

# Inhibition of signal transducer and activator of transcription 1 attenuates allergen-induced airway inflammation and hyperreactivity

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**Background:** Transcriptional factors of the signal transducer and activator of transcription (STAT) family play an important role in orchestrating immune reactions.

**Objective:** The aim of the current study was to investigate the role of STAT-1 in murine allergen-induced sensitization and development of airway inflammation (AI) and airway hyperreactivity (AHR), cardinal features of bronchial asthma.

**Methods:** BALB/c mice were systemically sensitized to ovalbumin and challenged with ovalbumin through the airways. Decoy oligonucleotide (ODN) specific for STAT-1 was applied once locally to the airways of sensitized animals before allergen airway challenges.

**Results:** Single application of decoy ODN markedly and significantly reduced numbers of eosinophils and lymphocytes in bronchoalveolar lavage fluids compared with those seen in sensitized and challenged animals receiving mutant control ODN. Associated with this decrease in eosinophilic AI were significantly reduced levels of IL-5 in BAL fluid, of CD40 expression in peribronchial infiltrates, and of vascular cell adhesion molecule 1 expression in vascular endothelial cells, respectively. In addition, development of AHR after allergen sensitization and airway challenges was effectively abolished after local STAT-1 decoy ODN treatment.

**Conclusion:** The data indicate that a decoy ODN neutralizing STAT-1 effectively inhibits allergen-induced AI and AHR, probably by attenuating upregulation of costimulatory and adhesion molecules, and suggest a significant role of STAT-1 in asthma pathology. (*J Allergy Clin Immunol* 2004;114:288-95.)

**Key words:** CD40, vascular cell adhesion molecule 1, asthma, bronchial hyperreactivity

Bronchial asthma is characterized by a marked inflammatory response in the airways, leading to infiltration of eosinophils and lymphocytes, key cells inducing, regulating, and sustaining bronchoconstriction and airway

## Abbreviations used

AHR: Airway hyperreactivity  
AI: Airway inflammation  
APC: Antigen-presenting cell  
BALF: Bronchoalveolar lavage fluid  
CD40L: CD40 ligand  
ODN: Oligonucleotide  
OVA: Ovalbumin  
STAT: Signal transducer and activator of transcription  
VCAM: Vascular cell adhesion molecule

hyperreactivity (AHR). This inflammatory cascade is controlled by numerous mediators, such as cytokines, chemokines, and adhesion molecules, orchestrating a targeted infiltration of specific cells to specific organ sites. The expression and production of these mediators is determined by intracellular pathways transducing signals from membrane-expressed receptors downstream to the nucleus, leading to transcription and translation of specific genes.

A novel intracellular signaling pathway was discovered in the early 1990s, which, after phosphorylation of cytokine receptors by cytoplasmic Janus kinases, leads to activation of latent factors that were later termed signal transducer and activator of transcription (STAT).<sup>1,2</sup> STAT molecules are involved in the regulation of a multitude of physiologic and pathophysiologic conditions, including cellular and humoral defense,<sup>3</sup> growth,<sup>4</sup> lactation, and normal mammary gland development.<sup>5,6</sup> Two members of the family, STAT-4 and STAT-6, are centrally involved in regulating T-cell differentiation toward the T<sub>H</sub>1 and T<sub>H</sub>2 phenotypes by inducing the production of IL-12 and IL-4, respectively.<sup>4,7,8</sup> Accordingly, the absence or blockade of the STAT-6 signaling pathway inhibits the development of allergen-mediated T<sub>H</sub>2 responses, such as airway inflammation (AI) and AHR.<sup>9,10</sup> Sampath et al<sup>11</sup> provided the first evidence for a possible role of another member of the family in allergic AI. They demonstrated that STAT-1 was constitutively expressed in epithelial cells of asthmatic patients and suggested that increased activation of STAT-1 might induce the upregulation of STAT-1-dependent molecules, such as CD40.<sup>11-13</sup> CD40 is a 50-kd protein of the TNF receptor family, which was first identified on B cells as a counterpart of the CD40 ligand (CD40L) in T-cell-B-cell interaction.<sup>14</sup> The importance

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of CD40-CD40L interaction in allergic diseases was elegantly revealed by Lei et al,<sup>15</sup> showing that allergen-induced eosinophilic AI after allergen sensitization and airway challenges was unable to develop in CD40L-deficient mice. In this and other studies, the reduced leukocyte airway infiltration was attributed to a decreased expression of vascular cell adhesion molecule (VCAM) 1 in endothelial cells, which is critical for the infiltration of eosinophils and lymphocytes in the late phase of an asthmatic airway reaction.<sup>16</sup> Interestingly, full activation and expression of VCAM-1 is also dependent on STAT-1 signaling.<sup>17,18</sup>

