

Airway Epithelial STAT3 Is Required for Allergic Inflammation in a Murine Model of Asthma¹

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The STAT3 transcription factor is critical for cytokine signaling and the acute phase response, but its role in allergic asthma is largely undefined. To investigate the role of STAT3 in mediating allergic inflammation, we used chemical and genetic approaches to inactivate STAT3 in the airway epithelium of mice. In a murine model of chronic asthma, we demonstrate that the administration of house dust mite (HDM) leads to robust STAT3 activation in the airway epithelium, smooth muscle, and immune cells in the lungs of C57BL/6 mice. To investigate the role of STAT3 in HDM-induced airway inflammation, a conditional knockout of STAT3 in the airway epithelium was generated, e-STAT3^{-/-}. We determined that e-STAT3^{-/-} mice had a significant decrease in HDM-induced airway eosinophilia, lung Th2 accumulation, and chemokines compared with wild-type animals. Importantly, the e-STAT3^{-/-} mice had a significant decrease in airway hyperresponsiveness to methacholine. The administration of two STAT kinase inhibitors diminished STAT3 activation and markedly abrogated the HDM-induced lung inflammation. These findings suggest that STAT3 acts as a novel epithelial regulator of the allergic response by altering Th2 cell recruitment and effector function, and thus, targeting this molecule may provide the basis for a novel asthma therapy. *The Journal of Immunology*, 2007, 178: 6191–6199.

Asthma is a chronic inflammatory disease characterized by mucus hypersecretion, airway hyperresponsiveness (AHR),³ and airway wall remodeling that results in variable airway obstruction in genetically susceptible individuals (1). Aberrant Th2 immune responses to common Ags in the environment results in the generation of cytokines such as IL-4, IL-5, and IL-13, with the subsequent recruitment and activation of eosinophils and mast cells in the airway (2). Although multiple cell types, such as APCs and lymphocytes, are critical in the allergic response, the bronchial epithelium bridges the external and internal environments and is one of the first points of contact for allergens and thus likely plays a crucial role in establishing airway inflammation. Complex allergens, such as house dust mites (HDMs), which contain both proteases and endotoxin, induce an immune response by damaging the airway epithelium and by activating surface receptors, such as the pattern recognition receptors or the protease-activated receptors (3). These events potentially lead to enhanced allergen contact with APCs and the production of cytokines, growth factors, and repair proteins by the epithelium (4). Some studies suggest that the bronchial epithelium

may even participate in Ag presentation to lymphocytes via class II Ags on their surface (5). The precise signaling pathways by which aeroallergens modulate epithelial cell function, however, remain incompletely defined.

The STATs are a family of cytokine and growth factor-inducible transcription factors that are pivotal in immune and inflammatory responses (6, 7). Upon cytokine stimulation, STATs become activated by phosphorylation of tyrosine residues by receptor-associated Jak kinase family members. The Src family of kinases has also been shown to mediate STAT tyrosine phosphorylation by growth factors, and in some instances, by cytokines (8). This phosphorylation results in conformational change via P-Tyr-SH2 domain interactions, resulting in translocation of STATs to the nucleus where they associate with sequence-specific, DNA-binding elements to regulate transcription (9, 10). Both STAT6 and STAT1 have been shown to be important in the regulation of lung inflammation in response to allergens and viruses in murine models and in humans with asthma (11–14). STAT3 was identified initially as an acute phase response gene in the liver and has a pivotal role in directing inflammatory responses by inducing the gene expression of cytokines, chemokines, and adhesion molecules (15). Although prior studies suggest that STAT3 has a largely anti-inflammatory role in innate immune responses, elucidation of the role of STAT3 in biologic responses in general has been hindered by the fact that deletion of the *STAT3* gene results in embryonic lethality (16–18). Much less is known about the role of STAT3 in mediating allergic responses (19, 20).

HDM is one of the most common aeroallergens and is implicated in allergy and asthma symptoms in ~10% of the population (21). Chronic exposure to HDM extract in mice has been shown to lead to persistent airway inflammation, AHR, and airway remodeling, making this an ideal model for the study of asthma (21, 22). In this study, we use a HDM model of asthma to study the role of STAT3 in the airway epithelium in the regulation of allergic inflammation. To this end, we compared the effects of chronic HDM exposure on the development of airway eosinophilia, lung Th2 cell accumulation, airway cytokine and chemokine levels, and AHR in mice with a STAT3 conditional knockout (KO) in the airway epithelium

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; e-STAT3, epithelial STAT3; HDM, house dust mite; i.n., intranasal(ly); KO, knockout; MCh, methacholine; p-STAT3, phospho-STAT3; TARC, thymus and activation-regulated chemokine; TyrA1, tyrphostin A1; WT, wild type.

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and their wild-type (WT) littermates. Our studies demonstrate that airway epithelial STAT3 (e-STAT3) is essential for all of these features of chronic asthma. Moreover, chemical inactivation of epithelial STAT3 signaling was also highly effective at preventing HDM-induced inflammation in mice. Thus, these data have identified STAT3 as a novel epithelial regulator of the allergic response that may be suitable as a target for future asthma therapies.

Materials and Methods

Generation of CC10-Cre/STAT3^{flox/flox} mice

CC10-Cre transgenic mice were generated as previously described and provided on a C57BL/6 background by T. Mariani (Harvard University, Boston, MA) (23). STAT3^{flox/flox} mice were generated as previously described and provided on a C57BL/6 background by S. Akira (Osaka University, Osaka, Japan) (23, 24). These mice were crossed together to generate a lung airway epithelial STAT3 conditional KO mouse CC10-Cre/STAT3^{flox/flox} called e-STAT3^{-/-}. Mice were genotyped from DNA isolated by tail clips with PCR primers to the STAT3 flox gene and the Cre gene as described previously (18, 24). Successful KO was verified by immunohistochemistry as described below. All experiments were done using age-matched WT littermate control mice. All animal studies were performed in accordance with the guidelines of the Tufts University Institutional Animal Care and Use Committee.

Chronic HDM model

Eight-week-old BALB/c (inhibitor studies) mice or C57BL/6 (e-STAT3^{-/-} studies) mice were exposed to 40 μ g of purified HDM extract (Greer Laboratories) without exogenous adjuvant in 25 μ l of saline intranasally (i.n.) 5 days/wk for up to 7 consecutive wk (21). For each treatment, 8–10 mice were used per group.

