

Decoy oligodeoxynucleotide against STAT transcription factors decreases allergic inflammation in a rat asthma model

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ABSTRACT

Leukocyte infiltration and activation of the CD40-CD40 ligand costimulatory pathway may promote inflammatory processes such as asthma. The aim of this study was to investigate whether a single intratracheal application of a decoy oligodeoxynucleotide (ODN) specific for signal transducer and activator of transcription (STAT) family members 1 and 3 influences leukocyte influx and pulmonary CD40 expression in a rat model of allergic airway inflammation. In comparison with the corticosteroid budesonide, the authors investigated the efficacy of the STAT decoy ODN in ovalbumin-induced allergic asthma in a Brown Norway rat asthma model. Leukocytes of the bronchoalveolar lavage (BAL) and lung tissue were analyzed and expression of CD40 was assessed by Western blotting. Single administration of the STAT decoy ODN but not of a mutated control ODN or budesonide resulted in a significant decrease of eosinophils and T lymphocytes in the BAL fluid. Cell numbers of CD4+ and CD8+ lymphocytes were significantly decreased in the lung tissue after decoy ODN application. CD40 expression in protein extracts from lung tissue was also reduced significantly following STAT decoy ODN treatment. These findings indicate that a single, local application of a transcription factor decoy ODN specific for STAT1 and STAT3 caused an attenuation of the allergen-induced cellular inflammatory reaction and is at least as effective as a topical steroid.

KEYWORDS asthma, decoy, inflammation, STAT

Over the past few years, allergic diseases have constituted the main focus of respiratory research. A dysregulated immune response leads to the loss of tolerance to environmental allergens in bronchial asthma. In addition to airway hyperresponsiveness (AHR), the characteristics of asthma comprise an increased level of antigen-specific immunoglobulin E (IgE) and the

recruitment of eosinophils and other immune cells to the lung (reviewed in [1]). Many studies have also shown that lymphocytes, especially T helper (Th)2 cells, which produce cytokines such as interleukin (IL)4, IL5, and IL13, play a critical role in the development of allergic inflammation (reviewed in [2]). As common therapy, corticosteroids, antihistamines, and bronchodilators have been established to alleviate the allergic symptoms and inflammatory reactions. However, more research is necessary to develop new specific immunotherapy (reviewed in [3]), because these conventional drugs only act against the general symptoms of an allergic disease. The aim of this study was to find an approach to interfere with basic mechanisms of allergic inflammation.

Received 31 October 2008; accepted 25 June 2009

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The authors thank Britta Wenske and Manuela Höfer for their expert technical assistance and AVONTEC GmbH, Martinsried, Germany, for providing the study medication. The authors thank Sheila Fryk for correction of the English.

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Therefore, signal transducer and activator of transcription (STAT) proteins seem very important. STATs are a group of transcription factors that contribute a key molecular link between the binding of a cytokine to its cell surface receptor and the induction of specific genes. This means that they have the ability to transmit signals from the extracellular milieu to the nuclei of cells [4]. The dysregulation of STAT signaling can lead to allergic inflammation (reviewed in [5]), and in particular STAT6 seems to play an important role therein [6, 7]. STAT6-deficient mice, for example, do not develop a Th2 cytokine response or an allergen-specific IgE response [8]. However, other STAT molecules are also important for inflammation. Sampath *et al.* [9] have demonstrated that STAT1 is constitutively expressed in epithelial cells of asthmatic patients and permanent activation of STAT1 may lead to an up-regulation of STAT1 dependent molecules, such as CD40 [10]. Together with its ligand CD154, CD40 is involved in the activation of the immune response as a costimulatory signal. Lei *et al.* have demonstrated that it is not possible to induce an eosinophilia in CD154-deficient mice [11]. Moreover, it has been shown that STAT3 acts as an epithelial regulator of the allergic response in a mouse model of house dust mite-induced asthma by altering Th2 cell recruitment and effector function [12].