Targeting STAT-1 might thus provide an interesting and novel way to interfere with the expression of a variety of molecules, including costimulatory and adhesion molecules, possibly affecting a wide spectrum of allergen-mediated inflammatory responses. Here we show for the first time that disruption of STAT-1 signaling by local application of STAT-1 decoy oligonucleotides (ODNs) inhibited the development of allergen-induced eosinophilic AI and increased *in vivo* airway reactivity, indicating a significant role for STAT-1 in allergen-mediated airway diseases.

## METHODS

### Animals

All experiments were approved by the animal care facility (Berlin Office for Occupational Safety, Protection of Health and Technical Safety—LaGetSi). Female BALB/c mice, 20 to 23 g (obtained from the Federal Institute for Health Protection of Consumers and Veterinary Medicine [BgVV], Berlin, Germany), arrived free of pathogen and were maintained on an ovalbumin (OVA)—free diet in cages equipped with filter hoods.

### Decoy ODN technique

Double-stranded ODNs were prepared from complementary single-stranded phosphorothioate-bonded ODNs (Eurogentec, Köln, Germany) by melting at 95°C for 5 minutes, followed by a cool-down phase of 3 to 4 hours at ambient temperature. The efficiency of the hybridization reaction was verified with 2.5% agarose gel electrophoresis and usually found to exceed 95%. The sequences of the single-stranded ODNs were as follows (underlined letters denote phosphorothioate-bonded bases): STAT-1 consensus decoy ODN, CATGTTATGCATATTCCTGTAAGTG; mutant control ODN, CATGTTATGCAGACCGTAGTAAGTG.

The final concentration of the ODN dissolved in TEN buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 150 mmol/L NaCl, pH 7.5) was 0.4 mmol/L. Decoy ODNs enter target cells without the assistance of vectors and are protected by phosphorothioate modification against degradation, thus preventing gene transcription for at least 72 hours. The specificity of ODNs was analyzed by means of gel shift analysis (EMSA) with nuclear extracts from cultured mouse synovial fibroblasts, demonstrating the absence of the STAT-1 band only in the presence of the consensus but not the mutant ODN (data not shown). Mutant control ODNs lacking specificity for the consensus DNA-binding motif of the STAT-1 protein but otherwise being chemically identical were used to demonstrate the specificity of the decoy ODN approach.<sup>19,20</sup>

Consensus decoy ODNs were coupled with fluorescent Texas red and used at the same final concentration to verify *in vivo* uptake of

ODN decoy by airway cells. Cells that internalized ODN decoy were visualized by using a fluorescence microscope with an adequate filter.

### Treatment

Animals were sensitized to OVA (20 µg per injection; Sigma, Deisenhofen, Germany) adsorbed to 2 mg of Al(OH)<sub>3</sub> (Pierce, Rockford, Ill) administered by means of intraperitoneal injections on days 1 and 14. On days 28 and 29, animals were challenged for 20 minutes with 1% OVA (wt/vol) diluted in PBS (PAA Laboratory, Pasching, Austria) delivered by means of aerosolization, as previously described.<sup>21</sup> Twenty-four hours before the first airway allergen challenge, animals intranasally received 20 µL of decoy ODN (OVA-decoy), mutant control ODN solution (OVA-mutant), or vehicle alone (OVA-vehicle). The negative control group was sham sensitized and provoked with PBS and additionally treated intranasally with vehicle according to the same protocol (PBS-vehicle). For analysis of AI and AHR, animals were analyzed 24 hours after the last challenge. Some groups were challenged only once intranasally with 2 × 25 µL of PBS containing 50 µg of OVA (positive control and ODN treated animals) or saline alone to detect early regulatory changes. These animals were analyzed 12 hours after allergen challenge.

For *in vivo* uptake experiments, 20 µL of fluorescent Texas red—labeled consensus decoy ODN was administered intranasally once to naive mice. One hour later, animals were killed, and lungs were removed and shock frozen in liquid nitrogen. Ten-micrometer sections were cut on a cryostat, mounted on glass slides, rinsed with PBS, and analyzed for fluorescence accumulation by using epifluorescence microscopy (Leica DMR2; Leica, Weilburg, Germany).

