic activation by APCs induces translocation of GATA-3 from the cytoplasm to the nucleus, with binding to the promoter region of Th2 cytokine genes, as determined by chromatin immunoprecipitation. GATA-3 nuclear translocation is dependent on its phosphorylation on serine residues by p38 MAPK, which facilitates interaction with the nuclear transporter protein importin-α. This provides a means whereby allergen exposure leads to the expression of Th2 cytokines, and this novel mechanism may provide new approaches to treating allergic diseases. The Journal of Immunology, 2007, 178: 2491–2498.

Allergic inflammation in asthma, rhinitis, and atopic dermatitis, as well as humoral immunity against parasites, is regulated via the release of cytokines from Th2 lymphocytes. IL-4 and IL-13 regulate the synthesis of IgE, whereas IL-5 is critical for eosinophil differentiation. In mice, these Th2 cytokines are regulated by the zinc finger transcription factor GATA-3, which is predominantly expressed in Th2 cells (1). GATA-3 determines Th2 cell differentiation and selectively activates the promoters of IL-4, IL-5, and IL-13 through chromatin remodeling (2–5). The key role of GATA-3 in allergic airway inflammation has been demonstrated in mice by the reduction and release of Th2 cytokines in animals treated with dominant-negative mutants of GATA-3 and by local application of antisense oligonucleotides to GATA-3 (6, 7). Furthermore, conditional knockout of the GATA-3 gene in mice reduces expression of Th2 cytokines in vitro and in vivo (8), and similar results are seen in isolated murine CD4⁺ lymphocytes (9).

Enhanced nuclear localization of GATA-3 following TCR activation was first demonstrated in murine T cells (10). GATA-3 contains a classical nuclear import signal (11), and its size exceeds the m.w. limit for passive diffusion across the nuclear membrane (12). Nuclear import of proteins is an active process for proteins that contain a nonconsensus basic targeting sequence or nuclear localization sequence (NLS) (13). Nuclear import proteins, such as importin-α (karyopherin-α), play a critical role in transporting large proteins from the cytoplasm into the nucleus (14, 15). Importin-α has been shown to play a critical role in the nuclear import of transcription factors such as NF-AT, NF-kB, and STAT-1 in Jurkat T lymphocytes (16). A previous study has shown that deletion of a region encompassing the GATA-3 NLS region (aa 249–311) prevents nuclear localization of the overexpressed protein (11). The affinity of the importin-α-NLS interaction is a critical parameter in determining nuclear transport efficiency and may be influenced directly by phosphorylation (13, 15, 17).

We have determined the molecular mechanisms involved in nuclear translocation of GATA-3 and its regulation of Th2 cytokine genes in human T cells (HUT-78 cells and primary peripheral blood T cells), and have demonstrated a critical role for phosphorylation of GATA-3 by p38 MAPK, which is activated by TCR costimulation with anti-CD3 and anti-CD28 Abs.

Materials and Methods

Abs and reagents
The mAbs against human CD3, CD28, and importin-α were purchased from BD Biosciences. Rabbit Abs against human GATA-3 were from Santa Cruz Biotechnology; polyclonal rabbit Abs against phospho-p38 MAPK and phospho-activated transcription factor (ATF)-2 were from Cell Signaling Technology (New England Biolabs); mAbs against phosphoserine (clone 4H4) were from Affiniti Research Products; and Abs against rabbit IgG-conjugated tetramethylrhodamine isothiocyanate were from DakoCytomation. All other reagents were purchased from Sigma-Aldrich.

Cell culture and PBMC isolation
Human T cells (HUT-78), purchased from European Collection of Cell Culture, were cultured, as previously described (18). PBMC from patients with asthma were isolated by density centrifugation over Ficoll-Hypaque (density, 1.077 g/ml; Amersham Biosciences) (19). In some experiments, human T cells, PBMC isolation
Human T cells (HUT-78), purchased from European Collection of Cell Culture, were cultured, as previously described (18). PBMC from patients with asthma were isolated by density centrifugation over Ficoll-Hypaque (density, 1.077 g/ml; Amersham Biosciences) (19). In some experiments, the mAbs against human CD3, CD28, and importin-α were purchased from BD Biosciences. Rabbit Abs against human GATA-3 were from Santa Cruz Biotechnology; polyclonal rabbit Abs against phospho-p38 MAPK and phospho-activated transcription factor (ATF)-2 were from Cell Signaling Technology (New England Biolabs); mAbs against phosphoserine (clone 4H4) were from Affiniti Research Products; and Abs against rabbit IgG-conjugated tetramethylrhodamine isothiocyanate were from DakoCytomation. All other reagents were purchased from Sigma-Aldrich.