LPS administration

C57BL/6 WT and KO mice were given 12 mg/kg LPS by i.p. administration. Mice were sacrificed 2 h later, and lungs were inflated with 10% formaldehyde for paraffin embedding. Immunohistochemistry was performed as below.

Inhibitor administration

BALB/c mice were given HDM 5 days/wk i.n. for 3 wk. At $t = 3$ wk, mice were given i.n. tyrphostin A1 (TyrA1) (1 mg/kg) or SU6656 (8 mg/kg), both inhibitors together, or vehicle (DMSO) 5 days/wk for 3 wk along with concurrent HDM treatment (6 wk total HDM).

Bronchoalveolar lavage fluid (BALF) collection

Three days after the last HDM exposure, mice were sacrificed, and their tracheas cannulated with a 20-gauge catheter for BALF collection. The lungs were lavaged twice with 1 ml of a 0.1% BSA saline solution. Supernatant was preserved for cytokine analysis, and cell pellet was resuspended in 1 ml of saline solution for differential cell analysis according to morphologic criteria.

BALF cytokine and chemokine analysis

Cytokine levels were measured in triplicate samples of BALF or in total lung lysates from each animal by ELISA as described previously (25). IL-13, thymus and activation-regulated chemokine (TARC), KC, eotaxin, TNF- α , and IFN- γ ELISA kits were purchased from R&D Systems. IL-5 and IL-4 ELISA kits were purchased from BD Biosciences.

Histology and immunohistochemistry

Mice were sacrificed, and the lungs were perfused with PBS. Lungs were inflated and fixed in 10% formaldehyde at a pressure of 25 cm of water. Tissues were cut mid-sagittally and embedded in paraffin 24 h after fixation. Serial sections were obtained for histological analysis. Sections were stained with H&E. For immunohistochemistry slides were stained with a phospho-STAT3 (p-STAT3) (Tyr⁷⁰⁵) Ab (Cell Signaling Technology) and a peroxidase-based assay with diaminobenzidine as the chromagen as described previously (26). Five high-powered fields from each animal were examined, and the images shown are representative of the overall experimental results (eight animals per group).

Western blotting

Mice were sacrificed, and lungs were isolated and crushed in a high-salt lysis buffer. Total protein was run out on an SDS-Page gel. Abs against

p-STAT3 (Tyr⁷⁰⁵) or STAT3 (Cell Signaling Technology) were used to measure STAT3 activation.

Lung mononuclear cell isolation

The whole right lung was used for isolation of mononuclear cells by digestion with 150 U/ml type III collagenase (Sigma-Aldrich) at 37°C for 1 h. Lungs were crushed through sterile metal screens followed by filtration through nylon membranes (55 μ m) and centrifugation at 1000 rpm for 10 min at 4°C. The resulting pellet was resuspended in 5 ml of HBSS and layered on a double Percoll gradient consisting of a bottom layer of 60% Percoll and a top layer of 30% Percoll. Samples were centrifuged at 2000 rpm for 25 min. After centrifugation, the interface containing the lung mononuclear cells was collected, washed, counted, and pooled for FACS as previously described by others (27).

Flow cytometry

Lymphocyte gates were identified based on cell size and granularity and were verified in forward-vs-side scatter plots by backgating these histograms from single-stained Thy1.2⁺ cells. Lung mononuclear cells were stained with three fluorescently conjugated Abs: Thy 1.2-allophycocyanin (BD Pharmingen), CD4-PE (BD Pharmingen), and T1/ST2-FITC (MD Biosciences). To minimize nonspecific binding, 4×10^5 mononuclear cells were preincubated with Fc block (BD Pharmingen). A minimum of 50,000 events were counted on a FACSCalibur. Analysis was performed using Summit software (DakoCytometry).

Measurement of pulmonary function

After 5 wk of HDM administration, airway resistance was assessed using a Flexivent apparatus (SCIREQ). Following anesthesia with pentobarbital an endotracheal tube was inserted into the trachea of the mice and connected to a volume-cycled ventilator. Mice were placed under a light to maintain body temperature. Mice were ventilated at a rate of 160–200 breaths/min, a tidal volume of 0.2 ml with positive end expiratory pressure of 2–3 cm H₂O. Once baseline airway resistance was established, increasing doses of nebulized methacholine (MCh) (Sigma-Aldrich) from 3.12 to 50 mg/ml was administered, and airway resistance was measured for 6 min by the forced oscillation technique as previously described by others (28).

Intraperitoneal HDM sensitization

C57BL/6 mice were given 25 μ g of purified HDM extract by i.p. injection without exogenous adjuvant (day 0). On day 10, mice were challenged i.n. with 25 μ g of HDM extract. Mice were sacrificed 48 h later, and BALF was obtained for cell count and differential.

Statistical analysis

Data are expressed as means $\pm$ SD. Each experiment was repeated at least three times. All data points represent an average of a minimum of six mice per group. Students t test was performed on the means of two sets of data. Data was considered significant if the p value was $p < 0.05$.

Results

STAT3 activation in response to chronic HDM

To generate allergic inflammation in mice, we used a recently characterized model of chronic i.n. HDM administration, without an exogenous adjuvant, 5 days/wk for 5 wk (21, 22). To determine whether chronic HDM treatment resulted in STAT3 activation in the lung, Western blot analysis was performed on whole lung lysates using a p-STAT3 Ab that recognizes only the activated form of STAT3. As seen in Fig. 1a, chronic HDM treatment resulted in STAT3 phosphorylation in whole lung lysates. To identify in what cell types of the lung HDM-mediated STAT3 activation was occurring, immunohistochemistry was performed. In PBS-treated mice, there was minimal baseline STAT3 activation seen in the lungs (Fig. 1b). HDM administration in WT mice, however, resulted in significant STAT3 activation in the airway epithelium, the airway smooth muscle, the perivascularature, and in the immune cells surrounding the airway (Fig. 1b).