### Preparation of bronchoalveolar fluid

Twenty-four hours after the last airway challenge, animals were killed, and the tracheas were cannulated. Airways underwent lavage twice with 1 mL of ice-cold PBS, and cell numbers and cytokine contents were determined as described previously. Total leukocytes, lymphocytes, eosinophils, and macrophages were differentiated in bronchoalveolar lavage fluid (BALF) according to morphologic criteria.<sup>21</sup> In total, 12 mice were analyzed in each group in 3 independent experiments. Cell-free BALF was frozen at 20°C until further analysis.

### Determination of cytokines from BALF

Cytokine levels were determined by means of ELISA, as previously described.<sup>21</sup> Sensitivities were 10 ng/mL for IL-4, 15 ng/mL for IL-5, and 39 ng/mL for IFN-γ. In total, 12 mice were analyzed in each group in 3 independent experiments.

### Extraction of total RNA and mRNA quantification

A standard protocol was used for RNA extraction from whole lung cells. RNA was quantified by using real-time PCR. For details, refer to the e-text in the Journal's Online Repository at [www.mosby.com/jaci](http://www.mosby.com/jaci).

### Determination of airway reactivity

*In vivo* airway reactivity was measured in unrestrained animals by means of barometric whole-body plethysmography (corresponding to the Buxco system provided by EMKA Technologies, Paris, France), as previously described.<sup>21</sup> This method detects increases in airway reactivity to methacholine after sensitization and allergen airway challenges of mice, and measurements obtained with this technique closely correlate with the results of invasive measurements of intrapleural pressure and airway resistance.<sup>22</sup> In brief, pressure differences were measured between the main chamber of the

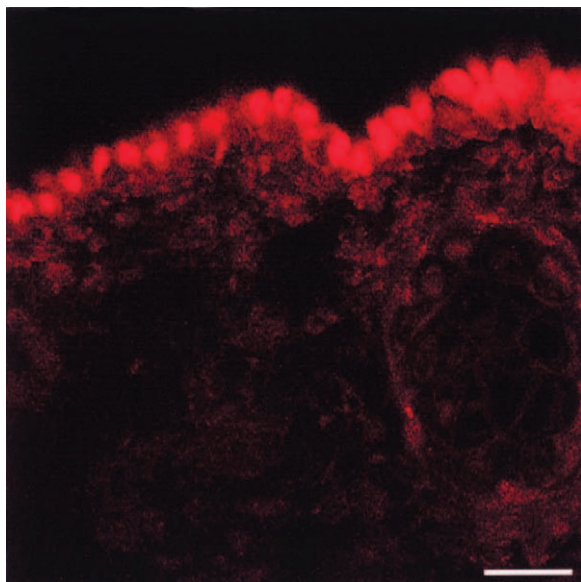


**FIG 1.** Uptake of decoy ODN by airway cells. Texas red–coupled decoy ODNs were delivered intranasally to untreated animals, as described in the Methods section. One hour after application of decoy, animals were killed. The scale bar represents 50  $\mu$ m.

plethysmograph, containing the animal, and a reference chamber (generating a box pressure signal). Mice were challenged with aerosolized PBS or increasing doses of methacholine (6–50 mg/mL) for 3 minutes, and readings were taken and averaged for 3 minutes after each nebulization. Data are expressed as a fold increase of baseline values (PBS challenge) by using the dimensionless parameter enhanced pause. In total, 12 mice were analyzed in each group in 3 independent experiments.

### Immunofluorescence analysis

Immunostaining was carried out as described previously<sup>23</sup> on cryostat-cut, transversal, 8- $\mu$ m-thick lung sections of (1) sham-sensitized, saline-challenged, and vehicle-treated animals (PBS-vehicle); (2) OVA-sensitized, OVA-challenged, and STAT-1 decoy ODN–treated animals (OVA-mutant); and (3) mutant control ODN–treated animals (OVA-decoy), respectively. Sections were mounted, fixed, washed in PBS, and incubated with 0.1 mol/L phosphate buffer containing 1% BSA and 10% normal swine serum for 1 hour at room temperature. After repeated washing steps in PBS, the tissues were incubated with a previously described monoclonal rat anti-mouse CD40 antibody clone, 3/23 (PharMingen),<sup>24,25</sup> diluted 1:50 or a rat anti-mouse VCAM-1 antibody clone, M/K-2 (R&D),<sup>26</sup> diluted 1:100 in the preincubation solution at 4°C. After overnight incubation and washing steps in PBS, the VCAM-1 signal was detected by means of incubation with a Cy-3–labeled secondary antibody (Amersham, Braunschweig, Germany) for 1 hour at ambient temperature. Slides were mounted in carbonate-buffered glycerol (pH 8.6) and viewed by using epifluorescence microscopy. The CD40 signal was detected by using a previously described alkaline phosphatase–antialkaline phosphatase technique with new fuchsin as a chromogen.<sup>27</sup> Endogenous alkaline phosphatase was quenched with levamisole, and nonspecific binding of the secondary antibody was blocked by means of preincubation of the slides with normal rabbit serum. Specificity of the antibody reaction was verified in parallel sections, which were incubated with rat IgG (clone 1D10) as the primary antibody.