Thus, in contrast to conventional treatments, a specific treatment with a decoy oligodeoxynucleotide (ODN), blocking a transcription factor, may constitute a new alternative therapy in the future. Desmet *et al.* have shown that the selective blockade of nuclear factor (NF)- κ B [13] as well as the blockade of activator protein-1 [14] results in an attenuation of the pathophysiologic features of experimental asthma. In addition, in a mouse model Quarcoo *et al.* [15] inhibited the allergen induced airway inflammation and hyperresponsiveness by intranasal application of a decoy ODN specific for STAT1. It is known that STAT transcription factors bind to similar (not identical) DNA binding sequences. The decoy ODN used in this study blocks STAT1 as effectively as STAT3 as shown previously with 3 different hapten-induced contact hypersensitivity animal models [16] and in murine antigen-induced arthritis (AIA) as a model for human rheumatoid arthritis [17]. The aim of this study was to investigate whether a single intratracheal application of a double-stranded decoy ODN-specific for STAT1 and STAT3 influences the leukocyte infiltration and pulmonary CD40 expression in a rat model of allergic airway inflammation.

MATERIAL AND METHODS

Animals

Male Brown Norway rats (mean weight $230\text{g} \pm 3.4\text{g}$, $n = 5\text{--}6$ animals per group) were obtained from Charles River (Sulzfeld, Germany) and kept in the central animal laboratory facility (Medical School of Hannover, Germany). Food and water were provided *ad libitum*. Rats were maintained in a separated minimal barrier sustained facility and were microbiologically monitored according to the Federation of Laboratory Animal Science Association (FELASA) recommendations. All procedures were approved by the Lower Saxony district government in Hannover, Germany.

Decoy ODN technique

Double-stranded ODNs were prepared from complementary single-stranded phosphorothioate-bonded ODNs obtained from Eurogentec (Köln, Germany) as previously described [18]. In contrast to the allergic asthma study in mice published previously by Quarcoo *et al.* [15], another STAT decoy ODN sequence with improved cellular uptake (unpublished data, Wagner *et al.*) and proofed efficacy in a rodent model of contact hypersensitivity [17] has been used. The sequences of the single-stranded plus strands were as follows: STAT consensus dODN, $\text{T}^*\text{G}^*\text{T}^*\text{GAATTACCGGAA}^*\text{G}^*\text{T}^*\text{G}$ (*denotes phosphorothioate-bonded bases: underlined letters denote the STAT binding motif), STAT control ODN $\text{T}^*\text{G}^*\text{T}^*\text{GGACCGTAGGAA}^*\text{G}^*\text{T}^*\text{G}$ (bold letters denote substitutions in the STAT binding motif). As shown previously [17], the control ODN in which the consensus binding GAS element TTC(N2–4)GAA has been mutated cannot neutralize STAT1 or STAT3.

Electrophoretic mobility shift analysis (EMSA)

Preparation of nuclear extracts from the cultured human umbilical vein endothelial cells (HUVECs) and subsequent nondenaturing 4% polyacrylamide gel electrophoresis were carried out as described previously [10]. The Jurkat T-cell line nuclear extract (sc-2132) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The above-described STAT consensus ODN was end-labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by using the 5'-end labeling kit from Amersham GE Healthcare. To ascertain the binding specificity, 1.0 to $2.0\text{ }\mu\text{L}$ of the appropriate gel supershift antibody (2 mg mL^{-1} ; Santa Cruz Biotechnology) per 6.0 to 7.0

μL of nuclear extract (3 to 10 μg protein) were preincubated overnight at 4°C or at room temperature for 60 minutes before the EMSA was performed [18].

Treatment

On days 0 and 7, all rats were sensitized with 1 mg ovalbumin (OVA) (Sigma, Deisenhofen, Germany) and 200 mg of $\text{Al}(\text{OH})_3$ (Sigma) in 1 mL 0.9% (sterile, pyrogen-free) NaCl (Braun Melsungen, Germany) applied subcutaneously (s.c.). As second adjuvant, concentrated preparations of 5×10^9 heat-killed *Bordetella pertussis* bacilli (kindly donated by the manufacturers, Chiron Behring, Marburg, Germany) in 0.4 mL 0.9% NaCl were given intraperitoneally at the same time. On day 13, the animals were challenged intratracheally (i.t.) with 250 μL 0.5% OVA. Under short ether (Baker, Deventer, The Netherlands) anesthesia, they were suspended in a hanging position by a rubber band fixed to the teeth of the upper jaw. After intubation of the trachea via the oral cavity, the solution was instilled. The correct position of the tube was checked by blowing air into the lung before application.