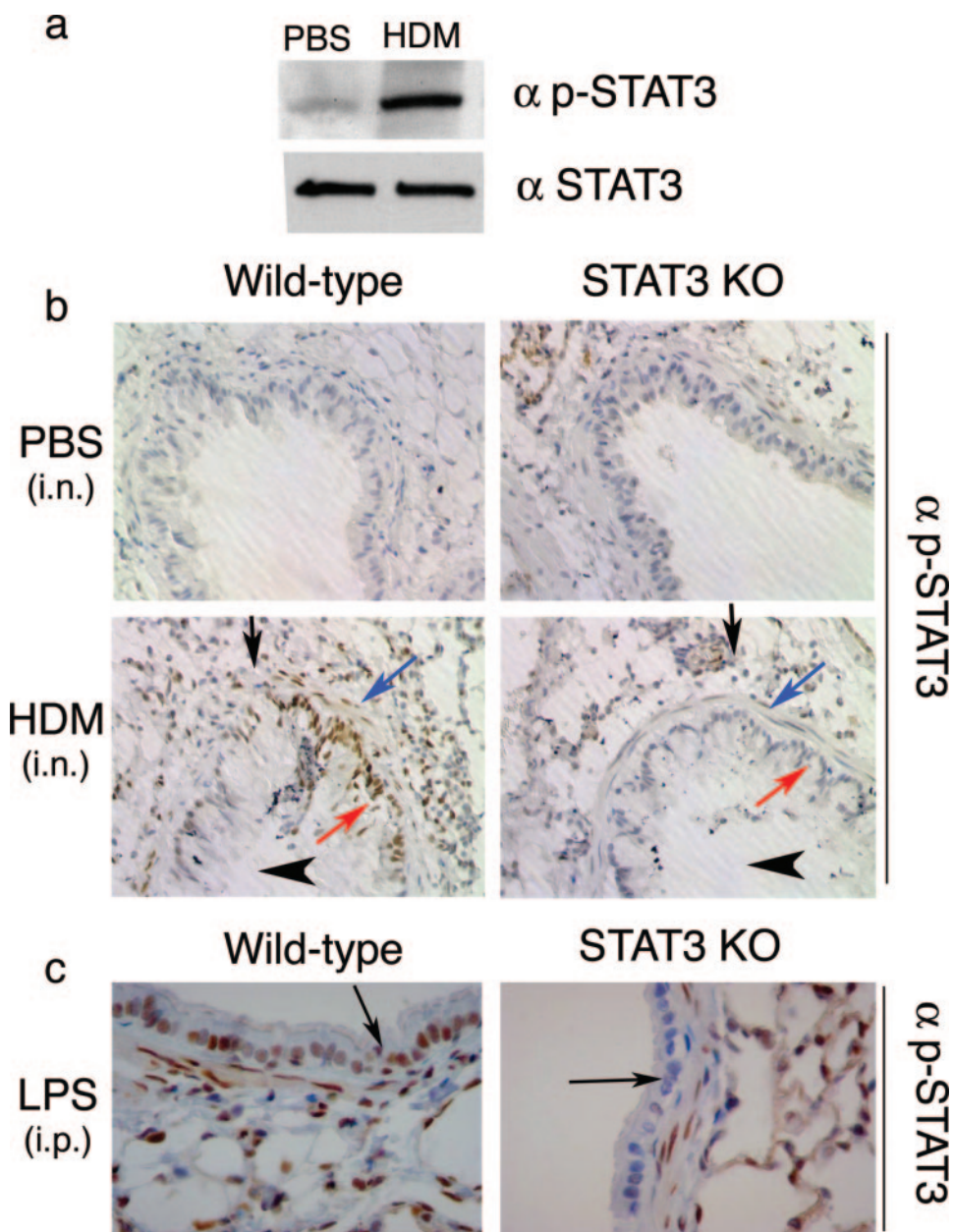


FIGURE 1. STAT3 activation in lungs from WT and e-STAT3^{-/-} mice following chronic HDM administration. WT and e-STAT3^{-/-} mice (KO) were given PBS or 40 μ g of HDM i.n. 5 days/wk for 5 total wk. Lungs were processed for (a) Western blotting using both p-STAT3 and STAT3 Abs and (b) immunohistochemistry using a p-STAT3 Ab. WT and KO mice were given a single dose of 12 mg/kg i.p. LPS and sacrificed 2 h later. Lungs were processed for (c) immunohistochemistry using a p-STAT3 Ab. Arrowheads = airway lumen, red arrows = epithelial staining, the blue arrow = smooth muscle staining, and black arrow = staining in surrounding immune cells. Data are representative of results from two separate experiments (six total mice).

