Original article

Signal transducer and activator of transcription 1 decoy oligodeoxynucleotide suppression of contact hypersensitivity

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Background: Cytokines play a pivotal role in allergy development through activating signaling mechanisms, such as the Janus kinase/signal transducer and activator of transcription (STAT) pathway, which controls the expression of numerous proinflammatory genes.
Objective: In comparison with 2 different corticosteroids and a calcineurin inhibitor, the efficacy of a STAT1 decoy oligodeoxynucleotide (dODN)–containing ointment on hapten-induced contact hypersensitivity was examined in 3 different animal models.
Methods: After sensitization, the test compounds were administered before hapten challenge, after hapten challenge, or both to different sites of the animal skin. Subsequent erythema and edema formation was scored macroscopically, microscopically, or by a shift in ear weight. Biopsy specimens were taken and processed for histopathology, immunohistochemistry, and real-time PCR analyses.
Results: Treatment with the STAT1 dODN but not the corresponding control ODN markedly improved the clinical signs of inflammation in all 3 animal models in a dose-related manner. In guinea pig skin this was accompanied by a distinct decrease in leukocyte infiltration into the dermis after 24 hours. In addition, expression of CD40, IFN-γ, IL-1β, IL-8, IL-12, and TNF-α was strongly attenuated. The dODN was equally effective in the domestic pig model when administered therapeutically, and its preventive effect in the mouse model lasted for more than 48 hours.
Conclusions: Altogether, treatment with the dODN proved to be at least as effective as treatment with the reference compounds.
Clinical implications: Topical administration of a STAT1 dODN might constitute a novel approach to the treatment of allergic contact dermatitis and related inflammatory skin diseases. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Contact hypersensitivity, allergic contact dermatitis, signal transducer and activator of transcription 1, decoy oligodeoxynucleotide, gene expression*

Contact hypersensitivity (CHS) clinically presenting as allergic contact dermatitis (ACD) is a delayed-type hypersensitivity (DTH) reaction in the outermost layers of the skin that is mediated by hapten-specific T cells.[1] The cutaneous proinflammatory response to the sensitizing agent in experimental CHS depends on the haptenated antigen and thus can be regarded as a cellular immune response mediated either by IFN-γ–producing T_H1 cells and T cytotoxic type 1 (T_C1) cells or by IL-4– and IL-10–producing T_H2 cells.[2] As a result, animal experimental CHS models have been applied for years in relation to psoriasis and atopic dermatitis drug discovery.[3] They are considered particularly useful when it comes to evaluating broad-acting anti-inflammatory drugs. Most popular are rodent CHS models because of the fact that the underlying pathogenesis is well known; they are highly reliable and require only minimal manipulation and treatment. Their disadvantage is that rodent skin is built up rather differently than human skin, so that a rodent CHS model frequently is complemented by its porcine equivalent.[4]

After appropriate sensitization, topical administration of strong lipophilic haptens, such as nitrohalobenzenes, to either species results in the presentation by skin dendritic cells but also keratinocytes of haptenated peptides to already primed T_H1 and T_C1 cells that patrol in the skin.[2,3] These in turn produce type 1 cytokines, namely IFN-γ, which stimulate resident skin cells, primarily keratinocytes, to produce various cytokines and chemokines that, within less than 12 hours, provoke the PMN infiltrate in the dermis and epidermis that is typical for this form of CHS.[5] In the intermediate and late elicitation phases, this is followed by a progressive accumulation of mononuclear cells.[6]

Apparently, it is the combined action of only a few pivotal transcription factors, among which is signal transducer and activator of transcription (STAT) 1, that mediates proinflammatory gene expression in psoriasis,[7] which, just like the aforementioned CHS response, is T_H1/T_C1

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Abbreviations used
ACD: Allergic contact dermatitis
AP-1: Activator protein 1
APC: Antigen-presenting cell
CHS: Contact hypersensitivity
DNCB: 2,4-Dinitrochlorobenzene
DNFB: 2,4-Dinitrofluorobenzene
dODN: Decoy oligodeoxynucleotide
DTH: Delayed-type hypersensitivity
EMSA: Electrophoretic mobility shift analysis
FITC: Fluorescein 5-isothiocyanate
NF-κB: Nuclear factor κB
PAEC: Porcine aortic endothelial cell
STAT: Signal transducer and activator of transcription
T_C1: Cytotoxic type 1

cell dominated. Increased expression of STAT1 and STAT1-dependent proinflammatory gene products also has been documented in a T_H1/T_C1-biased rodent model of CHS.[8] In the absence of long-term safety data for the latest biologic agents, calcineurin inhibitors and synthetic corticosteroids still represent the most commonly used anti-inflammatory drugs in dermatology. Topical corticosteroids, which are widely used in the therapy of ACD, exert their anti-inflammatory effects mainly through inhibition of the transcription factors activator protein 1 (AP-1) and nuclear factor κB (NF-κB).[9] However, long-term treatment with these drugs might lead to skin atrophy,[10] and it was such drawbacks that spurred the search for alternative treatment modalities.

Cutting-edge developments focus on small-molecule and nucleic acid–based drugs.[11] Because of limited oral bioavailability, topical administration of these drugs is preferred, even though the barrier properties of the skin might impede intradermal delivery. Decoy oligodeoxynucleotides (dODNs) are short double-stranded DNA molecules that seem to penetrate the skin rather easily, presumably because of an active transcellular transport. They mimic the genomic binding site of their target transcription factor so that its activity is blocked and therewith the expression of its target genes.[12] By using such a dODN directed against STAT1, we could already verify the broad anti-inflammatory effects of this novel drug class in different *in vivo* models of disease.[13-15] We have now examined the efficacy of an ointment containing a related dODN in 3 different models of CHS in comparison with established reference compounds.