One day before challenge (day 12), rats were treated i.t. with 2 different doses of STAT consensus decoy ODN (100 ng and 33 μg , decoy/OVA group), the mutant control decoy ODN (mutant/OVA group), the decoy ODN vehicle (vehicle/OVA group), and glucocorticoid budesonide (1 mg kg^{-1} of body mass; budesonide/OVA group). The PBS/PBS control group was treated on days 12 and 13 with phosphate-buffered saline (PBS). The animals were sacrificed 24 hours after challenge. Additional animals ($n = 5$) were treated with the higher dosage of 100 μg consensus decoy ODN or vehicle for histology after induction of allergic inflammation.

Preparation of tissue and cells

On day 15, the animals were sacrificed under deep ether anesthesia, the abdominal wall was opened, and the animals were killed by aortic exsanguination. The trachea was dissected and a cannula was inserted in situ. The lungs were lavaged with portions of 5 $\times$ 5 mL cold (4°C) 0.9% NaCl. The fluid was recovered by gentle aspiration. The lavage fluid was pooled, and an average of over 90% of the volume was retrieved from all animals. The pooled bronchoalveolar lavage (BAL) fluid was centrifuged (400 $\times g$, 10 min), washed, again centrifuged, and the resulting cell pellet resuspended in 1 mL PBS (containing 1% bovine serum albumin (BSA) and 0.1% sodium azide). For lung cell extraction, a mechanical method was used. To this end, the left lung was passed through a metal

sieve with 2 rounded tweezers. The sieve was rinsed with 40 mL PBS and the suspension was centrifuged, washed, and resuspended as described above. For histology, lungs were filled with optimal cutting temperature (OCT) gel for immunohistochemistry or with 4% buffered formalin for paraffin embedding and the preparation of hematoxylin and eosin (H&E) staining.

Differential cell counts

The total cell numbers in the BAL and the lung interstitium were determined in a Neubauer counting chamber using standard staining with Tuerk's solution (Merck, Darmstadt, Germany). Eosinophils, neutrophils, and monocytes/macrophages were assessed on slides prepared by centrifuging 1×10^5 cells in anticoagulant (8.8 g NaCl [Merck] + 0.99 g EDTA + 50 g BSA; in 1 L distilled water) for 5 minutes at 200 $\times g$. The slides were fixed in acetone for 10 minutes. Cells were morphologically identified after Diff-Quik (DQ; Dade Behring, Switzerland) staining. At least 300 cells were differentiated on each slide.

For flow cytometry, cells were transferred into microtiter plates (1×10^6 per well). The staining was performed as described previously [19]. In brief, after the cells were washed twice with PBS (containing 1% BSA and 0.1% sodium azide) the first antibody was incubated for 20 minutes at 4°C. The procedure was repeated with the secondary antibody. The cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, USA) focusing on the lymphocyte cluster. CD8+ T cells were identified as R73/Ox8 double positive; CD4+ T cells as R73 positive/Ox8 negative. Activated T cells were identified as CD4/CD25 double-positive cells, B cells as Ox12-positive cells. All antibodies were monoclonal mouse anti-rat (Serotec, Oxford, UK). Unconjugated antibodies were detected with phycoerythrin (PE)-conjugated secondary antibody (kappa PE; Dianova, Hamburg, Germany) and biotinylated primary antibodies with Red 670-streptavidin (Gibco, Gaithersburg, MD, USA). Isotype-matched antibodies served as control.

Western blot analysis of CD40 protein

The CD40 expression was analyzed in whole-tissue homogenates by Western blot according to standard protocols [20]. A polyclonal rabbit anti-rat CD40 antibody (Biovision, San Diego, USA) was used as the primary antibody at 1:500 dilution. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Sigma-Aldrich; 1:3000 dilution) was used as the secondary