Generation and confirmation of a STAT3 lung epithelial KO mouse

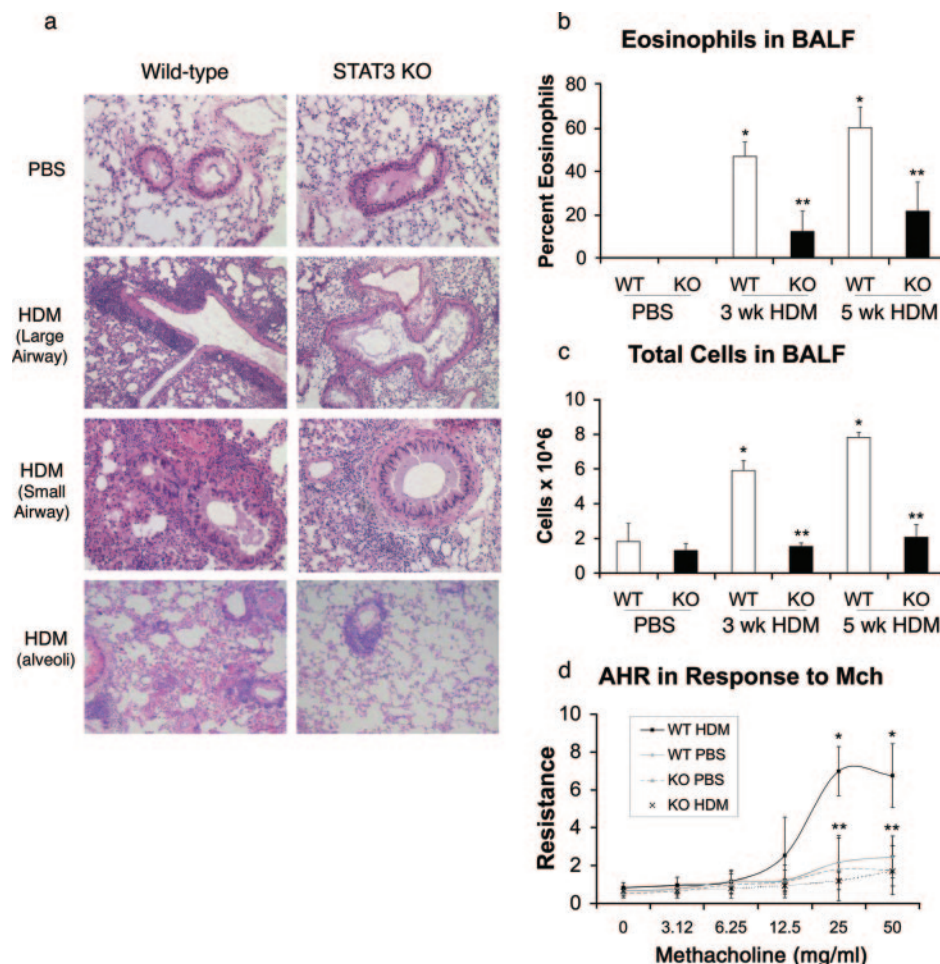
Since the STAT3 KO mouse is an embryonic lethal, a conditional KO approach was taken to determine the role of STAT3 in the airway epithelium in the regulation of allergic inflammation (29). We crossed STAT3^{Flox/Flox} mice with CC10-Cre mice in which Cre recombinase expression was under the control of the Clara cell secretory protein promoter (CC10) to generate a cohort of conditional mutant mice deficient in STAT3 in the airway epithelium, CC10-Cre/STAT3^{Flox/Flox}, to be called e-STAT3^{-/-} mice. In CC10-cre mice, Cre expression has previously been shown to be limited to the lung bronchiolar epithelium, as determined by the analysis of mice from a CC10-Cre/ROSA-Stop-LacZ cross (24). The Flox sites delete the exon in the *STAT3* gene where tyrosine phosphorylation occurs; therefore, we would expect these mice to have no STAT3 phosphorylation in response to agonists (18). To confirm that STAT3 activation was absent in the airway epithelium, mice were given i.p. LPS, which we previously have shown induces rapid and robust STAT3 activation in the lung (26). LPS

administration resulted in intense STAT3 activation in the airway epithelium, smooth muscle, and inflammatory cells in WT mice (Fig. 1c). In contrast, in e-STAT3^{-/-} mice, there was no LPS-induced STAT3 activation in the airway epithelium, whereas STAT3 activation in the other lung cell types remained unchanged. Treatment with HDM in the e-STAT3^{-/-} mice similarly prevented epithelial STAT3 activation (Fig. 1b). In contrast to what was seen with LPS, however, the KO mice had less HDM-induced STAT3 activation throughout the lung in multiple cell types. This decreased overall lung activation after HDM exposure likely relates to the decreased inflammation found in the e-STAT3^{-/-} mice.

Allergic inflammation in HDM-treated e-STAT3^{-/-}

As previously shown by Johnson et al. (21) in BALB/c mice, 5 wk of HDM administration in C57BL/6 WT mice led to a significant increase in peribronchial and perivascular inflammatory infiltrates compared with PBS-treated animals as determined by histologic

FIGURE 2. Lung/airway inflammation and AHR in HDM-treated WT and e-STAT3^{-/-} mice. WT and e-STAT3^{-/-} (KO) mice given either PBS or HDM i.n. 5 days/wk for 3 or 5 wk. Lungs were processed for (a) histology (H&E) and BALF was isolated for (b) airway eosinophils and (c) total airway cell number. Following HDM administration, mice were ventilated and (d) AHR following MCh challenge was measured. Airway resistance values were measured and graphed as cm H₂O/ml/s. In H&E images, original magnification was $\times 40$. Values in graphs illustrate the mean $\pm$ SD and represent $n = 6$ –8 animals for each group. (*, $p < 0.05$ vs PBS-treated mice; **, $p < 0.05$ when compared with WT HDM-treated mice).



analysis (Fig. 2a). In comparison, the e-STAT3^{-/-} mice had significantly less inflammation at all sites examined after HDM treatment. To determine whether e-STAT3^{-/-} mice had less airway eosinophilia, we analyzed the cells in the BALF after a time course of HDM exposure. In WT mice treated with HDM for 3, 5, or 7 wk, there was a significant increase in the percentage of airway eosinophils (Fig. 2b). In contrast, HDM-treated e-STAT3^{-/-} mice had significantly less eosinophils compared with WT animals at all time points (3 wk, WT = 45% vs KO = 12%; 5 wk, WT = 60% vs KO = 21%; 7 wk, WT = 76% vs KO = 33%; $p < 0.05$). Decreased total cell number was also found in the BAL fluid from e-STAT3^{-/-} mice compared with controls (Fig. 2c). Thus, these studies demonstrate that airway epithelial STAT3 is required for HDM-induced lung and airway inflammation.

Role of STAT3 in HDM-induced AHR

To determine whether STAT3 deletion in the airway epithelium altered AHR, we evaluated changes in airway resistance in response to increasing doses of MCh. As seen in Fig. 2d, WT and e-STAT3^{-/-} mice had no significant difference in their baseline airway resistance. However, WT animals treated with HDM for 5 wk exhibited a significant increase in airway resistance in response to MCh compared with PBS-treated mice. The response to MCh in HDM-treated e-STAT3^{-/-} mice was the same as PBS-treated e-STAT3^{-/-} or PBS-treated WT mice, demonstrating that STAT3 deletion from the epithelium protects mice from HDM-induced increases in AHR.

Impact of STAT kinase inhibition on HDM-induced inflammation

We have previously determined that the STAT kinase inhibitors TyrA1 (Jak inhibitor) and SU6656 (Src inhibitor) effectively block LPS-induced STAT3 activation in vivo (25). To determine whether these inhibitors would also prevent HDM-mediated STAT3 activation, we administered the inhibitors i.n. to mice already treated for 3 wk with chronic HDM. Administration of TyrA1, SU6656, or a combination of both inhibitors significantly decreased HDM-induced STAT3 activation as seen by Western blot analysis (Fig. 3a). In addition, administration of the inhibitors also resulted in a marked decrease in HDM-induced lung inflammation, as determined by pathologic analysis (Fig. 3b). Administration of TyrA1, or both inhibitors together, significantly inhibited HDM-induced airway eosinophilia whereas SU6656 had no significant impact (Fig. 3c). These results demonstrate that the local delivery of STAT kinase inhibitors effectively attenuated HDM-induced STAT3 activation and lung inflammation. Additionally, these data complement our genetic findings, suggesting that inactivation of lung STAT3 significantly abrogates allergic inflammation in response to HDM.