METHODS

Preparation of dODN ointment

Double-stranded ODNs were prepared from complementary, single-stranded, phosphorothioate-bonded ODNs obtained from Eurogentec (Cologne, Germany), as previously described.[16] The sequences of the single-stranded plus strands were as follows: STAT1 consensus dODN, T*G*T*GAATTACCGGAA*G*T*G (*denotes phosphorothioate-bonded bases); STAT1 control ODN, T*G*T*GGACCGTAGGAA*G*T*G (underlined letters denote substitutions in the STAT binding motif). As shown extensively in the past,[13-16] the control ODN in which the consensus-binding gamma-activated site element TTC(N2-4)GAA has been mutated cannot neutralize STAT1.

Between 0.13 and 8 mg of either ODN in TEN buffer (40 mmol/L solution) or TEN buffer alone (10 mmol/L Tris, 1 mmol/L EDTA, and 150 mmol/L NaCl) was added directly to 0.4 g of ointment base (Unguentum emulsificans DAB, obtained from a local pharmacy) in a sterile mortar. They were properly mixed, yielding a 0.02% to 1% ODN-containing hydrophilic cream (Unguentum emulsificans/TEN buffer ODN solution 1:1 [wt/vol]). All solutions and the ointment base, pestles, and mortars were prewarmed to 37°C. After stirring for 10 to 15 minutes at 37°C, the ointments were allowed to cool down to ambient temperature and stored at 4°C until use on the same day.

Isolation and culture of porcine aortic endothelial cells

Endothelial cells were isolated from porcine aortas, plated on 6-well plates, and cultured in Dulbecco's modified Eagle's medium/Ham F12, as described previously.[17] They were used as primary culture to demonstrate efficacy of the STAT1 dODN against porcine STAT1. For this purpose, porcine aortic endothelial cells (PAECs) were preincubated with the STAT1 dODN (4 hours at 10 μmol/L), stimulated with porcine IFN-γ (1000 U/mL medium) for 30 minutes, and subsequently prepared for electrophoretic mobility shift analysis (EMSA).

Guinea pig CHS model

Male Dunkin-Hartley guinea pigs (Charles River; body weight, 350-450 g) were sensitized with 50 μL of 10% 2,4-dinitrochlorobenzene (DNCB; Sigma-Aldrich, Deisenhofen, Germany) in acetone/olive oil 1:1 (vol/vol) administered to the dorsal side of the right auricle first and to the left auricle 24 hours later. On day 7, they were boosted on the neck with 15 μL of 2% DNCB in acetone/olive oil 95:5 (vol/vol). Seven days later, 6 test sites (1 cm^2 each) on the shaved backs of the animals were treated with one of the following incorporated into the standard dermal formulation (60 mg per test site): STAT1 dODN (n = 18 per dose), control ODN (n = 18), clobetasol propionate (0.25%, n = 6), or vehicle (n = 18). CHS was elicited with 10 μL of 0.5% DNCB in acetone/olive oil 95:5 (vol/vol) 4 hours later. Another 24 hours later, severity of erythema and edema formation was evaluated on a scale of 0 to 3 (0 = no visible erythema, 3 = severe erythema and focal edema), according to the method of Frosch and Kligman,[18] with the investigators blinded toward the treatment regimen. Thereafter, the animals were killed, and skin biopsy specimens were taken from each of the test sites. The study protocol was designed in accordance with German law on the protection of animals and approved by local authorities.

Domestic pig CHS model

Female domestic pigs weighing 12 to 15 kg were sensitized by means of epicutaneous administration of 500 μL of 10% 2,4-dinitrofluorobenzene (DNFB; Sigma-Aldrich) in a 1:5:1:3 (vol/vol) mixture of DNFB/acetone/dimethyl sulfoxide/olive oil distributed evenly to both auricles and groins. On day 10, challenge was performed by administering 15 μL of a 1% DNFB solution (without dimethyl sulfoxide) to each of 24 test sites (2-cm diameter) arranged in 4 craniocaudal lines on both sides of the back of each animal. The test sites were treated either 6 hours before and 30 minutes after or 30 minutes before and 6 hours after the DNFB challenge with 60 mg of ointment containing either 0.13% or 1% STAT1 dODN, 1% Elidel (pimecrolimus, SDZ ASM 981),[19] 1% hydrocortisone acetate, or the appropriate placebo (ethanol/propylene glycol 3:7 [vol/vol] in the case of hydrocortisone). Test sites were clinically examined 24 hours after the DNFB challenge at the peak of the inflammatory response.

TABLE I. Scoring of the clinical signs of CHS in the domestic pig model 24 hours after the DNFB challenge

Score	Erythema/intensity	Erythema/extent	Induration
0	Absent	Absent	Absent
1	Scarcely visible	Small spotted	Scarcely palpable
2	Mild	Large spotted	Mild hardening
3	Pronounced	Confluent	—
4	Severe (or livid discoloring)	Homogenous redness of test site	—

Changes were scored on a scale from 0 to 4, allowing a combined maximal score of 10 per designated site (Table I). In total, 8 animals were used in 2 independent series of experiments. The study protocol was approved by the local governmental authority (Landesregierung, Wien, Magistratsabteilung 58).

Mouse CHS model

Groups of 8 female BALB/c mice (Charles River, Sulzfeld, Germany) were sensitized with 50 μL of 0.5% fluorescein 5-isothiocyanate (FITC; Sigma-Aldrich) on the shaved ventral abdomen on days 1 and 2 and boosted on days 21 and 22. CHS was elicited with 10 μL of 0.5% FITC on the medial aspects of the right ears on day 28. Twenty-four or 48 hours thereafter, the animals were killed, and the individual differences in weights of the left (unchallenged) and right (challenged) ears in test and control animals were determined. Increase in ear weight was used as a parameter of inflammatory swelling. Topical treatment was performed 30 minutes before and 4 hours after challenge with 10 mg of the active (1% STAT1 dODN), control (control ODN), or placebo (vehicle) formulation. Hydrocortisone acetate at 1% was used as a comparator.

dODN penetration into guinea pig skin

Uptake of the nucleic acid into the guinea pig skin was verified by using a Texas Red–labeled dODN (Eurogentec), the sequence of which was identical to that of the unlabeled STAT1 consensus dODN. Permeation and distribution of the dODN in the dermis and epidermis was monitored for up to 6 hours by means of fluorescence microscopy. To this end, guinea pig skin biopsy specimens, frozen in liquid nitrogen and embedded in OCT Compound Tissue-Tek (Miles Laboratories, Elkhart, Ind), were cut into 10-μm sections with a cryostat (Leica, Wetzlar, Germany). Subsequently, fluorescence distribution was visualized with a MicroMax CCD camera (Princeton Instruments, Trenton, NJ) coupled to an Axiovert S100 TV microscope (Zeiss, Jena, Germany).