### Statistical analysis

Results are presented as means  $\pm$  SD. Means were compared by using the Student *t* test with the SPSS version 7.5.2G software package (SPSS, Chicago, Ill). *P* values of less than .05 were regarded as significant differences.

## RESULTS

### Uptake of decoy ODN by airway cells

Single intranasal application of Texas red–labeled STAT-1 consensus decoy ODN confirmed *in vivo* uptake by airway epithelial cells. Intracellular fluorescence accumulation was found as early as 1 hour after application of the decoy ODN (Fig 1) and remained detectable 6 hours later (not shown).

### STAT-1 inhibition decreases eosinophilic airway infiltration

Accumulation of leukocytes, especially eosinophils and lymphocytes, in the BALF is a cardinal feature of allergen-mediated AI and bronchial asthma. Therefore we determined the influx of immune cells in the BALF of OVA-sensitized and OVA-challenged animals. Sensitization and airway challenges (OVA-vehicle) resulted in significantly higher numbers of total leukocytes, as well as eosinophils and lymphocytes, compared with those seen in sham-sensitized and challenged controls (PBS-vehicle; Fig 2). Treatment of sensitized mice with the STAT-1 decoy ODN before allergen airway challenges (OVA-decoy) significantly reduced total leukocyte counts, as well as the numbers of infiltrating eosinophils in the BALF, compared with those found in sensitized and challenged control animals treated with the mutant control ODN (OVA-mutant; Fig 2). Sensitized mice treated with the mutant control ODN before allergen airway challenges (OVA-mutant) showed similar differential cell counts as vehicle control animals. This reduction after STAT-1 decoy ODN treatment in infiltrating cells implied a critical role of STAT-1–dependent gene expression in the induction of AI in this model.

### STAT-1 inhibition decreases IL-5 levels in BALF

Another cardinal feature of allergic AI is the induction of a typical cytokine pattern characterized by predominant  $T_H2$  (IL-4 and IL-5) and rather little  $T_H1$  (IL-12 and IFN- $\gamma$ ) cytokine production in the targeted organ. In the BALF of sensitized and allergen-challenged mice (OVA-vehicle), IL-5 levels were significantly increased compared with those seen in negative control animals (PBS-vehicle; Fig 3). Animals treated with the STAT-1 decoy ODN once before repeated allergen airway challenges (OVA-decoy) showed a significant reduction in the BALF level of IL-5 compared with the mutant control ODN–treated (OVA-mutant) or vehicle-treated (OVA-vehicle) groups (Fig 3). There were no significant differences in the other cytokine levels determined.