Cell culture and PBMC isolation
Human T cells (HUT-78), purchased from European Collection of Cell Culture, were cultured, as previously described (18). PBMC from patients with asthma were isolated by density centrifugation over Ficoll-Hypaque (density, 1.077 g/ml; Amersham Biosciences) (19). In some experiments, the mAbs against human CD3, CD28, and importin-α were purchased from BD Biosciences. Rabbit Abs against human GATA-3 were from Santa Cruz Biotechnology; polyclonal rabbit Abs against phospho-p38 MAPK and phospho-activated transcription factor (ATF)-2 were from Cell Signaling Technology (New England Biolabs); mAbs against phosphoserine (clone 4H4) were from Affiniti Research Products; and Abs against rabbit IgG-conjugated tetramethylrhodamine isothiocyanate were from DakoCytomation. All other reagents were purchased from Sigma-Aldrich.

Cell culture and PBMC isolation
Human T cells (HUT-78), purchased from European Collection of Cell Culture, were cultured, as previously described (18). PBMC from patients with asthma were isolated by density centrifugation over Ficoll-Hypaque (density, 1.077 g/ml; Amersham Biosciences) (19). In some experiments, the mAbs against human CD3, CD28, and importin-α were purchased from BD Biosciences. Rabbit Abs against human GATA-3 were from Santa Cruz Biotechnology; polyclonal rabbit Abs against phospho-p38 MAPK and phospho-activated transcription factor (ATF)-2 were from Cell Signaling Technology (New England Biolabs); mAbs against phosphoserine (clone 4H4) were from Affiniti Research Products; and Abs against rabbit IgG-conjugated tetramethylrhodamine isothiocyanate were from DakoCytomation. All other reagents were purchased from Sigma-Aldrich.

Cell culture and PBMC isolation
Human T cells (HUT-78), purchased from European Collection of Cell Culture, were cultured, as previously described (18). PBMC from patients with asthma were isolated by density centrifugation over Ficoll-Hypaque (density, 1.077 g/ml; Amersham Biosciences) (19). In some experiments, the mAbs against human CD3, CD28, and importin-α were purchased from BD Biosciences. Rabbit Abs against human GATA-3 were from Santa Cruz Biotechnology; polyclonal rabbit Abs against phospho-p38 MAPK and phospho-activated transcription factor (ATF)-2 were from Cell Signaling Technology (New England Biolabs); mAbs against phosphoserine (clone 4H4) were from Affiniti Research Products; and Abs against rabbit IgG-conjugated tetramethylrhodamine isothiocyanate were from DakoCytomation. All other reagents were purchased from Sigma-Aldrich.

Cell culture and PBMC isolation
Human T cells (HUT-78), purchased from European Collection of Cell Culture, were cultured, as previously described (18). PBMC from patients with asthma were isolated by density centrifugation over Ficoll-Hypaque (density, 1.077 g/ml; Amersham Biosciences) (19). In some experiments, the mAbs against human CD3, CD28, and importin-α were purchased from BD Biosciences. Rabbit Abs against human GATA-3 were from Santa Cruz Biotechnology; polyclonal rabbit Abs against phospho-p38 MAPK and phospho-activated transcription factor (ATF)-2 were from Cell Signaling Technology (New England Biolabs); mAbs against phosphoserine (clone 4H4) were from Affiniti Research Products; and Abs against rabbit IgG-conjugated tetramethylrhodamine isothiocyanate were from DakoCytomation. All other reagents were purchased from Sigma-Aldrich.
Peripheral venous blood (150 ml) was collected, CD3\(^+\) T cells were isolated by negative selection (Miltenyi Biotec), and CCR5\(^+\) cells were further isolated by immunomagnetic beads (20). To drive peripheral blood T cells toward a Th2 phenotype, PBLs were prepared by negative selection and resuspended in RPMI 1640 containing 10% FCS. A total of 4 \(\times\) \(10^6\) cells was incubated for 5 days with human IL-4 (20 ng/ml; R&D Systems) and a neutralizing anti-IFN-\(\gamma\)-Ab (2.5 \(\mu\)g/ml; R&D Systems) to induce a Th2 phenotype. Control cells were incubated in medium alone for the duration of the experiment. At the end of this time, cells were stimulated with anti-CD3/CD28 (1 \(\mu\)g/ml each) for 1 h at 37°C to stimulate Th2 cytokine release. The patients with asthma were naive to corticosteroid therapy and treated with inhaled bronchodilators as required. All patients gave informed consent, and the study was approved by the Ethics Committee of the Royal Brompton and Harefield NHS Trust.