EMSA

Preparation of nuclear extracts from the cultured PAECs and guinea pig skin biopsy specimens followed by nondenaturing 4% polyacrylamide gel electrophoresis was carried out as described in detail elsewhere.[17] A [γ-^{32}P]Adenosine triphosphate–labeled sis-inducible element gel shift ODN (Santa Cruz Biotechnology, Heidelberg, Germany) was used for detection of STAT1 in nuclear extracts of the cultured PAECs. For homogenization of the skin biopsy specimens, frozen samples were pulverized with 5 or 6 strokes of a hammer, placed in a tube together with a stainless-steel bead, and homogenized in the Mixer Mill MM 301 (Retsch, Haan, Germany) for 30 seconds at 30 Hz. Labeled AP-1 and STAT1 gel shift ODNs (Santa Cruz Biotechnology) were used for transcription factor detection in the nuclear extracts of the biopsy specimens.

Quantitative real-time PCR

RNA isolation and reverse transcription were carried out as described previously.[16] Real-time PCR was performed in a Light-Cycler (Roche Diagnostics, Mannheim, Germany) by using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Details of the PCR conditions and primer sequences can be found in Table E1 in the Online Repository at www.jacionline.org. Recombinant standards were synthesized *in vitro* from the cloned PCR fragments in plasmid DNA (TOPO TA Cloning kit; Invitrogen, Karlsruhe, Germany). The identity of the amplification products was verified by means of direct sequencing with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif).

Histochemistry

Tissue samples were fixed with 4% formaldehyde and embedded in paraffin from which 5-μm-thick successive sections were cut, stained with Giemsa reagent (Merck, Darmstadt, Germany; 1:10 dilution for 60 minutes at ambient temperature), and mounted with Entellan (Merck). They were examined by an experienced pathologist in a blinded fashion. Changes were scored for epidermal spongiosis (0-3), dermal inflammatory infiltrate (0-3), and epidermal inflammatory infiltrate (0-3), allowing a combined maximal score of 9. In addition, the thickness of the stratum corneum and the cellular part of the epidermis was measured with a calibrated ocular micrometer by means of standard light microscopy.

Visualization of neutrophil infiltration was achieved by using the naphthol AS-D chloroacetate esterase kit (Sigma-Aldrich), according to the manufacturer's instructions. Sections were counterstained with acid hematoxylin and mounted with Aquamount (Gurr, Hanau, Germany). The degree of PMN infiltration was determined in a blinded fashion by counting naphthol AS-D chloroacetate esterase–positive red cells in 5 random fields per slide with the aid of a SPOT RT color CCD camera (Diagnostic Instruments, Sterling Hights, Mich) coupled to the Axiovert S100 TV microscope and the MetaMorph 6 software package (Molecular Devices, Downingtown, Pa).

Immunohistochemistry

Immunohistochemistry was performed on the aforementioned 5-μm-thick paraffin sections. Antigen retrieval was required for the detection of IL-8 (10 mmol/L sodium citrate; microwave oven set to 600 W for 30 minutes) and macrophages (30 minutes at 37°C with 0.1% trypsin in Tris-buffered saline with Tween 20). The following primary antibodies were used: rabbit anti-human IL-8 (dilution 1:10; Research Diagnostics, Concord, Mass), rabbit anti-human CD40 (dilution 1:400, Calbiochem/Merck Biosciences, Bad Soden, Germany), mouse anti-human monocyte/macrophages (clone MAC387, dilution 1:400; AbD Serotec), and mouse anti-guinea pig T-cells (clone CT5, dilution 1:1000; AbD Serotec, Düsseldorf, Germany). After incubation with the corresponding secondary horseradish peroxidase–labeled goat anti-rabbit or goat anti-mouse antibody (DAKO EnVision+, DAKO, Glostrup, Denmark), sections were exposed to the chromogenic substrate (DAKO AEC). After hematoxylin and eosin counterstaining, the sections were mounted with Aquamount. Positive immunoreactivity was documented with the SPOT RT color CCD camera, and the degree of macrophage and T-cell infiltration was monitored in a blinded fashion by counting 3 random fields per slide with the aid of the MetaMorph 6 software package.

Data analysis

Unless indicated otherwise, results are expressed as means ± SEM of *n* individual experiments. The Kruskal-Wallis test (nonparametric ANOVA) followed by a Dunnett post hoc analysis or the

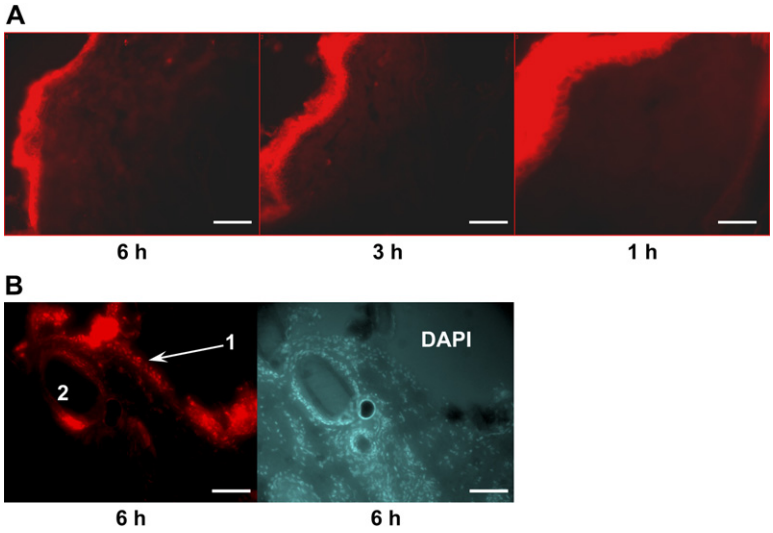


FIG 1. Detection of Texas Red–labeled STAT1 dODN penetration into guinea pig skin. **A**, Time-dependent uptake of the fluorescent dODN (0.2% ointment). **B**, Detection of the fluorescent dODN in the epidermis *(1)* and a hair follicle *(2)* 6 hours after topical application. The *right panel* shows 4′-6-diamidino-2-phenylindole dihydrochloride *(DAPI)*–stained nuclei. *Scale bar,* 100 μm.

nonparametric Wilcoxon rank sum test were used where appropriate to determine differences between the means and the corresponding control value, with a *P* value of less than .05 considered statistically significant.