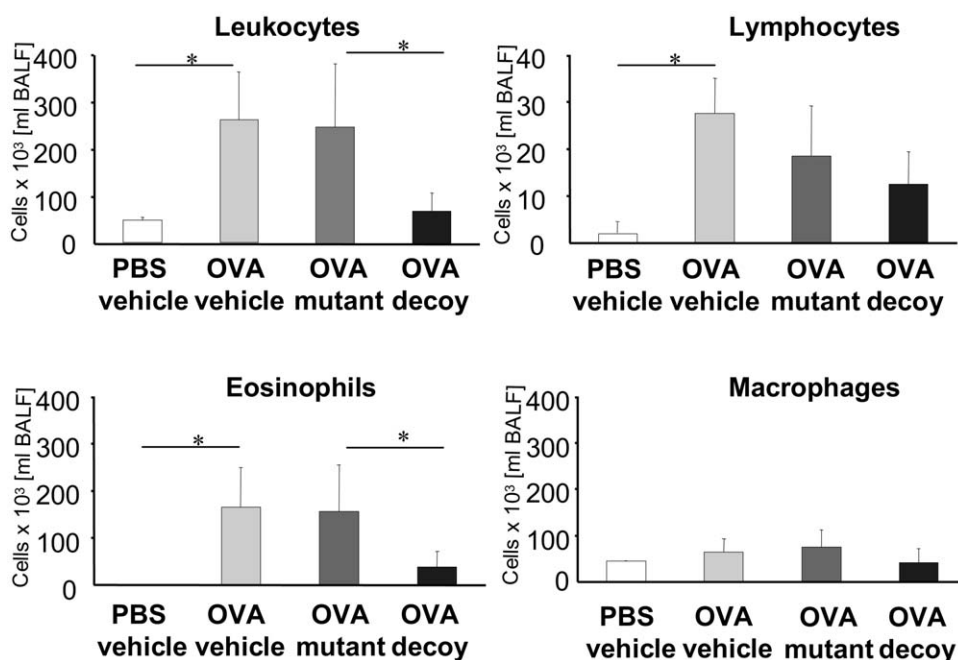


**FIG 2.** Cell differentiation in BALF. Total leukocytes, lymphocytes, eosinophils, and macrophages were differentiated in BALF of sham-sensitized, intranasally vehicle-treated (PBS-vehicle); OVA-sensitized, vehicle-treated (OVA-vehicle); STAT-1 decoy ODN-treated (OVA-decoy); or mutant control ODN-treated (OVA-mutant) mice 24 hours after the last aerosol challenge. Results are expressed as average values  $\pm$  SD. Asterisks indicate a statistically significant difference.

### STAT-1 inhibition decreases IL-5 mRNA production in lung tissues

In addition to cytokine protein levels in BALF, we assessed mRNA expression in lung tissues after single allergen challenge through the airways. Sensitization and airway allergen challenge (OVA-mutant) resulted in significantly enhanced IL-5 and slightly augmented IL-4 mRNA production in lung tissues compared with those seen in nonsensitized, challenged control animals (PBS-vehicle; Fig 4). In contrast, IFN- $\gamma$  mRNA production was rather decreased after allergen sensitization and challenge. Single local application of STAT-1 decoy ODN before airway allergen challenge (OVA-decoy) significantly inhibited IL-5 and showed a tendency toward reduced IL-4 mRNA production compared with that seen in sensitized, challenged animals. IFN- $\gamma$  mRNA production in the STAT-1 decoy ODN-treated group (OVA-decoy) was restored to the levels of nonsensitized control animals (Fig 4).

### Immunofluorescence analysis

Local inflammatory responses and infiltration of effector cells after allergen sensitization and airway challenges might be regulated by STAT-1-dependent upregulation of costimulatory and adhesion molecules. We therefore analyzed the expression and distribution of CD40 and VCAM-1 in lung tissues by means of immunofluorescence analysis. Allergen sensitization and airway allergen challenges (OVA-mutant) resulted in a marked upregulation of VCAM-1 expression in

endothelial cells of blood vessels in lung tissues compared with that seen in nonsensitized control animals (PBS-mutant), which showed nearly no VCAM-1 expression at all (Fig 5). In contrast to the sensitized and challenged control mice (OVA-mutant), this upregulation of VCAM-1 expression in endothelial cells was completely inhibited by a single and local STAT-1 decoy ODN application to sensitized mice before airway allergen challenge (OVA-decoy; Fig 5).

The costimulatory molecule CD40 is expressed early during the inflammatory process. Therefore staining of lung tissues was performed as early as 12 hours after a single intranasal allergen challenge of sensitized mice. In sensitized (OVA-mutant), but not in sham-sensitized, challenged animals (PBS-vehicle), we observed a marked upregulation of CD40 expression by infiltrating leukocytes in the peribronchial regions of the airways (Fig 5). Single and local treatment with STAT-1 decoy ODN of sensitized animals before allergen challenge (OVA-decoy) resulted in a near-complete inhibition of this increase in CD40 expression, attaining a level of immunofluorescence similar to that observed in sham-sensitized, challenged animals (Fig 5).

### Determination of *in vivo* airway responsiveness

In addition to AI, development of AHR is a main cardinal feature of allergen-induced bronchial asthma. *In vivo* airway responsiveness to aerosolized methacholine in spontaneously breathing and conscious mice was