Role of STAT3 in lung lymphocyte activation and Th2 cell accumulation

To determine whether there were differences in the lymphocyte populations of cells being activated or recruited to the lungs in the e-STAT3^{-/-} mice, we performed FACS analysis on lung mononuclear cells from HDM-treated mice. The outlined (R1)

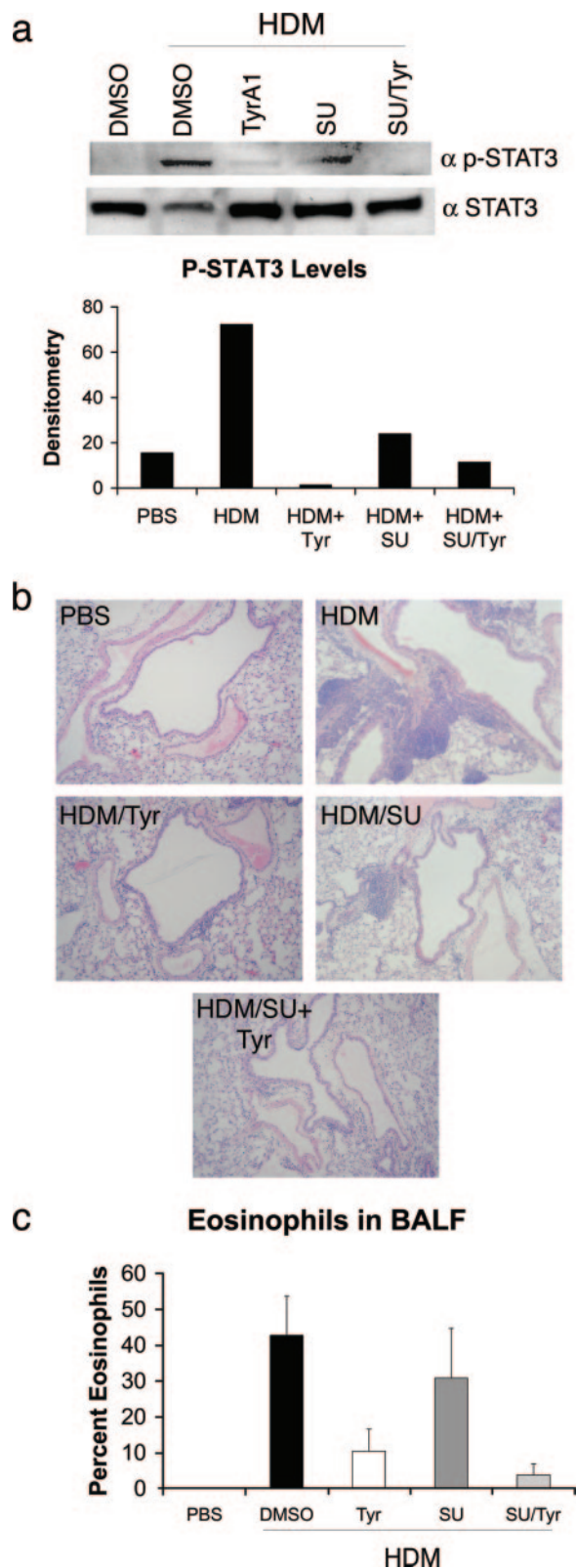


FIGURE 3. Effects of i.n. inhibitor administration on airway inflammation and STAT3 activation in BALB/c mice. BALB/c were given i.n. HDM continuously for 3 wk. Subsequently, mice were treated with i.n. HDM alone or HDM combined with either i.n. TyrA1 (Tyr) (1 mg/kg), SU6656 (SU) (8 mg/kg), or both inhibitors together. Lungs were processed for (a) Western blot analysis using p-STAT3 and STAT3 Abs (b) histology (H&E staining) and (c) airway eosinophils (*, $p < 0.01$ vs PBS-treated mice; **, $p < 0.05$ vs HDM-treated mice). Densitometry was performed on the p-STAT3 band, which was normalized to STAT3 and reported as fold STAT3 activation relative to control (PBS).