RESULTS

Time course of dODN penetration into guinea pig skin

After administration of the ointment containing the Texas Red–labeled STAT1 dODN (at a dose of 0.2%), a prominent fluorescence signal was detected first in the epidermis and then started to penetrate into the dermis after 3 to 6 hours (Fig 1, *A*) and appeared to be clearly cell associated (Fig 1, *B*).

Effects of the STAT1 dODN in the guinea pig CHS model

EMSA revealed a prolonged activation of the transcription factors AP-1 and STAT1 in guinea pig skin over the 24-hour exposure to DNCB (see Fig E1 in the Online Repository at www.jacionline.org). The macroscopic (Fig 2, *A*; for exemplary data, see Fig E2, *A*, in the Online Repository at www.jacionline.org) and histopathologic (Fig 2, *B*; for exemplary data, see Fig E2, *B*, in the Online Repository at www.jacionline.org) evaluation, as well as the epidermal thickness measurements (Fig 2, *C*), all show that application of the STAT1 dODN significantly attenuated the DNCB-induced inflammatory response in a dose-related manner, with the dose of 0.13% being as effective as 0.25% clobetasol. At the same dose, the mutated control ODN did not reveal any inhibitory effects (Fig 2). Histochemical/immunohistochemical analyses at this time confirmed a considerable decrease in the number of infiltrating neutrophils (Fig 3, *A*), T cells (Fig 3, *B*), and monocytes/macrophages (Fig 3, *C*) in the STAT1 dODN-treated skin sections (for exemplary data, see Fig E3 in the Online Repository at www.jacionline.org). Again, the dose of 0.13% proved to be as effective as 0.25% clobetasol, and the control ODN was ineffective. Real-time PCR analyses further corroborated the anti-inflammatory effects of the dODN, as well as those of the steroid, demonstrating an almost complete suppression of the DNCB-induced increase in IL-1β, IL-8, IFN-γ, TNF-α, and IL-12p40 mRNA abundance (Fig 4). This finding was confirmed on the protein level for CD40 and IL-8 by means of immunohistochemistry (Fig 5).

Effects of the STAT1 dODN in the domestic pig model of CHS

The efficacy of the STAT1 dODN was verified in a pig CHS model because porcine skin resembles human skin more closely than rodent skin.[4] The STAT1 dODN ointment was tested both in a preventive and in a therapeutic treatment arm against placebo and at least 1 active comparator. In the preventive treatment arm the STAT1 dODN was tested at a dose of 0.13% against vehicle and 1% Elidel in 3 animals (4 test sites each) and at a dose of 1% against vehicle and 1% hydrocortisone in 3 animals (2 test sites each). With this treatment regimen, the dODN at a dose of 0.13% was as effective as 1% hydrocortisone and at a dose of 1% attained more than 80% of the effect of 1% Elidel in blocking the clinical signs of cutaneous inflammation in this animal experimental model (Fig 6, *A*).

In the therapeutic treatment arm the STAT1 dODN was tested at a dose of 1% against vehicle and 1% hydrocortisone in 2 more animals (2 test sites each). Although the inhibitory potency of the dODN appeared to be similar under these conditions (35% ± 15% vs 42% ± 11% inhibition in the preventive treatment arm), the

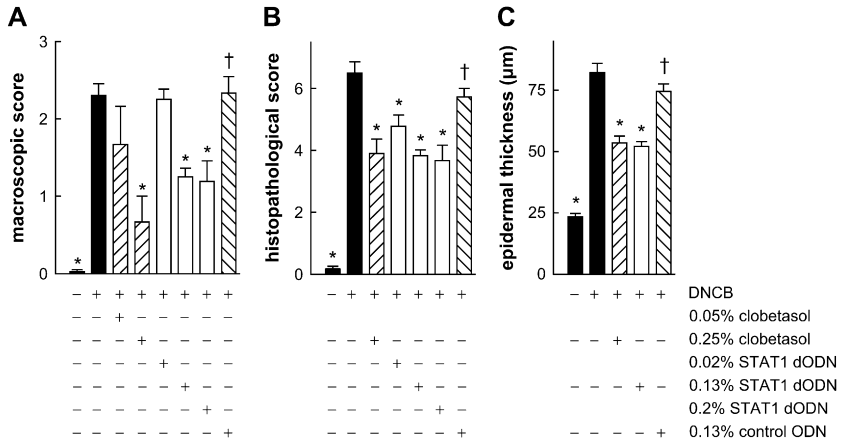


FIG 2. Topical STAT1 dODN treatment reduces the macroscopic **(A)** and histopathologic **(B)** signs of inflammation, as well as the increase in epidermal thickness **(C)**, in the guinea pig CHS model 24 hours after challenge with DNCB. Statistical summary of the evaluation of 18 and 6 (clobetasol) test sites, respectively, is shown. *$P < .05$ versus DNCB plus vehicle; †$P < .05$ versus 0.13% STAT1 dODN.