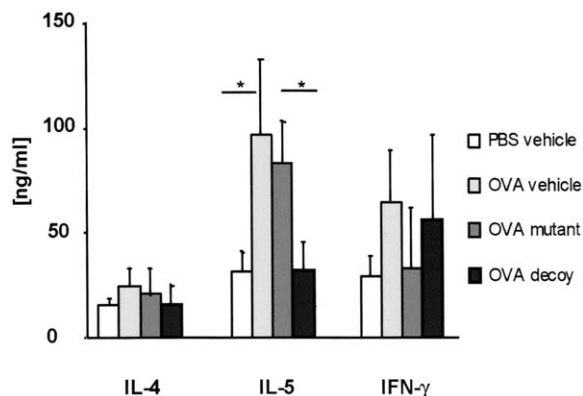


**FIG 3.** Cytokine level in BALF. IL-4, IL-5, and IFN- $\gamma$  levels were measured in BALF of sham-sensitized, intranasally vehicle-treated (PBS-vehicle); OVA-sensitized, vehicle-treated (OVA-vehicle); STAT-1 decoy ODN-treated (OVA-decoy); or mutant control ODN-treated (OVA-mutant) mice. Results are expressed as average values  $\pm$  SD. Asterisks indicate a statistically significant difference.

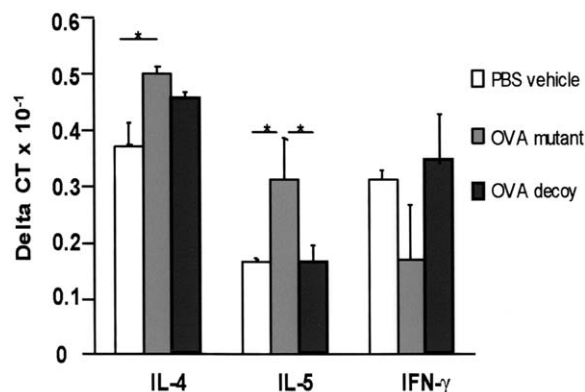


**FIG 4.** Cytokine mRNA expression in lungs. RNA levels of IL-4, IL-5, and IFN- $\gamma$  mRNA in whole lung extracts of sham-sensitized, intranasally vehicle-treated (PBS-vehicle); OVA-sensitized, vehicle-treated (OVA-vehicle); STAT-1 decoy ODN-treated (OVA-decoy); or mutant control ODN-treated (OVA-mutant) mice by means of real-time RT-PCR and expressed as  $\Delta$ CT  $\times 10^{-1}$ . Results are expressed as means  $\pm$  SEM of 3 independent experiments. Asterisks indicate a statistically significant difference.

assessed by means of barometric whole-body plethysmography.<sup>22</sup> OVA sensitization and airway allergen challenges (OVA-vehicle) significantly increased airway reactivity compared with that seen in sham-sensitized, challenged control animals (PBS-vehicle). This increase in airway reactivity was virtually abolished by single and local application of STAT-1 decoy ODN to sensitized, challenged mice (OVA-decoy), whereas treatment with mutant control ODN (OVA-mutant) had no effect (Fig 6).

## DISCUSSION

In the present study we investigated the effects of STAT-1 neutralization on allergen-induced inflammatory responses and development of AHR. We show here for the first time in a murine model of allergen-induced AI that single and local application of STAT-1 decoy ODN to sensitized animals before repeated allergen airway challenge attenuates 2 major pathologic findings of bronchial asthma, AI and AHR, to a striking extent.

We used a decoy ODN approach with the benefit that this versatile tool not only antagonizes a single gene product but also inhibits transcription of all genes downstream of the transactivation of the specific transcription factor. Another advantage of the decoy ODN technique is the wide variety of potential target cells, namely all cytokine receptor-bearing cells, the signal transduction of which involves the STAT-1 pathway. This includes antigen-presenting cells (APCs) inducing immune reactions, T cells regulating inflammatory responses, and gatekeeper cells in endothelial and epithelial layers regulating the traffic of leukocytes across tissue borders. Furthermore, application of decoy ODN is unproblematic and fast because no additional vectors are required to transport the decoy ODN into the target cell,<sup>19,20</sup> where it unfolds its effects immediately by blocking its target transcription factor, even in quiescent

cells.<sup>28</sup> Finally, decoy ODNs are highly specific for their target transcription factor, although we cannot fully exclude that STAT-3 (TRANSFAC matrix V\$STAT 3.01), because of sequence homologies in the core DNA-binding region, might also be neutralized by the STAT-1 decoy ODNs used, whereas interference with both STAT-5 (V\$STAT5.01) and STAT-6 (V\$STAT6.01) is unlikely.