**Knockdown of GATA-3 expression**

RNA interference was used to specifically suppress expression of GATA-3 in HUT-78 cells. Four ds21–23mer RNA oligonucleotides directed against GATA-3 were synthesized by Dharmacon and used in combination (100 nM each) to suppress GATA-3 expression. Target sequences are as follows: AAGAaAGAGUGCCUCaAGuaC and AAaUCGAGACCAaAG ACCAaAA. Cells were transfected with small interfering RNA (siRNA) using Gene Silencer (Gene Therapy Systems), as described by the manufacturer, and GATA-3 expression was monitored by RT-quantitative PCR (described, using cDNA probes (22). Primers for IL-4, IL-5, and IL-13 were as follows: forward, GCC-3\(^b\) -TTATCTAGATTAGCTGTAACCCAT\(^b\) and reverse, 5\(^b\)-AAATCATTCAA and reverse, 5\(^b\)-TTGGAGACTCCTCACGCATGT. For relative quantification, RT-PCR was conducted, as previously described (21).

**Multiplex detection of Th1/Th2 cytokines**

The levels of human Th1/Th2 cytokines and chemokines were detected in HUT-78 culture medium before and after 18-h stimulation with anti-CD3/CD28 using the Beadlyte Human 22-Plex MultiCytokine Detection System (Upstate Biotechnology), according to the manufacturer’s instructions. The sensitivity obtained for each cytokine and chemokine was 1.9 pg/ml.

**Construction of an \(\Delta\text{NLS-GATA-3}\) mutant**

p-YFP-GATA-3 was constructed by inserting the full-length GATA-3 cDNA (provided by A. Winoto (University of California, Berkeley, CA) into the EYFP-C1 vector (BD Clontech). Full-length GATA-3 was isolated by PCR using the following primers: forward, 5'-CTCAGATCTAAAGC and reverse, 3'-CTGATTAGCCTTGGAAA. The resulting product was purified and ligated into the EYFP-C1 vector (BD Clontech). An NLS-deficient GATA-3 (\(\Delta\text{NLS-YFP-GATA-3}\)) was obtained by removal of the previously described NLS domain (aa 249–311) by excision with XcmI and PsiI.

**Transfection**

HUT-78 cells were transfected with EGF-GATA-3 or \(\Delta\text{NLS-EGF-GATA-3}\) using lipofectamine, according to the manufacturer’s instructions (Invitrogen Life Technologies); before cells were serum deprived for 24 h. Cells were subsequently stimulated with anti-CD3/CD28. Cytosins were prepared and GATA-3 localization was determined by confocal microscopy, as previously described (21).

**RT-PCR**

Total RNA was extracted using lysis buffer (Rneasy kit; Qiagen). During RNA purification, genomic DNA was digested with RNase-free DNase (Amersham Biosciences). Next, 0.5 \(\mu\)g of total RNA was reverse transcribed using the avian myeloblastosis virus reverse transcription (Promega). For relative quantification, RT-PCR was conducted, as previously described, using cDNA probes (22). Primers for IL-4, IL-5, and IL-13 were.