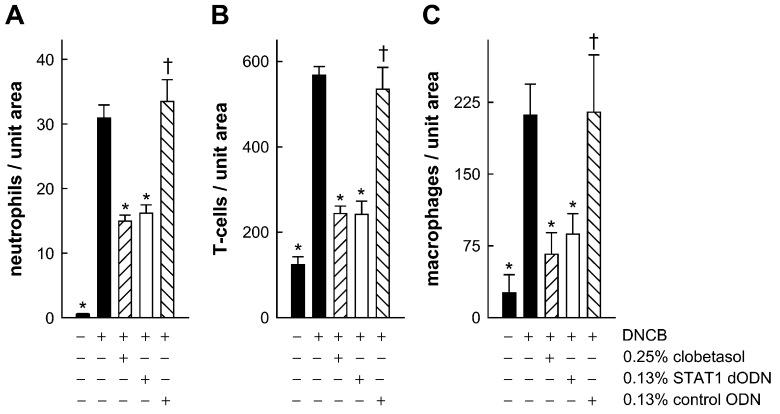


FIG 3. STAT1 dODN attenuation of neutrophil **(A)**, T-cell **(B)**, and monocyte/macrophage **(C)** infiltration into the guinea pig skin 24 hours after DNCB challenge. Statistical summary of the mean cell count per unit area in 3 to 5 random fields of view of 18 and 6 (clobetasol) specimens, respectively, is shown. *$P < .05$ versus DNCB plus vehicle; †$P < .05$ versus 0.13% STAT1 dODN.

corticosteroid seemed to be less effective (16% ± 11% vs 27% ± 11% inhibition).

Nuclear extracts of IFN-γ–stimulated cultured PAECs were analyzed to verify that the dODN used blocks porcine STAT1 equally well. As shown in Fig E4 in the Online Repository at www.jacionline.org, the STAT1 dODN, but not the control ODN, significantly blunted the nuclear translocation of STAT1 in these cells.

Effects of the STAT1 dODN in the mouse CHS model

Finally, a third animal experimental model of CHS was used, which, in contrast to the previous 2 models, is driven by a T_H2-type rather than a T_H1-type immune response[20,21] and thus more closely resembles ACD or atopic dermatitis.[22] To this end, BALB/c mice were sensitized to and challenged with FITC, and the ensuing ear swelling at 24 and 48 hours after the FITC challenge was taken as a readout for the ongoing inflammatory reaction. As shown in Fig 6, *B*, pretreatment with the STAT1 dODN at a dose of 1% but not with the control ODN was similarly effective as in the other 2 CHS models after 24 hours (51% inhibition). This inhibitory effect was maintained after 48 hours (43% inhibition), demonstrating that this was not due to delaying the onset of the cutaneous inflammation. Unexpectedly, hydrocortisone at the same percentage (but not on a molar basis) was more effective than the dODN in this animal experimental model (79% and 89% inhibition, respectively).

DISCUSSION

The present findings demonstrate that topical application of a dODN directed against STAT1, with at least the same efficacy as a mild (hydrocortisone) or an ultrapotent topical corticosteroid (clobetasol), as well as a highly

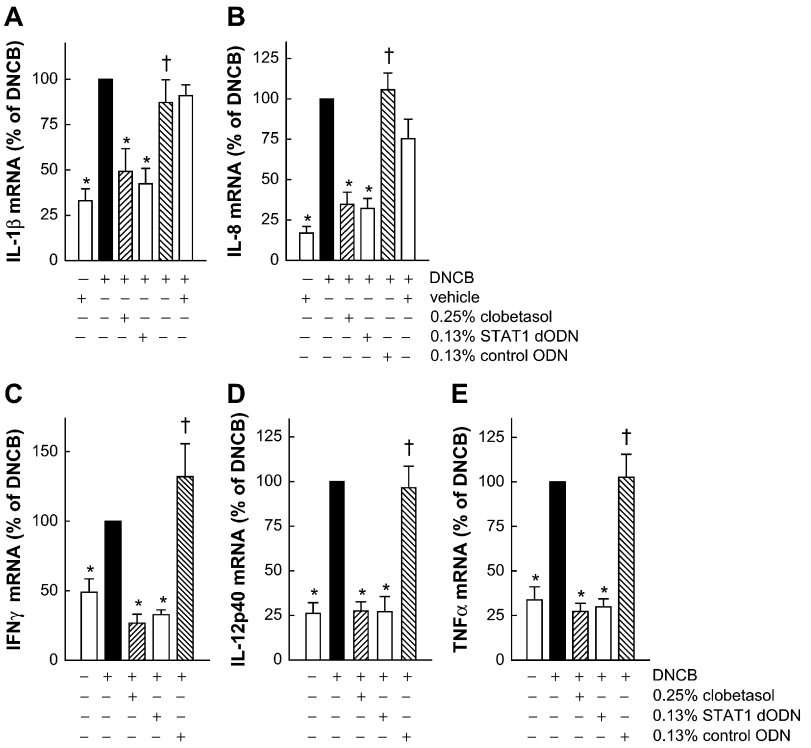


FIG 4. STAT1 dODN suppression of DNCB-induced mRNA expression of IL-1β **(A)**, IL-8 **(B)**, IFN-γ **(C)**, IL-12p40 **(D)**, and TNF-α **(E)** in the guinea pig skin 24 hours after challenge. Statistical summary of real-time PCR data obtained with 18 and 6 (clobetasol) biopsy specimens, respectively, is shown. *$P < .05$ versus DNCB plus vehicle and 0.13% control ODN; †$P < .05$ versus STAT1 dODN.