STAT-1 is generally associated with the transduction of signals mediated by IFN receptors. Although T<sub>H</sub>1 cytokines, like IFN- $\gamma$ , are normally thought to antagonize allergen-mediated immune responses, there are accumulating data suggesting a critical involvement of IFN- $\gamma$  in the development of bronchial asthma. IFN- $\gamma$  was found to be increased in serum and BALF of asthmatic individuals,<sup>29</sup> and the development of AHR in mice was attributed to the presence of IFN- $\gamma$ .<sup>30</sup> Along the same line, evidence for a pathophysiologic role of this cytokine in allergen-induced airway disease was provided by studies with allergen-specific IFN- $\gamma$ -producing T<sub>H</sub>1 cells, demonstrating that transfer of these cells into naive mice resulted in development of increased AI and AHR on subsequent allergen airway challenges.<sup>11,31</sup> Therefore disrupting STAT-1 signaling might inhibit expression and production of factors downstream of IFN receptors that might be pivotal for the development of AI and AHR.

One likely candidate is CD40, a major costimulatory molecule expressed in APCs and B cells that is critically involved in T-cell activation and T-cell-B-cell cooperation for Ig-isotype switch.<sup>14</sup> Twelve hours after a single allergen airway challenge, we observed a marked upregulation of CD40 in lung tissues of sensitized animals, indicating a central role of CD40 costimulation in the induction of allergen-mediated airway immune responses. This suggestion is supported by several murine studies showing that blocking of CD40 on APCs or of the corresponding T-cell receptor CD40L causes a striking

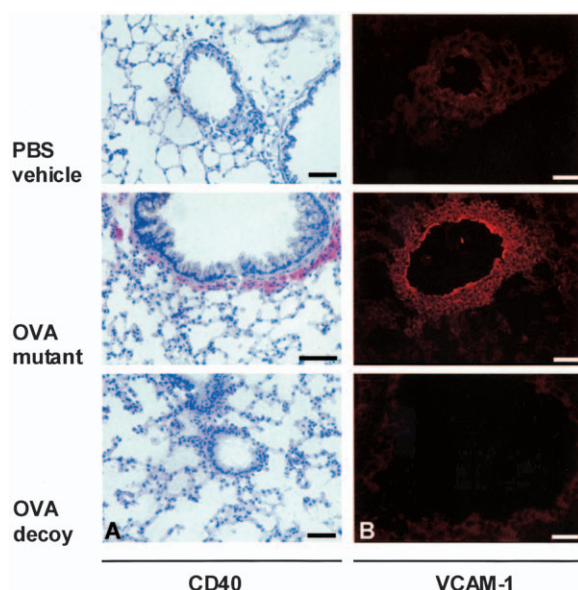


**FIG 5.** Immunohistochemistry. CD40 (A) and VCAM-1 (B) immuno-reactivity in lungs of sham-sensitized, intranasally vehicle-treated (PBS-vehicle); mutant control ODN-treated (OVA-mutant); or STAT-1 decoy ODN-treated (OVA-decoy) mice. The scale bar represents 50  $\mu$ m.

attenuation of the inflammatory responses ensuing after allergen sensitization and airway challenges.<sup>15,32,33</sup>

Similar to *in vitro* data demonstrating an abrogated expression of CD40 after STAT-1 decoy ODN treatment,<sup>13</sup> we observed complete inhibition of *in vivo* upregulation of CD40 in peribronchial cells after local application of STAT-1 decoy ODN to sensitized animals before airway allergen challenge.

As a consequence of abrogated CD40 expression caused by disruption of STAT-1 signaling, T-cell activation and cytokine production critical for development of AI and AHR might be impaired. Indeed, we found increased production of IL-5 in BALF and of IL-5 mRNA in lung tissues in sensitized and challenged animals that was significantly reduced after single and local treatment of sensitized mice with STAT-1 decoy ODN before airway challenges. This might be the result either of direct effects of STAT-1 decoy ODN on T cells leading to decreased cytokine production or because of abolished T-cell activation after impaired expression of CD40.<sup>34-36</sup>

In addition to IL-5,<sup>16</sup> several other factors are pivotal for the induction of eosinophilic airway infiltration. Peripheral blood eosinophils are guided through blood vessels into the tissues of target organs by means of interaction with specific adhesion molecules. In the case of eosinophils, interaction between cell-bound very late antigen 4 with VCAM-1 expressed on the endothelium is crucial for targeted migration into tissues.<sup>16</sup> The expression of VCAM-1 is under the control of another  $T_H2$  cytokine, IL-4, and is found to be upregulated in lung tissues of asthmatic patients.<sup>37,38</sup> Accordingly, we observed increased expression of VCAM-1 in lung tissues of sensitized mice after allergen airway challenges, facilitat-