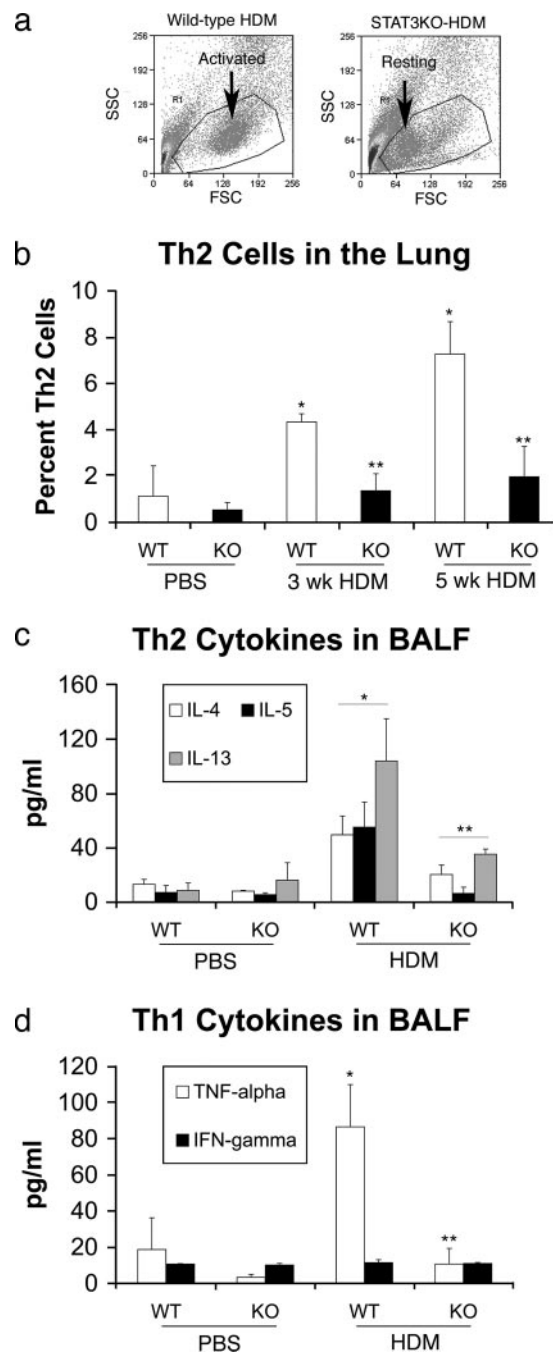
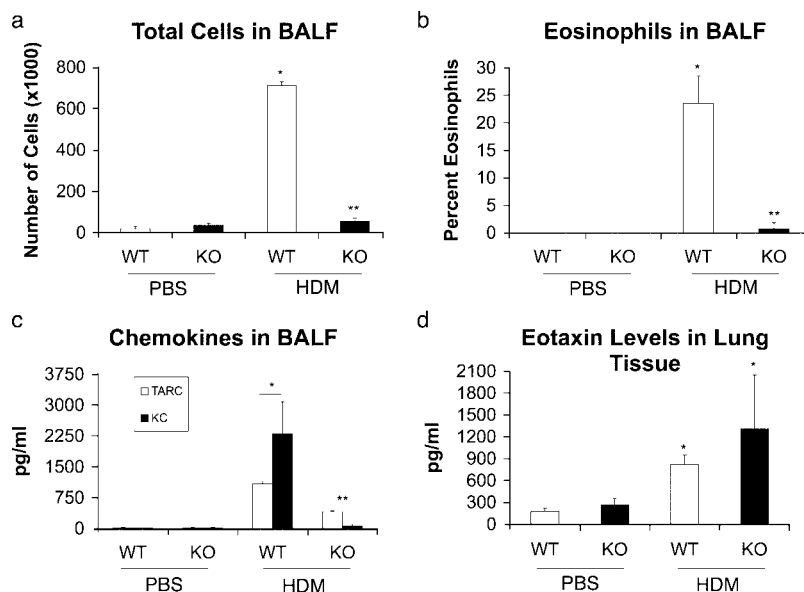


FIGURE 4. Percentage of Th2 cells and levels of cytokines in e-STAT3^{-/-} mice following HDM exposure. HDM was administered i.n. continuously for 3 or 5 wk to WT and e-STAT3^{-/-} (KO) mice. a, Activated vs resting lymphocytes were measured and compared by FACS. b, Percentage of Th2 cells in the lungs of mice were determined by FACS with three different fluorescently conjugated Abs; Thy1.2-allophycocyanin, CD4-PE, and T1ST2-FITC (*, $p < 0.05$ vs PBS-treated mice; **, $p < 0.05$ when compared with HDM treatment in WT mice). c, BALF from 5-wk, HDM-treated mice was analyzed for Th2 cytokines IL-4, IL-5, and IL-13, and (d) Th1 cytokines TNF- α and IFN- γ by ELISA (*, $p < 0.05$ vs PBS mice; **, $p < 0.05$ when compared with WT HDM-treated mice).

population of cells represents all living lymphocytes. All other dots represent dead or dying cells or debris. FACS analysis of the isolated lymphocyte population revealed a significant increase in the total number of activated lymphocytes, or cells greater in size and granularity, in the lung tissue of 3- (data not shown) and 5-wk, HDM-treated WT mice (Fig. 4a). Analysis of

FIGURE 5. Airway eosinophilia after i.p. HDM sensitization and chemokine production following chronic HDM administration. WT and e-STAT3^{-/-} (KO) C57BL/6 mice were sensitized with 25 μ g of HDM i.p. on day 0 and were challenged with 25 μ g of HDM i.n. on day 10. BALF was analyzed for (a) total cell number and (b) percent airway eosinophilia 48 h after challenge (*, $p < 0.05$ compared with PBS-treated mice). c, WT and KO mice were treated with i.n. HDM for 1 or 5 wk. TARC was measured after 1 wk of HDM, and KC was measured after 5 wk of HDM by ELISA using BALF (*, $p < 0.05$ compared with PBS treatment; **, $p < 0.05$ vs HDM treatment in WT mice). d, Eotaxin levels in crushed lung tissue from WT and KO treated with 1 wk of HDM were analyzed by ELISA (*, $p < 0.01$ compared with PBS treatment).



lung mononuclear cells from the e-STAT3^{-/-} mice treated with HDM, however, revealed cells with decreased forward and side scatter characteristics, consistent with a resting state when compared with WT mice. To examine whether e-STAT3^{-/-} mice had an altered lung Th2 cell population, FACS analysis was performed on lung mononuclear cells that were triply stained with Abs to Thy1.2, CD4, and T1/ST2. HDM exposure led to a significant expansion of Th2 cells at both 3- and 5-wk time points as compared with control animals (Fig. 4b). In contrast, the Th2 cell population in the lungs of e-STAT3^{-/-} mice treated with HDM was significantly less at both time points examined compared with WT mice (3 wk, WT = 4.32% vs KO = 1.36%; 5 wk, WT = 7.26% vs KO = 1.96%; $p < 0.01$ for both time points). These studies demonstrate that HDM-induced lymphocyte activation and Th2 cell accumulation in the lung is dependent on airway epithelial STAT3.

Role of STAT3 in HDM-induced cytokine production

Since the lung Th2 cell population was significantly decreased in HDM-treated e-STAT3^{-/-}, we evaluated whether Th2 cytokine production in the BALF of these mice was also diminished. All Th2 cytokines evaluated, IL-4, IL-5, and IL-13, were significantly increased in the BALF of WT mice after 5 wk of HDM treatment as compared with control mice (Fig. 4c). The production of all these Th2 cytokines was reduced significantly in the BALF of HDM-treated e-STAT3^{-/-} mice when compared with WT control mice. There was no significant difference between the WT and the e-STAT3^{-/-} in the basal levels of these cytokines.