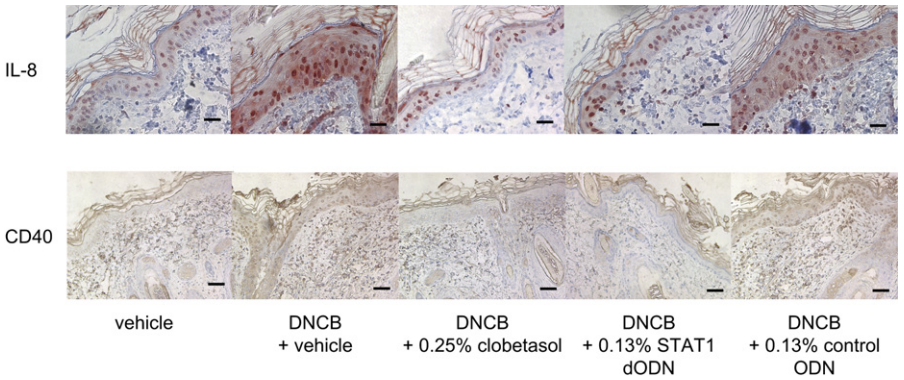


FIG 5. STAT1 dODN–mediated decrease in IL-8 and CD40 protein abundance in guinea pig skin biopsy specimens taken 24 hours after challenge with DNCB. Representative immunohistochemistry analyses are shown. *Scale bar*, 50 μm (IL-8) and 100 μm (CD40), respectively.

active topical immunosuppressive (Elidel), impairs the cutaneous inflammatory reaction in 3 different animal experimental models of CHS. Because of its greater molecular weight, efficacy of the dODN treatment appears to be even more impressive on a molar basis (ranging from 1 to 2 orders of magnitude when compared with Elidel and clobetasol or hydrocortisone, respectively). In contrast to the rodent models, the domestic pig model of CHS used has a high predictability for the topical activity of a test compound in human subjects. Thus cyclosporin A, which is highly active in rodent CHS models, is inactive in the domestic pig model, as well as in patients with ACD, whereas the other calcineurin inhibitors, FK 506 and Elidel, as well as most corticosteroids, tend to be as effective in pig models as in human subjects.[19,23-25]

Because these potent anti-inflammatory drugs are capable of inhibiting ACD, just like psoriasis or atopic dermatitis, the STAT1 dODN might likewise be effective in these genuine immune-mediated skin diseases. For atopic dermatitis, which primarily comprises a T_H2 cell but

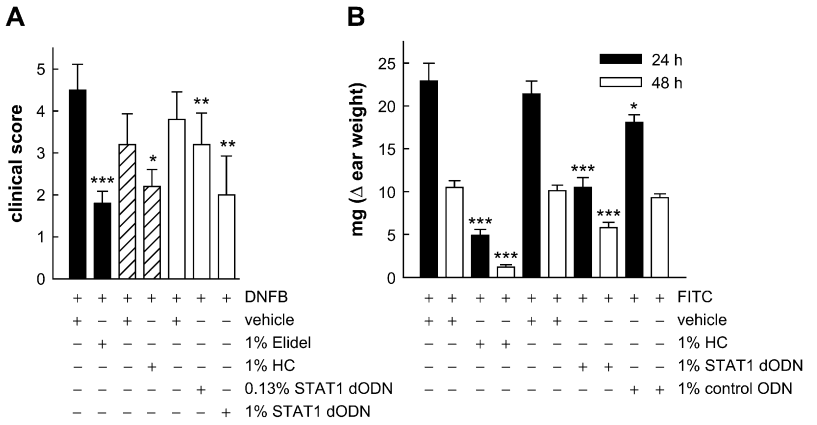


FIG 6. Topical STAT1 dODN treatment diminishes the clinical signs of CHS in the domestic pig model **(A)** and edema formation in the mouse model **(B)**. Statistical summary of the scoring of 6 to 12 test sites in the preventive treatment arm 24 hours after DNFB challenge is shown in Fig 6, *A*. $*P < .05$, $**P < .01$, and $***P < .001$ versus vehicle. Statistical summary of the changes in ear weight (difference in milligrams between challenged and unchallenged ear) in 8 animals each 24 and 48 hours after challenge with FITC is shown in Fig 6, *B*. $*P < .05$ and $***P < .001$ versus vehicle.

also a T_H1 cell component,[22] this assumption is supported by our findings in the mouse CHS model that also has a T_H2 bias.[20,21] For psoriasis, which mainly constitutes a T_H1/T_C1 cell–mediated and possibly also a T_H17 cell–mediated immune response,[7] our findings in the guinea pig and domestic pig models, which are both T_H1 biased,[4,26] might be considered predictive. However, the somewhat lower efficacy of the STAT1 dODN in the mouse model compared with the other 2 models along with its supposedly predominant interfering with IFN-γ–dependent proinflammatory gene expression suggest that dODN blockade of this transcription factor might be best suited for the treatment of T_H1-dominated immunologic disorders. In fact, injection of 0.25 nmol of a related STAT1 dODN, which on a molar basis corresponds to one thirtieth of the effective dose (0.13%) in the nitrohalobenzene-induced CHS models, into the knee joint of female C57BL/6 mice significantly blocked the antigen-induced cutaneous DTH response elicited 7 days later.[14]

Analogous to our findings in the T_H2-weighted mouse model (up to 48 hours follow-up), we assume that in both T_H1/T_C1-weighted CHS models, treatment with the STAT1 dODN also has resulted in a genuine inhibition of the inflammatory response rather than a delay in its onset. Moreover, in the therapeutic treatment arm of the domestic pig model, the dODN was first administered 30 minutes before challenge with DNFB, which initiates a rapid activation of the complement system, as well as mast cells.[27,28] This initiation step, which is clinically apparent in pigs immediately after application of the hapten,[4] most likely has occurred before the dODN could take effect. Furthermore, unlike ACD, the onset and duration of experimentally induced CHS is much shorter. In the domestic pig model, for example, early signs of inflammation can be observed within 6 hours after DNFB challenge that culminate at 24 hours and wane rapidly, so that after 72 hours, the test sites are virtually indistinguishable from normal skin.[4] Therefore the second series of experiments in this CHS model can be regarded as a real therapeutic treatment regimen.

