

Letter to the Editor

Efficacy of T-cell transcription factor-specific DNazymes in murine skin inflammation models*To the Editor:*

Most current therapies for inflammatory skin diseases are mainly symptomatic and do not interfere with the underlying pathomechanisms. In addition, they are often accompanied by moderate to severe adverse effects. Therefore, there is an unmet medical need for efficient and well-tolerated therapies that target disease-specific mechanisms. The involvement of different subtypes of T_H cells in skin inflammation has been intensively investigated¹ and different T_H subtype-specific transcription factors have been identified. These molecules orchestrate the differentiation and activation of the respective T_H subtype and influence the development of other subtypes, thereby modulating the nature of the inflammatory immune response.²

We generated deoxyribozymes (DNazymes) directed against the central transcription factors of T_H2- and T_H1-cell differentiation and activation, namely, guanine adenine thymine adenine sequence-binding protein 3 (GATA-3) for T_H2 and T-box transcription factor TBX21 protein (Tbet) for T_H1 cells (see Table E1 in this article's Online Repository at www.jacionline.org). 10-23 DNazymes represent a particular class of antisense molecules combining the specificity of antisense molecules with an inherent catalytic activity that makes them an attractive tool for highly specific interference with disease-causing target molecules. They are single-stranded DNA molecules with 2 sequence-specific RNA-binding domains flanking a central catalytic domain that exerts RNA cleavage activity after appropriate binding (see Fig E1 in this article's Online Repository at www.jacionline.org).³ Targeting the transcription factors of disease-mediating T_H cells might be a versatile tool for therapeutic interventions in inflammatory skin diseases such as atopic dermatitis, allergic contact hypersensitivity, or psoriasis. Actually, efficacy of the GATA-3-specific DNzyme approach has been shown in mouse models of allergic airway inflammation⁴ and most recently in a phase IIa clinical trial in which inhalation of this compound resulted in significant abrogation of allergen-induced early- and late-phase allergic responses in patients with asthma.⁵

To investigate DNzyme efficacy in inflammatory skin conditions, different mouse models were established. An oxazolone-induced contact hypersensitivity mouse model was modified from standard acute hapten-induced contact hypersensitivity mouse models to establish prolonged skin swelling reactions compared with acute models, thereby enabling the analysis of treatment effects on T-cell-mediated pathomechanisms (see Fig E2 in this article's Online Repository at www.jacionline.org). Three days after epicutaneous sensitization with the hapten oxazolone, mice were challenged by epicutaneous oxazolone application, and the prominent skin swelling reaction induced as a result is accompanied by an influx of inflammatory cells into the dermis consisting of mononuclear cells, eosinophils, and neutrophils, indicating a mixed T_H1/T_H2-type inflammatory response and mild hyperkeratosis. Treatment with DNazymes formulated in a

water-in-oil-in-water emulsion, specifically developed for penetration enhancement and DNzyme protection,^{6,7} was performed by topical application.

Prophylactic treatment with the GATA-3-specific DNzyme hgd40 significantly reduced oxazolone-induced skin swelling reactions compared with placebo and control DNzyme ODNg3 (Fig 1, A). Immunohistologic analysis of CD4⁺ T cells revealed significantly lowered cell numbers on hgd40 treatment in the dermis of oxazolone-induced mice (Fig 1, B). Target regulation was demonstrated by significantly reduced GATA-3 mRNA levels after topical application of hgd40 during the sensitization phase (Fig 1, C, and Table E2 in this article's Online Repository at www.jacionline.org). Inhibitory effects of hgd40 treatment on skin swelling reactions could also be detected after semi-therapeutic treatment starting a day before challenge in this model (Fig 1, D). These data are in line with the efficacy of inhaled hgd40 in mouse models of allergic airway inflammation,⁴ indicating favorable therapeutic effects of topically applied hgd40 irrespective of the target organ. Furthermore, human T_H2-polarized CD4⁺ T cells (see the Methods section and Tables E3 and E4 in this article's Online Repository at www.jacionline.org) were transfected with the GATA-3-specific DNzyme hgd40 (the corresponding mRNA sequence of which is 100% homologous between mouse and human). This resulted in a significant reduction in intracellular GATA-3 protein levels at 22 hours after transfection paralleled by a decreased release of the T_H2 cytokine IL-13 compared with control DNzyme-transfected cells (Fig 1, E).