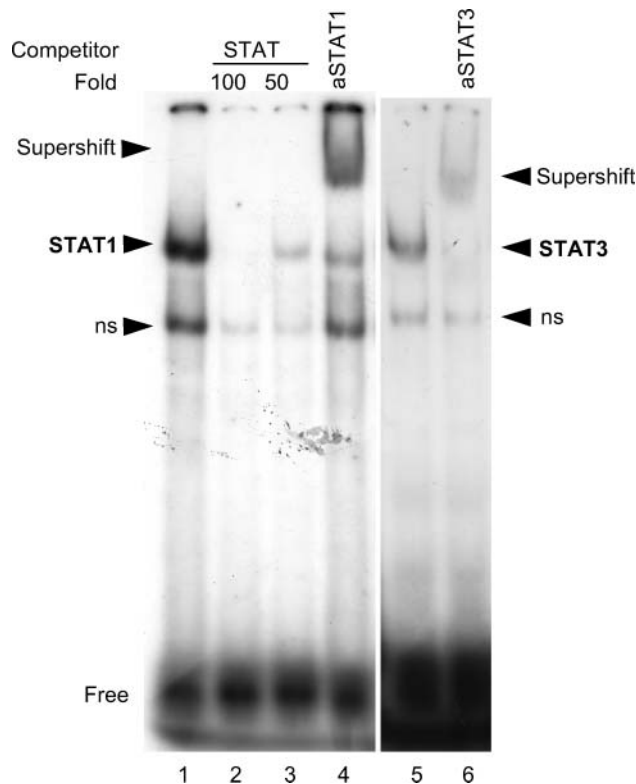


FIGURE 1 STAT1/STAT3. Electrophoretic mobility shift analysis (EMSA). EMSA with nuclear extracts from human cultured endothelial cells (HUVECs) stimulated by IFN- γ (1000 U/mL, 30 minutes, lanes 1 to 4) and human acute T-cell leukemia cells (Jurkat cell line, lanes 5 and 6). Specificity was confirmed by competition experiments using 50- or 100-fold excess of the unlabeled STAT decoy ODN (STAT) and gel supershift assay using a specific STAT1 (aSTAT1) or STAT3 antibody (aSTAT3) as described previously [10, 17]. (ns = nonspecific band; free = unbound decoy ODN).

antibody. Equal loading and transfer of protein was verified by reprobing the membrane with a monoclonal anti- β -actin antibody (Sigma-Aldrich; 1:3000 dilution). The intensity of the immunoreactive bands was quantified by densitometry.

Statistical analyses

Means, standard errors, and the level of significance were determined by GraphPad Prism 4.00 (GraphPad Prism Software, San Diego, USA). The Kruskal-Wallis test was used to compare the results of more than 2 experimental groups, the Mann-Whitney test was applied to compare 2 experimental groups. Values of $P < .05$ were taken as statistically significant.

RESULTS

STAT decoy ODN specificity

The efficacy of the used decoy ODN to bind and neutralize STAT1 and STAT3 transcription factors was

demonstrated with nuclear extracts of human endothelial cells and human Jurkat T cells (Figure 1). The specificity for STAT1 and STAT3 was confirmed by gel shift supershift analysis with specific IgG antibodies and a competition with excess unlabeled STAT decoy ODN.

Cell accumulation in the lung tissue and in the BAL

The allergic inflammation was indicated by a strong cell accumulation in the lung tissue as well as in the BAL of OVA-challenged rats. Total cell numbers in the PBS/PBS group (Figures 2A and 3A) were significantly lower in comparison to the OVA-treated rats. In addition, in the 3 different rat groups, which were all challenged with OVA, a significantly lower number of total cells was determined in the lung tissue but not in the BAL in the decoy ODN group treated with 33 μ g of the STAT decoy ODN. In contrast, no differences were found after treatment with 100 ng decoy ODN (data not shown). Furthermore, the number of eosinophils and neutrophils in the lung tissue were also significantly lower after treatment with the decoy ODN but not with the mutant control ODN or budesonide (Figure 2B, C). In the BAL fluid, only the number of eosinophils was significantly lower when compared to the vehicle group (Figure 3B). Moreover, there was a general tendency towards lower cell numbers in the decoy ODN-treated group.

Treatment with the decoy ODN also led to a significantly lower lymphocyte count, as compared to the other OVA-challenged groups in the lung tissue (Figure 2D). This was noticeable for the T-cell subsets (Figure 2F; CD4+ and CD8+ cells, Figure 2G, H) as well as the B cells (except for budesonide; Fig. 2E). In the BAL, the same significances were observed for smaller T-cell numbers after decoy ODN treatment. The number of activated CD4/CD25-positive T cells was similar in the different groups in the left lung and also in the BAL (Figure 2I, 3I).