As exemplified by the more detailed findings in the guinea pig model, the STAT1 dODN presumably exerts a rather broad anti-inflammatory effect. Thus the number of infiltrating neutrophils, T cells, and monocytes/macrophages into the epidermis and dermis was markedly reduced along with a near maximum blockade of the expression of IFN-γ, IL-1β, IL-8, IL-12p40, and TNF-α. IFN-γ can derive both from keratinocytes[29] and primed T_H1/T_C1 cells already present in the skin. Particularly IL-12p40, but also IL-1β, IL-8, and TNF-α, are likely to be expressed by skin antigen-presenting cells (APCs), including keratinocytes,[30] even though not all of these are controlled by STAT1. There was also a marked decrease in the abundance of the costimulatory molecule CD40, the expression of which is STAT1 dependent[16] and upregulated in response to IFN-γ by APCs, including keratinocytes,[31] as well as infiltrating monocytes/macrophages. Through receptor/ligand-mediated costimulation of CD154-expressing T_H1 cells (or T_H2 cells), these APCs are likely to fuel the cutaneous inflammatory reaction in experimental CHS.[32] Therefore disruption of CD40/CD154 costimulation can be regarded as a promising approach to the therapy of inflammatory skin diseases.[33] However, despite typifying one important target gene of the STAT1 dODN therapy, CD40 is only one among various adhesion molecules, cytokines, and chemokines.[13-15] This might also explain the marked inhibitory effect of the dODN treatment on leukocyte infiltration in the guinea pig CHS model.

In addition to STAT1, other transcription factor targets have been implicated in immune-mediated inflammatory skin diseases, such as NF-κB, STAT3, and possibly STAT6.[34] Topical administration of an NF-κB dODN has been successfully tested in a mouse model of atopic dermatitis[35] (in comparison with betametasone[36]), as well as in the DTH response of the same species,[37] but the potentially adverse proapoptotic effects of blocking

this transcription factor must be followed. Recently, dODN blockade of STAT3, which together with NF-κB and STAT1 is implicated in psoriasis,[7] has revealed its therapeutic potential in a genetically engineered mouse model of the disease.[38] Interestingly, the dODN used by us blocks STAT1 as effectively as STAT3,[14] so that it might be particularly suited for the topical treatment of patients with this inflammatory skin disease.

In summary, the aforementioned findings suggest that dODN-mediated blockade of STAT1 represents a novel option for the treatment of inflammatory skin diseases that presumably is more effective than state-of-the-art dermal anti-inflammatory drugs but less prone to side effects.

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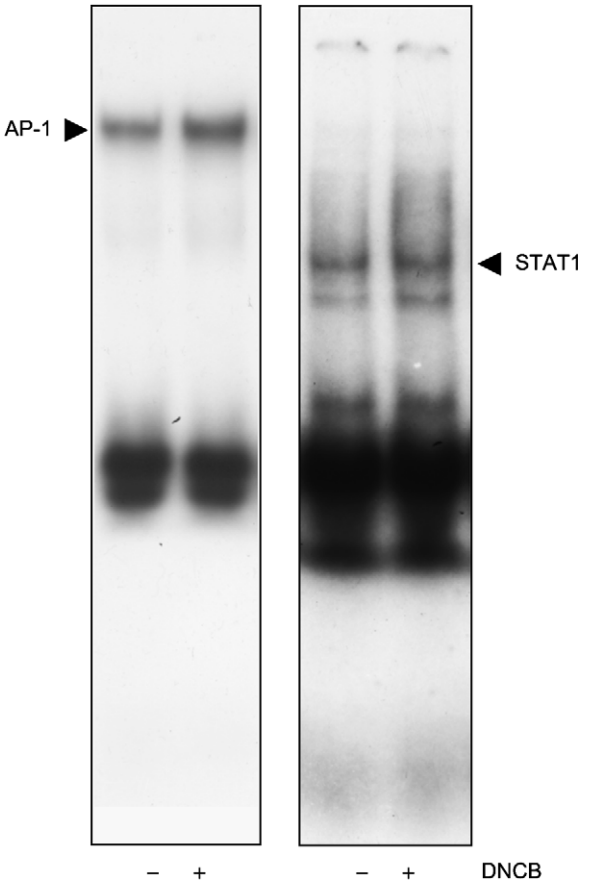


FIG E1. Activation of AP-1 and STAT1 in guinea pig skin biopsy specimens collected 24 hours after challenge with DNCB. Appropriate [γ-^{32P}]adenosine triphosphate–labeled gel shift oligonucleotides were used for detection of either transcription factor in nuclear extracts of these specimens (representative EMSA).

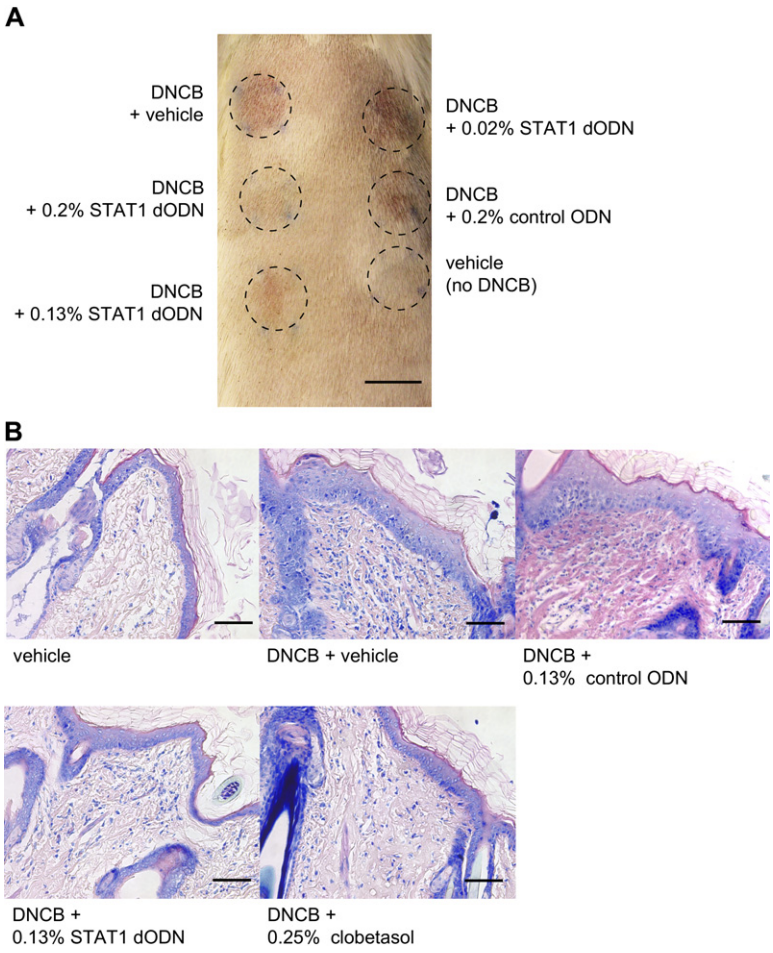


FIG E2. Exemplary results demonstrating the inhibitory effect of the topical STAT1 dODN formulation on the macroscopic **(A)** and histopathologic **(B)** signs of inflammation in the guinea pig model of contact hypersensitivity 24 hours after challenge with DNCB. In Fig E2, *B*, the comparable effect of 0.13% STAT1 dODN ointment and 0.25% clobetasol propionate on epidermal spongiosis and leukocyte infiltration is readily apparent.