To determine whether the e-STAT3^{-/-} mice had an altered Th1 response, we evaluated levels of IFN- γ and TNF- α in the BALF. Chronic HDM treatment resulted in a significant increase in TNF- α in the BALF of WT animals compared with PBS-treated mice. This increase in TNF- α was completely inhibited in e-STAT3^{-/-} mice (Fig. 4d). In contrast to TNF- α , IFN- γ generation was not significantly altered in either WT or e-STAT3^{-/-} mice after 5 wk of HDM treatment. Thus, STAT3 deletion from the epithelium attenuated both Th2 and Th1 cytokine responses after HDM administration.

Allergic inflammation in animals sensitized with i.p. HDM

To determine whether epithelial STAT3 deletion altered allergic inflammation due to ineffective T cell activation, we circumvented

the requirement for airway epithelial as the site of allergen sensitization by administering HDM i.p. Mice were sensitized with i.p. HDM on day = 0 and subsequently challenged with i.n. HDM on day = 10. In WT animals, there was a significant increase in both total cell number and eosinophils in the airway after HDM treatment compared with PBS animals (Fig. 5, a and b). In contrast, in e-STAT3^{-/-} mice treated with HDM there was a marked decrease in total cells and eosinophils in the airway compared with WT mice. These data suggest that Stat3 impacts on allergic airway inflammation by effects on cellular recruitment.

Role of STAT3 in HDM-induced chemokine generation

The level of TARC, a Th2 cell chemoattractant, was analyzed in BALF from HDM-treated WT and e-STAT3^{-/-} mice. After 1 wk of HDM treatment, there was a significant increase in TARC in the BALF of WT mice compared with PBS mice as determined by ELISA. In contrast, in HDM-treated e-STAT3^{-/-} mice, TARC production in the airway was attenuated significantly compared with WT mice (Fig. 5c). In addition, eotaxin levels were evaluated in the lung tissue of mice after 1 wk of HDM treatment. Eotaxin was increased significantly in the lungs of HDM-treated WT animals. In contrast to that seen with TARC, eotaxin levels were similar in the HDM-treated e-STAT3^{-/-} mice to that seen in WT mice (Fig. 5d). Chronic HDM administration for 5 wk also resulted in a significant increase in the α (CXC) chemokine family member KC in the BALF of WT animals (30). This increase in KC by HDM was blocked completely in the e-STAT3^{-/-} mice. These data suggest that HDM-induced generation of some chemokines is STAT3 dependent and is associated with a significant decrease in HDM-induced airway inflammation.

Discussion

Airway inflammation in response to aeroallergens requires the complex interplay between multiple cell types, including dendritic cells, lymphocytes, and the airway epithelium. The role of the airway epithelium in this process is not well defined and is currently being elucidated. As the bronchial epithelium is the first line of contact for aeroallergens, it likely plays a critical role in establishing and maintaining airway inflammation. In this study, we sought to characterize the role of airway epithelial STAT3 in

HDM-mediated allergic inflammation both by targeted gene deletion of STAT3 in the airway epithelium, as well as by small molecule inhibition of the STAT kinases. The HDM extract used in these studies is more representative of the complex aeroallergens found in the real world. Additionally, the i.n. route and prolonged course of administration better mimics the natural route and chronic nature of allergen exposure seen in asthma. Our data demonstrate that STAT3 is up-regulated in the airway epithelium of mice treated with chronic HDM. Furthermore, we show that STAT3 in the airway epithelium is required for HDM-mediated allergic inflammation and AHR. Importantly, we also demonstrate that HDM-induced STAT3 activation and lung inflammation can be reversed by the use of locally administered small molecule inhibitors of the STAT kinases Src and Jak. Finally, our data suggest that airway epithelial STAT3 may be critical for the regulation of immune cell recruitment during allergic inflammation.

Determining the role of STAT3 in mediating inflammatory responses has been hindered by the fact that knocking out STAT3 results in embryonic lethality (29). Cell-specific disruption of STAT3 in myeloid or endothelial cells suggests a largely anti-inflammatory role for STAT3 in innate immune responses (16, 17). Although STAT3 plays a role in dendritic cell function and T cell survival, its role in allergic immune responses is not well defined (18, 31). Chronic HDM treatment resulted in STAT3 activation in multiple cell types in the lung. Our results suggest that STAT3 normally plays a proinflammatory role in allergic responses as deletion of STAT3 in the airway epithelium significantly protected the mice from multiple features of HDM-induced inflammation. The subtle increases over time in both airway eosinophils and Th2 cells in the lungs of HDM-treated e-STAT3^{-/-} mice suggest that there are also STAT3-independent pathways that mediate HDM-induced inflammation. In support of this contention is the fact that other signaling molecules, such as NF- κ B and STAT6, have been shown to be up-regulated in the airway epithelium in allergic asthma (32–34). Since inflammation and AHR are usually correlated with one another, it was not surprising that epithelial STAT3 deletion also resulted in improved lung function in HDM-treated mice, as manifested by loss of AHR to MCh. While the mechanisms leading to AHR are still not entirely clear, Th2 cytokines such as IL-4, IL-5, and IL-13, which were decreased in e-STAT3^{-/-} mice, are thought to play a prominent role in the development of AHR. Thus, our findings identify a unique role for lung epithelial STAT3 in directing allergic inflammation and altering lung function in a chronic allergic asthma model.

Since cytokine pathways exert their cellular effects through tyrosine phosphorylation-dependent pathways, targeting tyrosine kinases would be an efficient way to dampen the allergic inflammatory cascade at multiple different sites (35–38). The efficacy of locally (i.n.) administered STAT kinase inhibitors in blocking HDM-induced STAT3 activation and allergic inflammation is proof of concept that targeting e-STAT3 might translate into an effective mode of therapy for asthma and further supports our findings in the e-STAT3^{-/-} mice. The more dramatic blockade of inflammation seen with the kinase inhibitors compared with the e-STAT3^{-/-} mice may be due to the inhibition of other STATs and/or the inhibition of STAT signaling in cells other than the epithelium (39). Other signaling cascades that rely on tyrosine phosphorylation, such as the MAPK pathway, could also be inhibited by these compounds as well. Importantly, these inhibitors were still effective at decreasing allergic inflammation even when first given 3 wk post-HDM treatment, a time point when airway inflammation is robust. This result is clinically relevant because patients with asthma typically present for treatment after inflammation is already established (21). These data also suggest that

STAT3 may have a more prominent role in the maintenance of allergic inflammation rather than its initiation. If STAT3 were just important in the initiation of allergic inflammation, such as dendritic cell activation and T cell priming, the inhibitors would not have been effective given the delayed administration relative to HDM. Thus, the local blockade of the STAT kinases and STAT3 activation may be an attractive strategy for the treatment of asthma in the future as this approach can significantly reverse HDM-induced inflammation.