**FIGURE 1.** The role of GATA-3 in Th2 cytokine expression demonstrated using siRNA in HUT-78 cells. A, Endogenous GATA-3 binds the native IL-4 and IL-5 promoters. ChIP assays were performed using an Ab to human GATA-3. Immunoprecipitated GATA-3 was analyzed by PCR for IL-4 and IL-5 gene. No association with DNA was observed in complexes immunoprecipitated with an irrelevant isotype control Ab (IgG). Input DNA samples were run as controls. B, RT-PCR demonstrates IL-4 and IL-5 mRNA expression following anti-CD3/CD28 Ab co-stimulation of HUT-78 cells for 14 h, in comparison with the mRNA expression in response to each stimulus applied separately. GAPDH was used to control for loading. Mean stimulation values \((n = 3–4)\) are given above mRNA lanes. C, Knockdown of GATA-3 mRNA expression compared with housekeeping gene GAPDH using siRNA (G3), with no effect of a scrambled siRNA control (Scr). Cell stimulation with anti-CD3/CD28 Abs had no effect on GATA-3 mRNA. Mean values \(\pm\) SEM of four independent experiments; **, \(p < 0.01\). D, Knockdown of GATA-3 mRNA expression inhibits IL-4 mRNA expression after stimulation with anti-CD3/CD28 Abs, whereas scrambled siRNA control had no effect. Mean \(\pm\) values are shown of four independent experiments; \(p < 0.05\). E, Knockdown of GATA-3 mRNA expression inhibits IL-13 mRNA expression after stimulation with anti-CD3/CD28 Abs, whereas scrambled siRNA control had no effect. Mean \(\pm\) values are shown of four independent experiments; \(p < 0.05\). F, Knockdown of GATA-3 mRNA expression has no inhibitory effect on IL-8 mRNA expression after stimulation with anti-CD3/CD28 Abs. Mean \(\pm\) values are shown of four independent experiments.
microscopy in the nucleus using an Ab specific for GATA-3 (red fluorescence with Texas Red staining) and 4',6-diamidino-2-phenylindole (DAPI) dye for DNA (blue fluorescence) at rest (t = 0 min; upper panel) and 30 min after stimulation with anti-CD3/CD28 Abs (t = 30 min; lower panel), showing translocation to the nucleus. Cells stained with isotopic specific polyclonal IgG were negative for GATA-3 (data not shown). B, Western blots showing increased GATA-3 in the nuclear fraction of anti-CD3/CD28 Ab-stimulated HUT-78 cells. Cytosolic (Cyt) and nuclear (Nucl) fractions were separated and subjected to GATA-3 immunoblotting. C, Histone H1α and MEK-1 were used to ensure equal loading of nuclear and cytoplasmic proteins, respectively. D, Removal of the putative GATA-3 NLS sequence prevents GATA-3 nuclear import following anti-CD3/CD28 Ab stimulation. Confocal microscopy images show cells transfected with wild-type GATA-3 labeled with a fluorescent tag showing yellow/green fluorescence in the cytoplasm of a transfected cell (left panel), but nuclear translocation after stimulation with anti-CD3/CD28 Abs (middle panel). Transfection with GATA-3 in which the NLS sequence has been deleted (ΔNLS-GATA-3) shows no nuclear localization after cell activation (right panel), indicating the importance of the NLS sequence for nuclear translocation of GATA-3. DAPI labels DNA to delineate the nuclei (blue fluorescence).

from Sigma-Genosys. Sequences of GADPH used were as follows (listed 5′–3′): 5′-CCACCTATGGCAAAATTTCCATGCC and 3′-TCTAGACCGGCAAGTCTAGGTCCAC.

Cell fractionation, immunoprecipitation, and Western blot analysis

Nuclear and cytoplasmic fractions were prepared, as previously described (22). Whole cell lysates were prepared in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl) in the presence of complete protease mixture inhibitor. Lysates were centrifuged at 4°C for 10 min at 12,000 rpm in an Eppendorf microfuge to remove cellular debris and immunoprecipitated with either 10 μl of Ab against GATA-3 or importin-α using A/G agarose slurry, in the presence of protease inhibitor using Catch and Release methodology (Upstate Biotechnology). Western blot analysis was performed, as described (22), using anti-GATA-3, anti-importin-α, anti-phospho-p38 MAPK, anti-phospho-ATF-2, and anti-phosphoserine Abs. Immunoreactive proteins were detected using an ECL kit (Amersham Biosciences).

Chromatin immunoprecipitication (ChIP)

ChIP was performed, as previously described (23), in HUT-78 T cells with 2 μg of anti-GATA-3 (Santa Cruz Biotechnology), or isotypic Ig G as a nonspecific control (Santa Cruz Biotechnology) overnight at 4°C. Promoter sequences were detected with PCR primers for the IL-4 promoter (−772 to +12), as follows: forward, 5′-GAAACCTAGAATGACCTAC-3′; reverse, 5′-GGTGAAGACCATTATAAGTTG-3′. Promoter sequences were detected with PCR primers for the IL-5 promoter, as follows: (−445 to +4); forward, 5′-TTAACCTAGCCAGTCATAAG-3′; reverse, 5′-GCTCTGAAACGTTCTG-3′. PCR was performed using a Hybrid Omnigene thermal cycler (Thermo Hybaid) with cycling parameters of 72°C for 10 min, 35 cycles at 94°C for 45 s, 52°C for 45 s, and 72°C for 45 s.