Western blot analysis of CD40 protein

The costimulatory molecule CD40 is expressed early during the inflammatory process. As immunohistochemical detection of the extent of CD40 in the dissected lung tissue proved to be variable, Western blot analysis of whole-tissue homogenates was performed instead. There was a marked reduction in CD40 expression in animals treated with the STAT decoy ODN as compared to the vehicle control or mutant control ODN-treated animals (Figure 4). The glucocorticoid budesonide had no significant effect on OVA-induced CD40

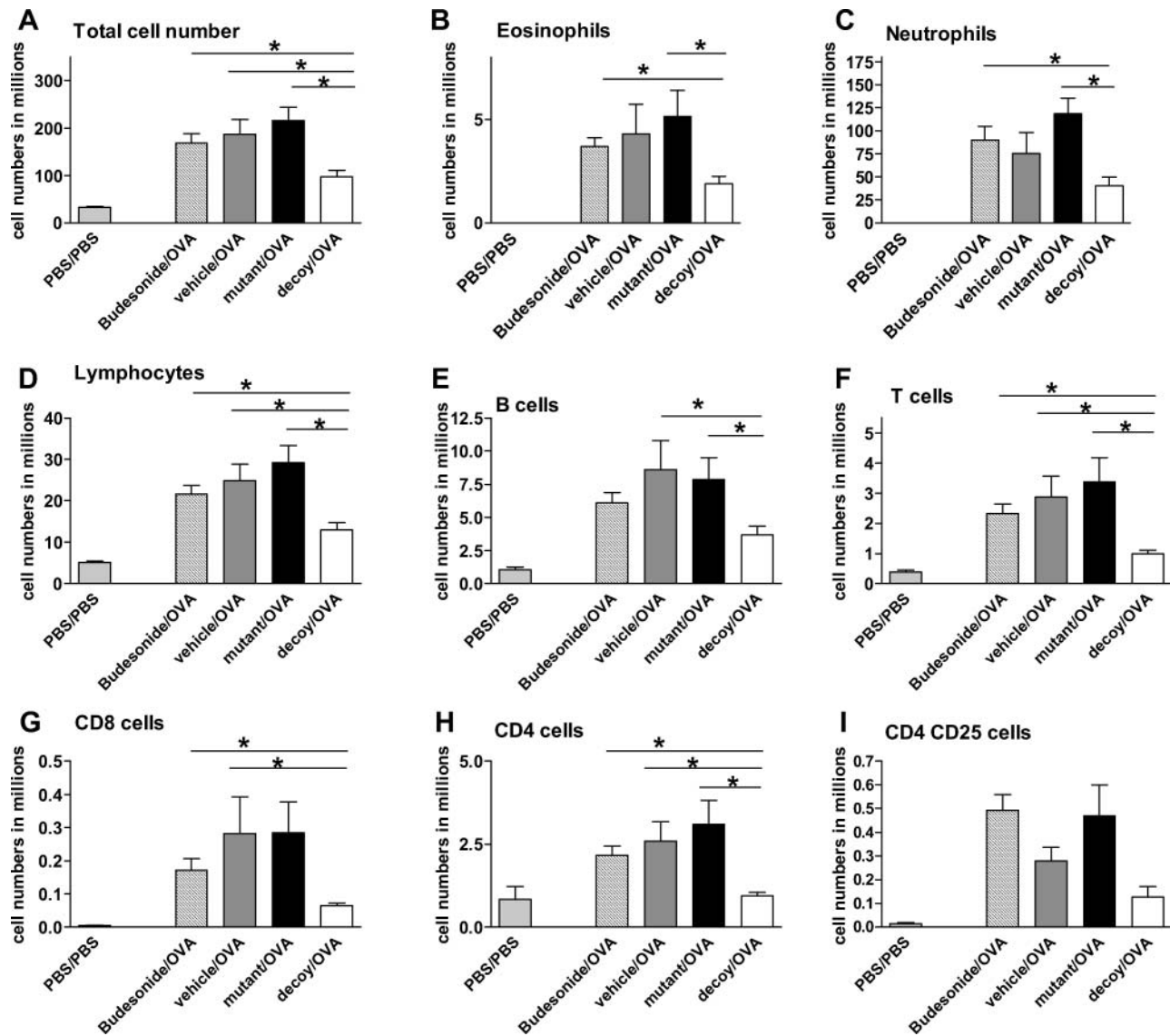


FIGURE 2 Lung tissue. Cell accumulation in the lung tissue of differently treated animal groups. All rats were sensitized with OVA on days 0 and 7. On day 12, rats were divided into 4 groups: The animals in the PBS/PBS group received PBS on day 12 and also on day 13. All other groups were treated with vehicle control, mutant control ODN, or decoy ODN on day 13 and with OVA on day 13. Rats were sacrificed 24 hours after the last treatment. Numbers of total cells (**A**) were determined in a Neubauer counting chamber, eosinophils (**B**) and neutrophils (**C**) were determined after Diff-Quik staining. Cell counts of the different lymphocyte subsets (**D–I**) were performed by flow cytometry. Data are means $\pm$ SEM ($n = 5–6$); $*P < .05$ between groups as indicated.

expression. In Brown Norway (BN) rat lungs after asthma induction and treatment with 100 μ g ODN, the cellular inflammation was clearly reduced (Figure 5).