Because the oxazolone model induced a mixed T_H1/T_H2 inflammatory phenotype based on T_H2- and T_H1-cell activities, potential effects of topical treatment with the mouse Tbet-specific DNzyme mtd32 were also investigated in this model. Prophylactic treatment with the mtd32 significantly reduced the skin swelling reaction over time compared with placebo and ODNg3 control DNzyme treatment (Fig 2, A). These data are in line with the finding of Jaceosidin-mediated reduction in ear swelling in a model of picryl chloride-induced contact dermatitis by inhibition of Tbet expression.⁸

To obtain further information on the efficacy of the Tbet-specific DNzyme in a predominantly T_H1-driven inflammatory response to a protein antigen, a new model was established using ovalbumin (OVA) as antigen. This model made use of DO11.10 mice transgenic for the OVA-specific T-cell receptor.⁹ OVA-specific immune responses are rapidly induced in these transgenic mice and easily provoked after challenge. Details on the protocol are provided in Fig 2, B, and the Methods section in the Online Repository. Briefly, sensitization was performed by a single subcutaneous immunization with OVA complexed with complete Freund's adjuvant while topical challenge was performed once daily starting 1 week after sensitization. This model is characterized by a significant skin swelling reaction and a prominent secretion and crusting of an inflammatory exudate accompanied by an influx of inflammatory cells into the dermis mainly consisting of mononuclear cells and neutrophils. Topical DNzyme treatment started a day before challenge. Twice-daily topical application of mtd32 significantly reduced the skin swelling reaction compared with placebo and control DNzyme