In vitro alkaline phosphatase treatment

Enzymatic dephosphorylation was performed, as described previously (24). Whole cell (100 μg) proteins from anti-CD3/CD28-treated cells were incubated with or without 30 U of calf intestinal alkaline phosphatases (New England Biolabs) in reaction buffer containing 50 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl, and 1 mM DTT (pH 7.9) for 1 h, at 37°C. As a control, the enzyme was coincubated with 50 mM sodium pyrophosphate (NaPi), a phosphatase inhibitor.

Statistical analysis

Data are represented as the mean ± SEM of at least three independent experiments and were compared using a two-tailed Student’s t test. The null hypothesis was rejected at p < 0.05.

Results

HUT-78 cells have a predominant Th2 phenotype

Using ChIP assays to examine transcription factor binding to native promoters, we showed that anti-CD3/CD28 Ab stimulation markedly enhanced GATA-3 enrichment at the IL-4 and IL-5 promoters (Fig. 1A) and that this was followed temporally by a 10- and 20-fold increase in IL-4 and IL-5 mRNA, respectively (Fig. 1B). HUT-78 cells were predominantly Th2-like in phenotype expressing IL-4, IL-5, IL-10, IL-13, and GM-CSF with no expression of IL-3, IFN-γ, or TNF-α. However, these cells did also express IL-2. Stimulation of HUT-78 cells with anti-CD3/CD28 enhanced the expression of IL-4 and IL-5 (Fig. 1) and of IL-13 (1528 ± 194 vs 516 ± 52 pg/ml; p < 0.05) and GM-CSF (440 ± 64 vs 94 ± 13 pg/ml; p < 0.05), but not of IL-10 (1855 ± 382 vs 1916 ± 157 pg/ml; p = NS).

GATA-3 is essential for Th2 cytokine production in human T cells

Interference RNA was used to selectively knock down GATA-3 expression in HUT-78 cells. Transfection of siRNAs resulted in
marked suppression of GATA-3 mRNA expression (Fig. 1C) within 48 h, whereas control scrambled siRNAs had no effect on its expression. GATA-3 knockdown completely suppressed anti-CD3/CD28-stimulated IL-4 mRNA expression (Fig. 1D), whereas control siRNAs had no effect. These results confirm that GATA-3 is essential for Th2 cytokine production in human T cells and support recent data from GATA-3 conditional knockout studies in mice (8). Similar results were obtained with IL-13 expression (Fig. 1E), but not with IL-8 expression (Fig. 1F).

GATA-3 nuclear translocation and Th2 cytokine gene regulation

GATA-3 immunoreactivity was predominantly localized to the cytoplasm before cell stimulation, but cell activation with anti-CD3/CD28 resulted in rapid (within 30 min) GATA-3 nuclear translocation.

---

**FIGURE 3.** Physical association between GATA-3 and importin-α in HUT-78 cells. A, Coimmunoprecipitation analysis demonstrating the time course of GATA-3/importin-α interaction following anti-CD3/CD28 costimulation, using anti-GATA-3 Ab to immunoprecipitate (IP) and anti-importin-α Ab to immunoblot (IB). B, Coimmunoprecipitation using anti-importin-α Ab to immunoprecipitate (IP) and anti-GATA-3 Ab to immunoblot (IB). C, Association between GATA-3 and importin-α was markedly increased after stimulation with anti-CD3/CD28 Ab (stim), but not in resting cells or with control Ab (unstim). Mean ± SEM values of three experiments are shown; p < 0.01. D, Anti-CD3/CD28-stimulated GATA-3 did not associate with the nuclear import protein transportin 1. Anti-GATA-3 immunoprecipitation and transportin 1 immunoblotting; the transportin positive control (PC) is also shown. E, Anti-CD3/CD28 stimulation did not affect the ability of transportin 1 to associate with the transportin-binding protein heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), as demonstrated by coimmunoprecipitation Western blot experiments. All above results are representative of at least three independent experiments.