DISCUSSION

Using a murine model of allergic asthma, we demonstrated previously that a single intranasal application of a STAT1 decoy ODN significantly reduced

the number of eosinophils and lymphocytes in bronchoalveolar lavage fluid [15]. In the current study, we evaluated in more detail the effect of 2 different dose regimens of the STAT decoy ODN by intratracheal administration in comparison with the potent corticosteroid budesonide [21] on the subsets of infiltrating leukocytes in BAL fluid and lung tissue in a rat model of allergic airway inflammation.

The previously observed lower number of infiltrating leukocytes was confirmed in the rat asthma model. The previously employed mouse model

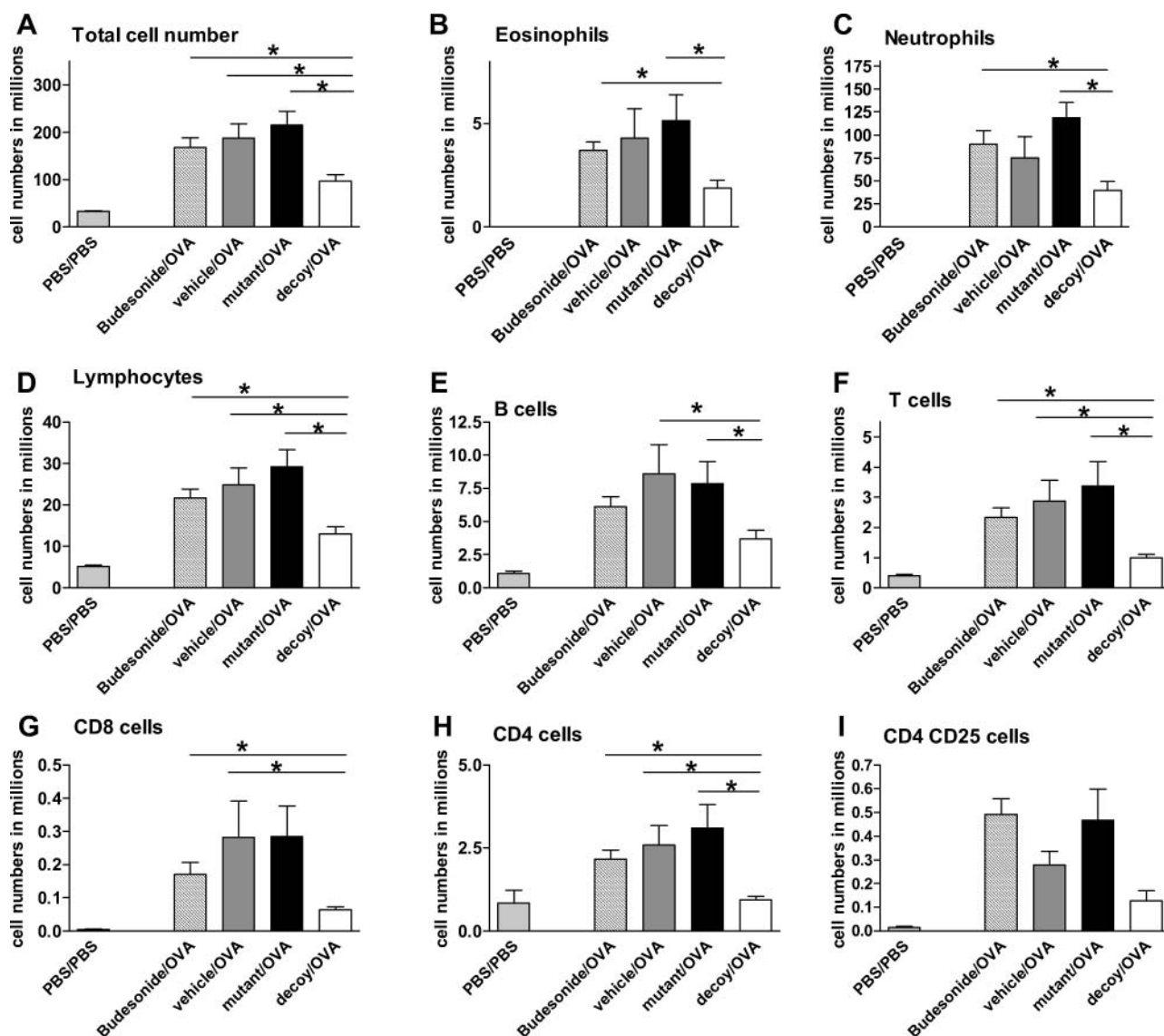


FIGURE 3 BAL fluid. Cell accumulation in the BAL fluid of differently treated animal groups. For details of the treatment regimen refer to Figure 1. Total cell numbers (A) were determined in a Neubauer counting chamber, eosinophil (B) and neutrophil (C) counts were determined after Diff-Quik staining, and those of the different lymphocytes subsets (D–I) by flow cytometry. Data are means $\pm$ SEM ($n = 5$ –6); * $P < .05$ between groups as indicated.

differs from the rat model in that intranasal versus intratracheal application and 1 challenge instead of 2 challenges were performed. Because the rat model has great advantages in asthma research, especially in terms of drug development [22], it was important to confirm the effect of STAT1 neutralization in the rat and to compare 2 dosages. Treatment with 33 μ g (2.5 nmol) but not with 100 ng (7.5 pmol) decoy ODN caused a significant decrease of eosinophils and T lymphocytes in the BAL fluid in comparison to vehicle control. This is the same dose range as used in a murine model of antigen-induced arthritis to significantly reduce local joint swelling and his-