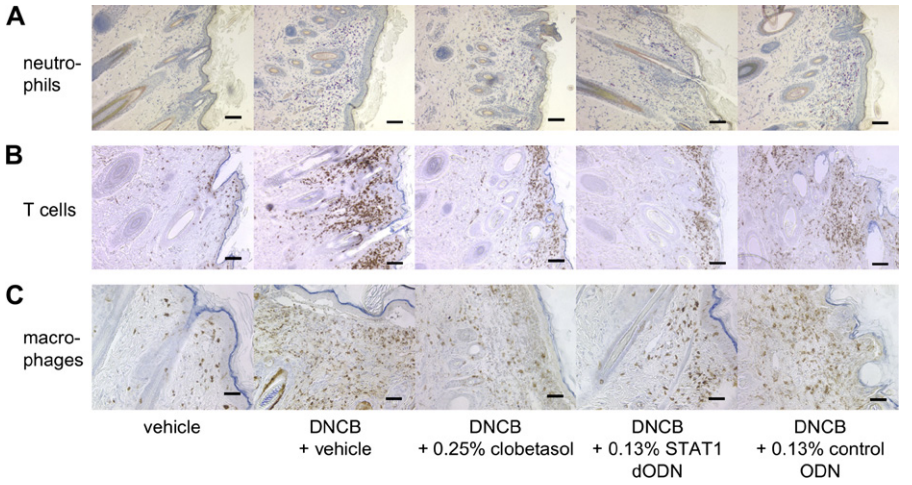


FIG E3. STAT1 dODN–mediated reduction in neutrophil **(A)**, T-cell **(B)**, and monocyte/macrophage **(C)** infiltration into guinea pig skin 24 hours after DNCB challenge. Representative images are shown. *Scale bar*, 100 μm.

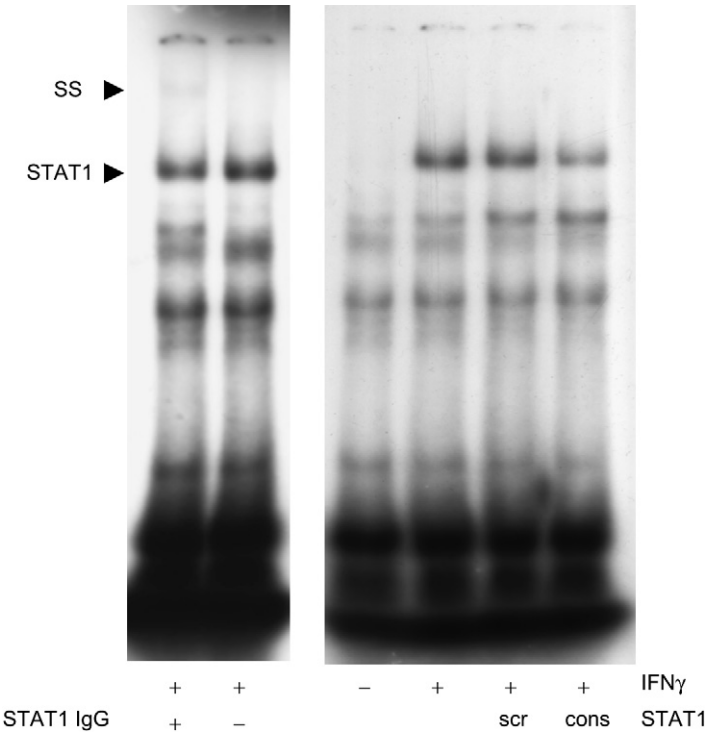


FIG E4. Specificity of the STAT dODN in cultured PAECs. IFN-γ (1000 U/mL, 30 minutes)–induced nuclear translocation of STAT1 is significantly reduced in cells pretreated with the consensus *(cons)* STAT1 dODN (10 μmol/L, 4 hours) but not the corresponding scrambled *(scr)* control ODN. A sis-inducible element gel shift oligonucleotide was used for detection of STAT1 in the nuclear extracts. Correct assignment of the STAT1-containing DNA-protein complex was confirmed by means of gel shift supershift *(SS)* analysis with a specific IgG antibody.

TABLE E1. Primers and PCR conditions

PCR product	Size (bp)	Annealing temperature	Forward/reverse primer 5′→3′	GenBank no. (sequence)
IL-1β	148	55°C	GGGATCAAGCTGCAAATCTCC AAGGGAAGCAAGGGTCTCAGG	AF119622 (250-398)
IL-12p40	68	55°C	TCAAACCAGACCCACCGAA GCTGACCTCCACCTGCTGA	AB025724 (686-754)
IL-8 (neutrophil attractant protein 1)	83	52°C	GCTGCGATGCCAGTGTATTAAG GGTCCACTCTCAATCACTTTCAGT	LO4986 (146-229)
TNF-α	132	60°C	CAATCCAGACAGGCAGGTTTGGTCC AAGTGCAGCAGGCAGAAGAGCGTGG	U77036 (15-147)
IFN-γ	52	58°C	CTAAGTTCTTCAAAGACAACAGCAG GTTTTTGAAAGCCTGCACCT	AY151287 (376-428)
GAPDH	137	62°C	GACCACAGTCCATGCCATCACTGC ATGACCTTGCCCACAGCCTTGG	DQ403052 (439-576)

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.