---

**FIGURE 4.** Activation of p38 MAPK by anti-CD3/CD28 Ab stimulation of HUT-78 cells. A, Anti-CD3 Ab caused a concentration (left) and time-dependent phosphorylation of p38 MAPK detected with an Ab against Thr<sup>180</sup> and Tyr<sup>182</sup>, which detected an activated form of p38 MAPK (p38Thr/Tyr-p). B, Anti-CD28 Ab caused a concentration (left) and time-dependent phosphorylation of p38 MAPK. C, Combined anti-CD3/CD28 Ab gives a greater increase in phosphorylated p38 MAPK with an effect persisting over 60 min. D, Phosphorylation of ATF-2 (p-ATF-2), a downstream target of p38 MAPK activation, by anti-CD3/CD28 Ab stimulation with onset by 1 h and persistence for over 6 h. E, Inhibition of ATF-2 phosphorylation after anti-CD3/CD28 stimulation by SB203580. Each result is representative of at least three independent experiments.
Western blotting confirmed these results and showed that GATA-3 nuclear localization was rapid and persisted for at least 14 h (Fig. 2, B and C). These results suggest that GATA-3 nuclear localization is a critical step in the regulation of Th2 cytokine gene expression.

Interaction of GATA-3 and importin-α

We investigated whether nuclear import of GATA-3 required an interaction with importin-α. Stimulation of HUT-78 cells caused a rapid (<10-min) interaction of GATA-3 with importin-α, which peaked at 20 min and returned to baseline by 60 min (Fig. 3, A and B). Anti-CD3/CD28 Ab markedly increased the association between GATA-3 and importin-α (Fig. 3C). Thus, rapid association with importin-α occurred before GATA-3 nuclear localization. Deletion of the GATA-3 NLS region prevented nuclear translocation of YFP-GATA-3 following anti-CD3/CD28 stimulation (Fig. 2D), providing further evidence for a role of importin-α in GATA-3 function. In contrast, GATA-3 did not interact with the non-NLS recognizing nuclear transport factor transportin-1 after cell stimulation with anti-CD3/CD28 Ab (Fig. 3D). This was not due to a lack of transportin-1 activity, because transportin-1 was seen to associate with associated factors such as heterogeneous nuclear ribonucleoprotein A1 (Fig. 3E).

Activation of p38 MAPK by anti-CD3/CD28 Abs

Using an Ab directed against Thr180 and Tyr182 to recognize the activated form of phosphorylated p38 MAPK, we showed that anti-CD3 Ab (Fig. 4A) and anti-CD28 Ab (Fig. 4B) independently increased p38 phosphorylation. Although the maximal induction of p38 phosphorylation was not increased by combined anti-CD3/CD28 Abs, the induction of p38 phosphorylation and activity occurred earlier (before 15 min) and was more sustainable (maximal activation still seen after 60 min) than by cross-linking of CD3 or CD28 alone (Fig. 4 and data not shown). ATF-2, a target protein downstream of p38 MAPK, was also phosphorylated by anti-CD3/CD28 Abs at 60 min, and this persisted for over 6 h (Fig. 4D). Phosphorylated ATF-2 was detected in nuclear fractions after anti-CD3/CD28 Ab stimulation, and this was inhibited by SB203580, a

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** GATA-3 phosphorylation is essential for GATA-3 nuclear import in HUT-78 cells. A, Immunoblotting analysis of anti-CD3/CD28 costimulation showing increased expression of serine phosphorylated (P-Ser) GATA-3 protein at 30 min. Whole cell extracts treated with CIP in the absence and presence of the phosphatase inhibitor NaPi (50 mM) were used as controls. B, Immunoblotting analysis of CIP-treated whole cell extracts from anti-CD3/CD28-costimulated cells revealed decreased interaction between GATA-3 and importin-α, which was reversed by NaPi. The above results are representative of at least three independent experiments.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** GATA-3 phosphorylation by p38 MAPK in HUT-78 cells. A, Western blot analysis of the effects of the selective p38 MAPK inhibitor (SB203580) on the expression of serine-phosphorylated GATA-3 protein (p-Ser) in anti-CD3/CD28-costimulated cells. SB203580 (1 μM) was preincubated for 30 min before stimulation, and proteins were extracted after a further 30 min. B, Coimmunoprecipitation of SB203580-treated cells demonstrating a reduced interaction between GATA-3 and importin-α (Imp-α) using immunoprecipitation with anti-GATA-3 Ab and immunoblotting with anti-importin-α Ab (upper blot) and immunoprecipitation with anti-importin-α Ab and immunoblotting with anti-GATA-3 Ab (lower blot). SB203580 was preincubated for 30 min before stimulation, and proteins were extracted after a further 20 min. C, Suppression of anti-CD3/CD28 Ab-mediated GATA-3 nuclear transport by SB203580, resulting in GATA-3 cytoplasmic retention. SB203580 was preincubated for 30 min before stimulation, and proteins were extracted after a further 60 min. MEK and histone H1° were used as markers for equal cytoplasmic and nuclear loading, respectively. D, Concentration response of the inhibitory effect of SB203580 on nuclear and cytoplasmic GATA-3 protein after stimulation with anti-CD3/CD28 Ab. Mean ± SEM of three to four separate experiments are shown.
selective inhibitor of p38 MAPK, with inhibition at a concentration of 0.01 μM (Fig. 4E).