tological signs of acute and chronic arthritis. It was achieved by treatment with 0.25 to 10 nmol STAT1 decoy ODN [17].

Several transcription factors have been implicated in the inflammatory process in asthma (reviewed in [23]). Among these, decoy ODN mediated local neutralization of activator protein (AP)-1 or NF- κ B, which both play a critical role in Th2-cell differentiation and associated cytokine expression, was associated with attenuation of all pathophysiological features of experimental asthma [13, 14]. A possible role for STAT1 activation through the Th1 cytokine interferon (IFN)- γ in combination

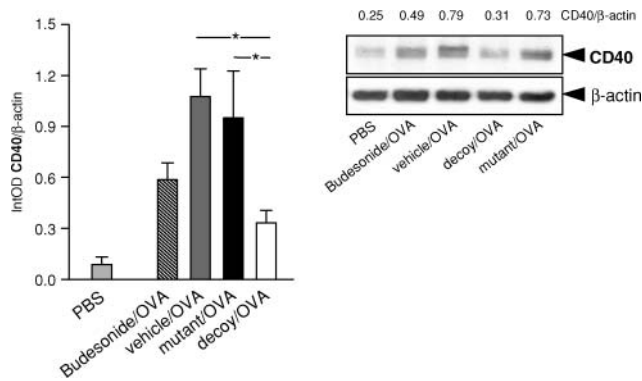


FIGURE 4 CD40 protein. Western blot determination of CD40 expression in dissected lung tissue of animals treated with budesonide, vehicle, mutant control ODN (mutant), or STAT1 decoy ODN (decoy) and of nonallergic (PBS) animals. For details of the treatment regimen refer to Figure 1. * $P < .05$ versus vehicle and mutant control ODN ($n = 6$ for each group). The inset shows an exemplary Western blot analysis.

with allergy-driven overproduction of Th2-type cytokines in allergic airway inflammation was suggested by Sampath et al. [9]. Moreover, STAT1 has been implicated in the cytokine-induced expression of the costimulatory molecule CD40 [10], which is up-regulated on inflamed airway epithelium [24]. Because CD40/CD40L costimulation is also involved in allergen-driven Th2 cytokine production, STAT1 seems to be centrally involved in the early regulation and manifestation of immune responses following either the Th1 or Th2 direction and plays a pivotal role in establishing chronic immune disease [25]. As noted before, the decoy ODN used by us blocks STAT1 as effectively as STAT3 [17] so that it might be particularly suited for the treatment of allergic asthma as it has been shown recently that airway epithelial STAT3 is required for allergic inflammation in a murine model of asthma [12].