Our data show that three critical Th2 cytokines, IL-4, IL-5, and IL-13, are down-regulated in the epithelial STAT3 conditional KO mouse, suggesting that STAT3 deletion in the epithelium dampens the signals required for robust Th2-driven responses to allergens in the lung. The fact that STAT3 deletion did not enhance IFN- γ generation and actually resulted in decreased TNF- α suggest that these mice have a decrease in multiple lung cytokines and not a skewing toward Th1 responses. Thus, e-STAT3 and the airway epithelium are critical players in HDM-induced cytokine responses. The mechanism of reduced allergic inflammation seen in the HDM-treated e-STAT3^{-/-} mice is likely complex given that STAT3 is activated by multiple inflammatory agonists and subsequently induces the expression of genes that participate in allergic inflammation, such as chemokines, adhesion molecules, and iNOS. In addition, TLR4 signaling has been shown to be required for allergic inflammation in the lung in response to allergens. Thus, it is possible that endotoxin, a known STAT3 agonist (Fig. 1c) found in HDM, is required for STAT3-induced allergic inflammation via a TLR4 signaling pathway.

In this allergic model, mice were sensitized and challenged with HDM by the i.n. route, and hence, e-STAT3 deletion could alter Ag presentation and T cell priming at the epithelium. Our results, however, demonstrate that e-STAT3^{-/-} mice sensitized by the i.p. route did not develop HDM-induced airway inflammation. These data suggest that cellular recruitment is inhibited when airway e-STAT3 is deleted, assuming equivalent priming is occurring in WT and e-STAT3^{-/-} mice. Supporting this contention is our result demonstrating that some critical chemokines were reduced significantly in the BALF of e-STAT3^{-/-} mice compared with WT mice treated with HDM. TARC is up-regulated in the bronchial epithelium and in immune cells in asthma and has been shown to be an important chemoattractant for Th2 cells in mice (40). Furthermore, the neutralization of TARC inhibited not only Th2 cell infiltration in the lung but also eosinophil accumulation and AHR in a mouse model of asthma (41). Consistent with this, we show that the e-STAT3^{-/-} mice have significantly less HDM-induced TARC in the BALF, and this is accompanied by decreased allergic inflammation and AHR in the lung. In addition to TARC, we also found that two other HDM-induced chemoattractants, KC and TNF- α , were decreased significantly in the e-STAT3^{-/-} mice compared with WT mice (42, 43).

In contrast to chemokines TARC and KC, eotaxin levels were unchanged in the lungs of e-STAT3^{-/-} mice, indicating that not all chemokines are decreased with epithelial STAT3 deletion. This result is not unexpected given that multiple cell types in the lungs can generate chemokines. Interestingly, eotaxin production was not sufficient to promote allergic airway inflammation in e-STAT3^{-/-} mice. As others have reported, either Th2 cells or IL-5 are required for maximal tissue eosinophilia to occur in allergic inflammation (44, 45). Our data are also consistent with work by others demonstrating that STAT6 signaling in the resident lung cells, but not in the Th2 cells, is critical for eosinophilic inflammation and maximal allergic inflammation in a murine model of asthma (46). Hence, our studies suggest that STAT3 in the airway epithelium is pivotal for the production of chemokines

and subsequent cellular trafficking and recruitment into the lung after both acute and chronic allergen challenge.

The exact mechanism by which HDM-induced STAT3 activation alters chemokine levels in the airways is unknown. Chemokine regulation is complex and occurs at several different levels that include both transcriptional and posttranscriptional modifications. HDM induces the generation of multiple cytokines that up-regulate chemokines and that also induce STAT3, including IL-5, IL-6, IL-13, and GM-CSF. STAT3 has been shown to regulate gene transcription of some chemokines, such as CXCL8 and CXCL11 (47, 48). In addition, the upstream promoter of TARC contains three STAT DNA-binding motifs. Two of these STAT sites have been shown to mediate IL-4-induced TARC gene expression via STAT6 (49). Whether STAT3 also binds these sites and is required for TARC gene expression is unknown and is currently an area of active investigation. It is also possible that STATs regulate chemokines posttranscriptionally by impacting on matrix metalloproteases or the decoy receptors known to modulate chemokine activity (50).

While our i.p. allergen sensitization result suggests that cellular recruitment is defective, it does not exclude the possibility that Ag processing at the epithelium is impaired in the e-STAT3^{-/-} mice. STAT3 deletion in the epithelium could alter the epithelial barrier, receptor signaling, or Ag presentation. Whether e-STAT3 acts to regulate critical dendritic cell growth and differentiation factors such as thymic stromal lymphopoietin and/or GM-CSF is currently an area of investigation in the laboratory (51, 52).

Finally, the role of STAT3 in human asthma is an area of active investigation in the laboratory. We have previously shown that STAT3 is both pro-proliferative and antiapoptotic in primary human airway smooth muscle cells in vitro, suggesting a critical role for STAT3 in the accumulation of smooth muscle mass, a major contributor to asthma pathogenesis (data not published) (53). Additionally, STAT3 single nucleotide polymorphisms were recently shown to be associated with decreased lung function in patients with asthma (54). These data, combined with our data from HDM treated e-STAT3^{-/-} mice, suggest a potential role for STAT3 in the pathogenesis of asthma in humans with more severe disease.

In conclusion, this study demonstrates the critical role of STAT3 in the airway epithelium in the regulating allergen-induced inflammation and AHR in a murine model of chronic asthma. In addition to its known ability to enhance T cell survival and dendritic cell differentiation, we have now shown that e-STAT3 plays a critical role for inflammatory cell trafficking to the lung in response to acute and chronic allergen challenge. Moreover, blocking the STAT kinases and STAT3 with small molecule inhibitors effectively decreased allergic airway inflammation in this model. Based on our data, targeting STAT3 and/or the STAT kinases in the airway may be a novel and effective therapeutic approach for the treatment of asthma.

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Disclosures

The authors have no financial conflict of interest.

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