Role of MAPK in GATA-3 translocation

The phosphorylation status of nuclear export substrates is required for optimal interaction with importin-α (12, 13). We investigated whether phosphorylation of GATA-3 affects its ability to interact with importin-α. Anti-CD3/CD28 Ab stimulation of HUT-78 cells induced GATA-3 serine phosphorylation (Fig. 5). In vitro treatment with calf intestinal phosphatase (CIP) to reduce phosphorylation markedly reduced GATA-3 phosphorylation (Fig. 5A), and caused the dissociation of GATA-3 from importin-α. In contrast, inhibiting CIP activity with NaPi prevented GATA-3 dissociation from importin-α. These results indicated that serine phosphorylation of GATA-3 is essential for its interaction with importin-α. In contrast, anti-CD3/CD28 stimulation had no effect on GATA-3 threonine phosphorylation or on importin-α phosphorylation status (data not shown).

Because p38 MAPK is activated by anti-CD3/CD28 Abs, we next investigated the effects of p38 MAPK on GATA-3 phosphorylation. Anti-CD3/CD28 Ab stimulation increased serine phosphorylation of GATA-3, and this was inhibited by SB203580 (1 μM) (Fig. 6A). SB203580 resulted in marked impairment of the association between GATA-3 and importin-α (Fig. 6B). Western blot analysis of fractionated protein extracts derived from HUT-78 cells showed that treatment of SB203580 caused a concentration-dependent increase in cytoplasmic expression of GATA-3 protein in parallel with decreased nuclear GATA-3 expression (Fig. 6C).

This effect of SB203580 was concentration related and peaked with a 15.8-fold rise in cytoplasmic expression of GATA-3 with SB203580 (1 μM) compared with a 4.2-fold increase at 0.01 μM (Fig. 6D).

Inhibition of the other MAPK pathways, ERK, and JNK with specific inhibitors (PD098059 and SP600125, both at 1 μM, respectively) did not affect GATA-3 phosphorylation, association with importin-α, or nuclear import (data not shown). Taken together, these data indicate that p38 MAPK-mediated phosphorylation facilitates GATA-3 nuclear import by promoting its interaction with importin-α.

GATA-3 in primary blood T lymphocytes

Peripheral blood T cells expressing GATA-3 purified from mild asthmatic subjects demonstrated both cytoplasmic (42%) and nuclear (58%) GATA-3 localization (Fig. 7A), confirming previous results (20). Stimulation with anti-CD3/CD28 caused nuclear translocation of GATA-3 at 60 min, demonstrated by both confocal microscopy (Fig. 7A) and Western blotting (Fig. 7B). This was associated with an earlier (30-min) interaction between GATA-3 and importin-α (Fig. 7C), and with GATA-3 phosphorylation (Fig. 7D), confirming the results seen in HUT-78 cells. Importantly, SB203580 (1 μM) attenuated anti-CD3/CD28-induced GATA-3 serine phosphorylation in these cells (Fig. 7D). Interestingly, when we cultured cells ex vivo, we observed a dramatic shift of GATA-3 from the cytoplasm to the nucleus whether cells were incubated in a Th2-inducing environment or not (Fig. 7E).
Discussion
In this study, we present evidence that the nuclear import of GATA-3 from its normal cytoplasmic location requires the direct interaction with the nuclear transport protein importin-α, following p38 MAPK-mediated serine phosphorylation. Costimulation with anti-CD3 and anti-CD28 mAbs, which mimics the activation of T cells by APCs, results in activation of the p38 MAPK signal transduction pathways. The resulting phosphorylation of GATA-3, interaction with importin-α, and nuclear localization result in binding of GATA-3 to the promoter region of Th2 cytokine genes and increased gene expression, thereby linking stimulation of T cells by APCs to Th2 cytokine secretion and allergic inflammation. Studies were initially conducted in HUT-78 T cells, which we show to have many characteristics of Th2 cells. Results in freshly isolated peripheral blood T cells confirmed the cytoplasmic localization of GATA-3 in 40% of these cells and the subsequent nuclear localization following anti-CD3/CD28 stimulation associated with changes in GATA-3 phosphorylation and interaction with importin-α.