One main finding of the present study is that cell numbers of neutrophils and CD4+ and CD8+ lymphocytes were significantly decreased in the lung tissue after application of the decoy ODN. Of those cells implicated in the inflammatory response in asthma, CD4+ Th2 cells, mast cells, and eosinophils are thought to play a crucial role [26]. This subset of CD4+ T cells has a distinct cytokine secretion pattern that leads to further priming and activation of other Th2 cells and aids in the induction of effector mechanisms carried out by other cells, for example the development of airway eosinophilia [27]. In turn, these cytokines prompt B cells, which were also found to be reduced in the lung tissue following decoy ODN treatment in this study, to generate allergen-specific IgE [28]. Moreover, it has been suggested

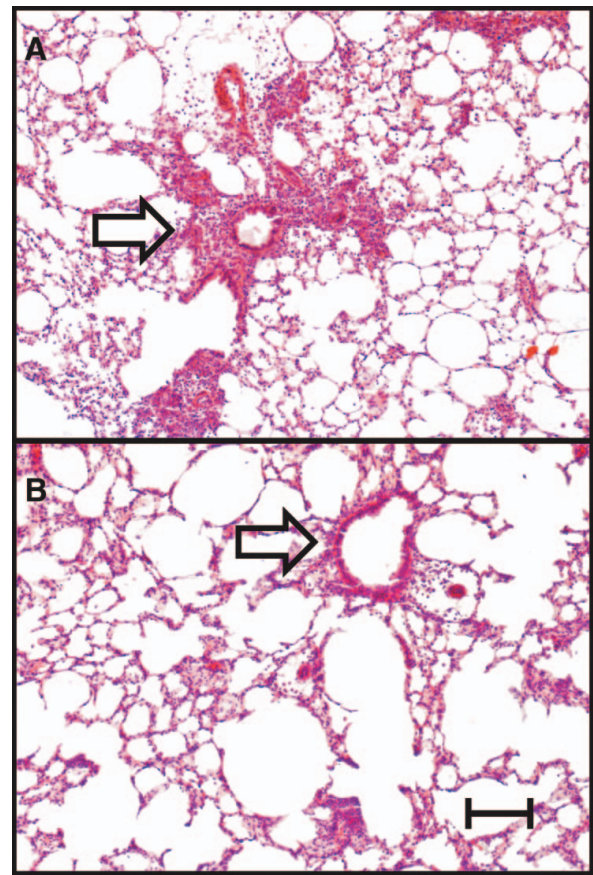


FIGURE 5 Histopathology. H&E staining of lung sections of BN rats after asthma induction with sham or decoy treatment (100 μ g). In asthma rats with sham treatment (A), the inflammation was obvious and prominent around the bronchi and vessels (A, arrow). After STAT1 inhibition (B), the peribronchial and perivascular infiltrations were clearly reduced (arrow). Bar represents 200 μ m.

that T-lymphocyte subpopulations and eosinophils communicate by means of both direct cell-cell interactions and through the secretion of inflammatory signals, which is critical to the onset and progression of pulmonary pathology [29]. A more chronic inflammation is characterized by an increased number of CD8+ Tc1 cells, a subpopulation of CD8+ cytotoxic T cells, and macrophages in the lung tissue as well as neutrophils in the airway lumen [30]. Thus, recruitment and homing of these different lymphocytes play a crucial role in the pathogenesis of asthma and can apparently be inhibited or prevented by single application of the STAT1 decoy ODN.

Glucocorticoids are highly effective in the control of diseases associated with T-cell activation [31]. The most striking result of this study is the minimal effect of the high dose (1 mg kg^{-1}) of budesonide, which is a commonly used intermediate potency oral steroid

in humans [32] and was used as a positive control in this study. This observation was validated by the lack of a significant effect of budesonide on CD40 abundance in dissected lung tissue of rats sensitized and challenged with ovalbumin. The failure to respond may result from reduced glucocorticoid binding to its cytosolic receptor, reduced glucocorticoid receptor expression, enhanced activation of inflammatory pathways, or lack of a corepressor activity [33]. Glucocorticoid receptors either bind to DNA and induce the expression of anti-inflammatory genes or act indirectly to repress the activity of the above mentioned transcription factors NF- κ B and AP-1. Glucocorticoid receptors are in fact down-regulated in hepatic T lymphocytes in rats with experimental cholangitis [34] and divergently expressed in human and murine monocytes and T cells [35]. Another explanation for the ineffectiveness of budesonide in this study may be that we performed only a single application 24 hours before and not a second application 1 hour before allergen challenge as published by others [20]. Thus, STAT1 decoy ODN treatment might be beneficial in patients who fail to respond well to therapy with high-dose glucocorticoids.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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