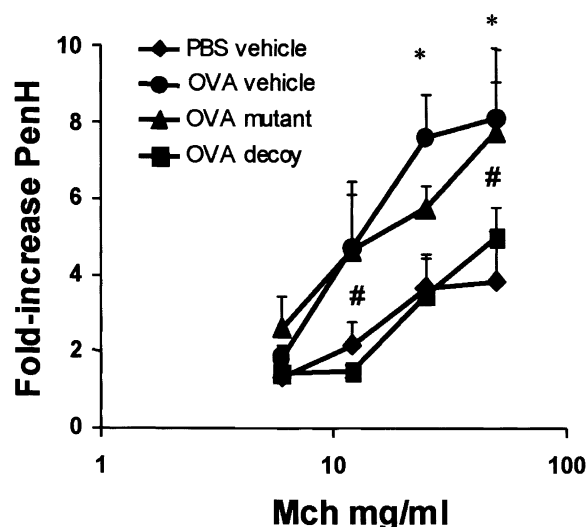


**FIG 6.** Airway responsiveness. Airway responsiveness to aerosolized methacholine was measured in unrestrained and conscious mice, and enhanced pause was determined. Expressed are the fold increase in enhanced pause values of sham-sensitized, intranasally vehicle-treated (PBS-vehicle); OVA-sensitized, mutant control ODN-treated (OVA-mutant); or STAT-1 decoy ODN-treated (OVA-decoy) mice. \*Significant difference between OVA-decoy/OVA-mutant groups; #significant difference between PBS-vehicle/OVA-vehicle groups.

ing the increased eosinophilic AI found in this group. In contrast, VCAM-1 expression was markedly reduced in lung tissues of sensitized animals treated with STAT-1 decoy ODN before airway allergen challenges. This fact might very well be an additional explanation for the decreased eosinophilic influx seen in the BALF of decoy ODN-treated animals.

In conclusion, the reduced inflammatory responses observed after STAT-1 decoy ODN application to sensitized mice before airway challenges might be the result of different and possibly synergistic mechanisms: (1) inhibition of T-cell-dependent eosinophilic airway infiltration (independently of IL-5) by means of down-regulation of T-cell costimulation through CD40<sup>39,40</sup>; (2) blockade of eosinophilic AI by means of decreased production of IL-5; and (3) blockade of eosinophilic airway infiltration by means of decreased expression of essential adhesion molecule, such as VCAM-1.<sup>41</sup>

AI is one of the cardinal features of allergic airway disease and might be very important for the development of AHR.<sup>42</sup> In the current study allergen sensitization and airway challenges induced significant increases of *in vivo* airway reactivity, which are indicated by a leftward shift and increase in the slope of the dose-response curve to inhaled methacholine. The development of AHR was inhibited by a single local application of the STAT-1 decoy ODN to sensitized animals before repeated airway allergen challenges. It seems reasonable to suggest that the reduction in leukocytic, and especially eosinophilic, AI was responsible for this inhibitory effect of STAT-1 decoy ODN on the development of AHR. However, it cannot be

ruled out that other factors that are affected by the inhibition of STAT-1 signaling might be involved in attenuating increased airway responsiveness because the development of AHR might occur independently of (eosinophilic) AI.<sup>43</sup> Nonetheless, the combination of a reduced level of (T-cell) activation and reduced numbers of leukocytes (eosinophils) in airway infiltrates after STAT-1 neutralization might cause the inhibition of a complex airway response, such as the development of AHR.

Inhibition of signaling pathways by application of decoy ODN raises additional questions regarding the further alteration of gene expression by this treatment. Increased STAT-1 signaling has been discovered in a variety of diseases and might lead to unphysiologic tissue responses to otherwise physiologic stimuli. Depending on the affected tissue, this might lead to very different diseases, such as dwarfism,<sup>44</sup> leukemia,<sup>45</sup> and bronchial asthma.<sup>11</sup> Nonphysiologic activation of STAT-1 by epithelial cells in patients with bronchial asthma might lead to the development of asthma-related symptoms. This might be rectified by neutralization of STAT-1 with a decoy ODN, as demonstrated in the present experimental model.

In conclusion, our data indicate that a single local application of a decoy ODN neutralizing the transcription factor STAT-1 effectively inhibits allergen-induced AI and AHR, most likely through attenuating the enhanced expression of CD40 and other effector molecules. These data suggest that STAT-1 might play a pivotal role in the development of allergen-mediated airway disease and points toward potential novel targets for molecular therapy in the treatment or prevention of bronchial asthma.

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