The expression of Th2 cytokines genes has previously been reported to be blocked by p38 MAPK inhibition (25–30). In murine T cells, both the TCR CD3 and coreceptor CD28 need to be ligated for maximal duration of p38 MAPK activation (31), and this is confirmed in our studies. In human T cells, Th2 cytokines (IL-4, IL-5, and IL-13) are more effectively inhibited by a p38 MAPK inhibitor after anti-CD3/CD28 stimulation than the Th1 cytokine IL-2, indicating some specificity of effect and consistent with an inhibitory effect on GATA-3 (32). The location of the serine residue(s) of GATA-3 that is phosphorylated by p38 MAPK is not yet determined, but there are at least 12 potential MAPK consensus sites. The role of ERK and JNK in the regulation of Th2 cytokines is less well defined and appears to be dependent on the stimulus used to activate T cells. CD3 activation alone results in the activation of ERK pathways (33, 34). Activation of CD28 alone weakly activates ERK pathways (35), whereas engagement of CD3 and CD28 as well as phorbol esters synergistically activates JNK pathways (33, 36). Activation of CD3 and another costimulatory molecule ICOS results in activation of ERK and JNK, but not p38 MAPK, in human CD4+ cells (30). Our results show that only p38 MAPK, but not ERK or JNK, is involved in the phosphorylation of GATA-3 and its interaction with importin-α. These data confirm a previous report of direct phosphorylation of GATA-3 by p38 MAPK (in response to cAMP stimulation) in murine T cells (37).

Protein phosphorylation of nuclear import substrates plays an important role in nuclear import of proteins, particularly for the optimal interaction with importin-α (17). Given that the phosphorylated serine sites located within the N-terminal of the NLS sequence in GATA-3 are largely negative in terms of charge, this may enhance interaction with the positively charged regions of importin-α (38).

Our results suggest that p38 MAPK plays a critical role in the regulation of Th2 cytokine genes and that inhibition of p38 MAPK may have therapeutic benefit in suppressing allergic inflammation. In OVA-sensitized and exposed mice and guinea pigs, a p38 MAPK inhibitor effectively suppressed eosinophilic inflammation in the lungs (39). An aerosolized p38α MAPK antisense oligonucleotide markedly reduced pulmonary eosinophilic inflammation, airway hyperresponsiveness, and mucus hypersecretion in OVA-sensitized mice after allergen exposure (40). This was accompanied by a significant reduction in IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid, indicating a likely inhibitory effect on Th2 cells. This suggests that p38 MAPK inhibitors may be of potential benefit in the therapy of asthma and other allergic diseases (41, 42).

Data in murine cells (8) indicate that conditional deletion of GATA-3 in polarized murine Th2 cells affects IL-5 and IL-13, but not IL-4 release, and is at odds with the results presented in this study. The same group has also recently presented data (43) indicating that GATA-3 was important for IL-4 production in naive murine CD4+ T cells following anti-CD3/CD28 stimulation and that IL-4 release could be attenuated by a MAPK inhibitor. The data presented in this study indicate that in HUT-78 cells, GATA-3 is involved in IL-4 production. Further studies using selective knockdown of GATA-3 in primary human peripheral blood Th2 cells are needed to confirm whether the differences between the results reported in this study and those seen in murine cells reflect a species difference or are unique to HUT-78 cells. Interestingly, we also demonstrated that culture of peripheral blood cells for 5 days in cell culture resulted in nuclear translocation of GATA-3 independent of whether Th2-inducing conditions were used or not. This suggests that either a component of the cell culture conditions drives GATA-3 translocation or that a constituent of plasma holds GATA-3 within the cytoplasm. An investigation of this effect is beyond the scope of the current manuscript.

In summary, our data provide the first evidence for an active contribution of phosphorylated GATA-3 nuclear import in the regulation of Th2 cytokine gene expression and the critical role of p38 MAPK. Prevention of phosphorylated GATA-3 interaction with importin-α, by inhibition of p38 MAPK or via other novel strategies, may provide a new approach for the treatment of allergic diseases (44).

Acknowledgment
We thank Professor Malcolm Johnson for helpful discussions.

Disclosures
P.J.B., I.M.A., and K.I. have received research funding from GlaxoSmithKline and AstraZeneca.

References