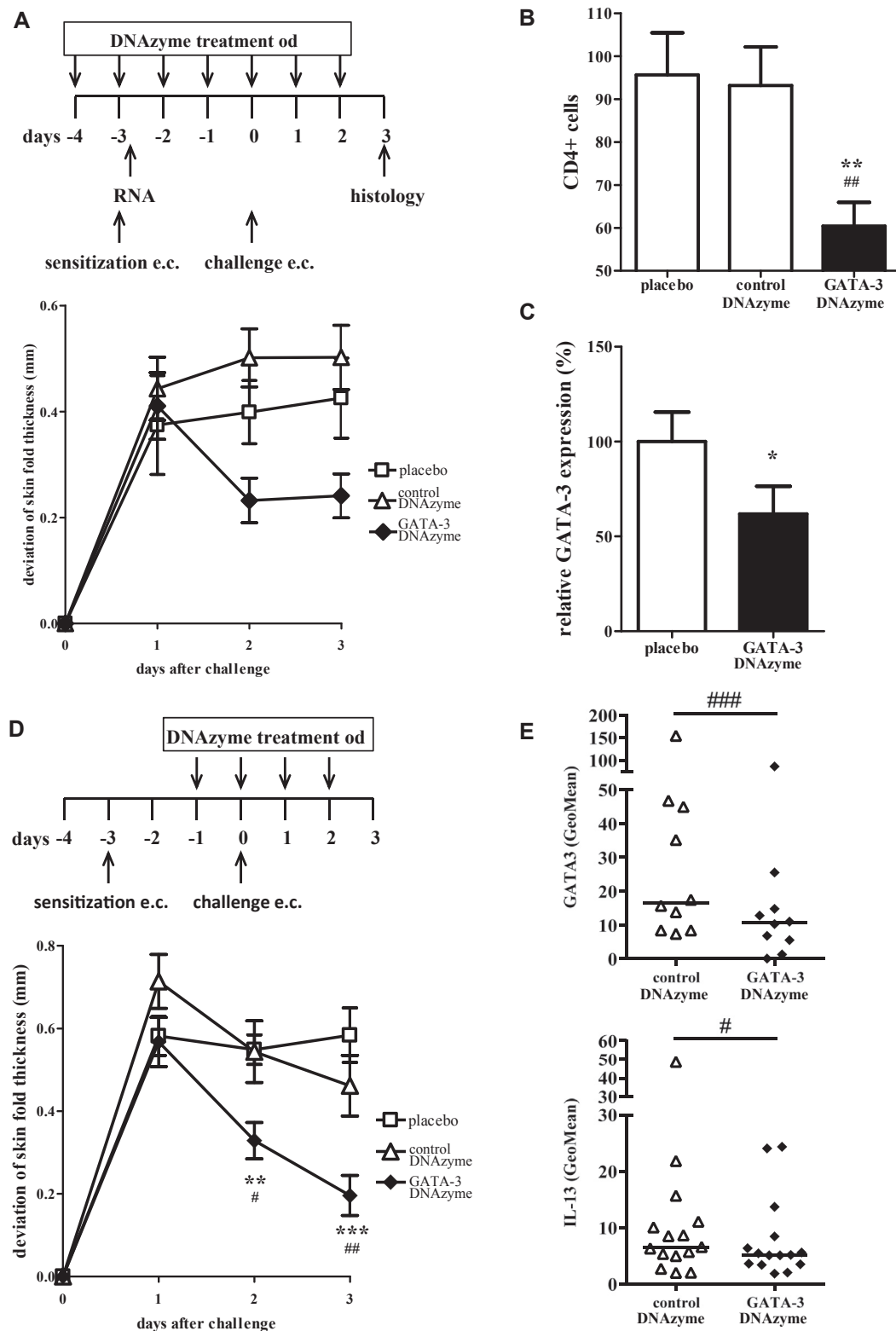


FIG 1. Efficacy of the GATA-3-specific DNzyme *in vivo* and *in vitro*. Prophylactic treatment of oxazolone-induced skin inflammation with the GATA-3-specific DNzyme hgd40 significantly inhibited skin swelling (**A**), reduced dermal CD4⁺ T-cell numbers (**B**), and inhibited GATA-3 mRNA expression at 6 hours after oxazolone induction (**C**). Semi-therapeutic topical treatment with hgd40 showed similar results (**D**). The GATA-3-specific DNzyme mediated significant reduction in GATA-3 protein levels and secretion of the T_H2 cytokine IL-13 in human polarized T_H2 cells (**E**).† Results are presented as mean ± SEM or individual data points. Data represent cumulative results of 3 (Fig 1, A and B) or 2 (Fig 1, C and D) independent experiments, with n = 8 animals per group. Significance is displayed as **P* < .05, ***P* < .01, and ****P* < .001 compared with placebo and #*P* < .05, ##*P* < .01, and ###*P* < .001 compared with control DNzyme ODNg3. e.c., Epicutaneous; od, once daily. †Extended data to data set shown in Krug et al.⁵

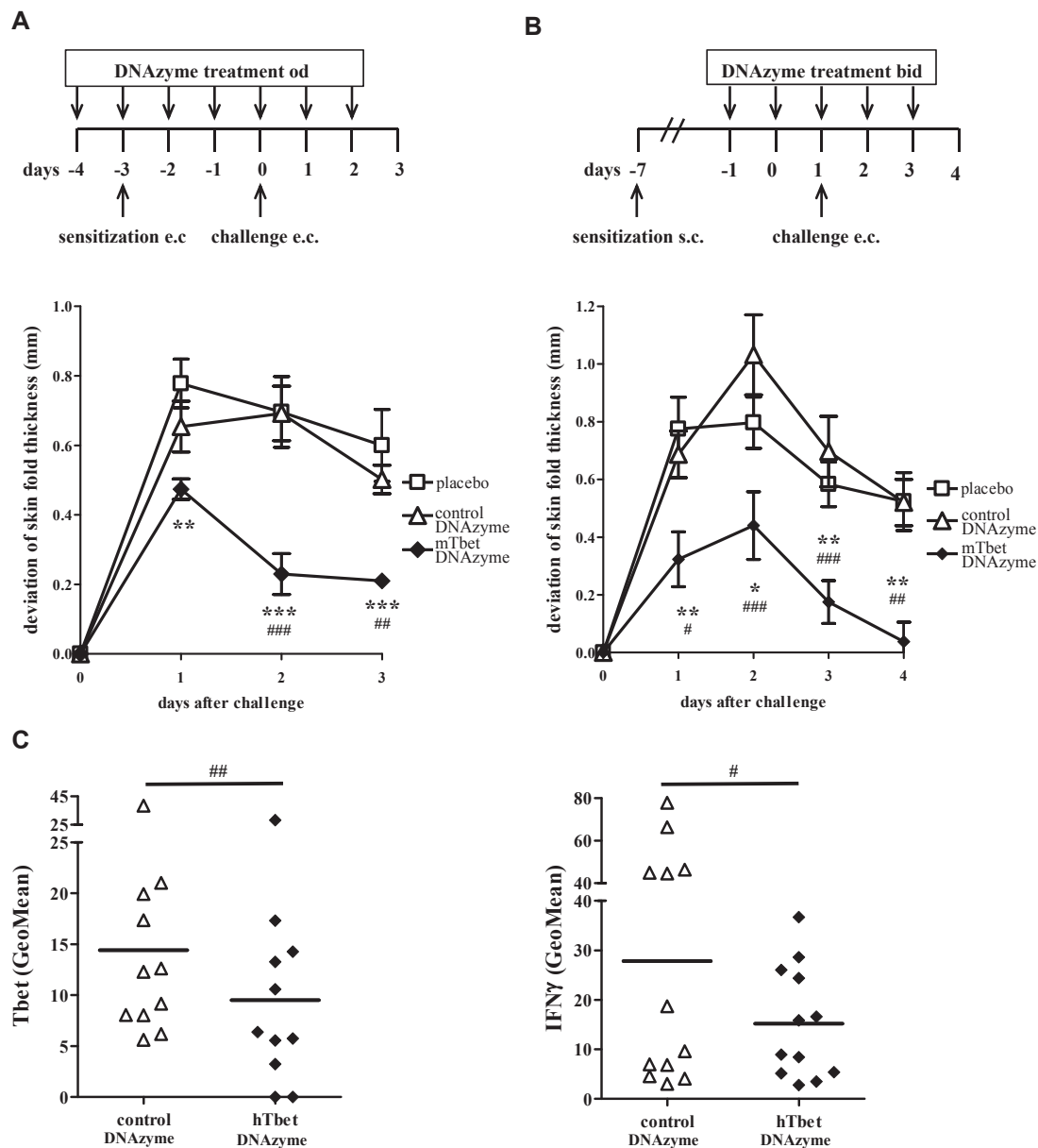


FIG 2. Efficacy of Tbet-specific DNazymes *in vivo* and *in vitro*. Topical treatment with the murine mTbet-specific DNzyme mtd32 significantly inhibited skin swelling responses on prophylactic treatment in an oxazolone-induced (**A**) and on semi-therapeutic treatment in an OVA-induced skin inflammation model (**B**). The human hTbet-specific DNzyme htd70 mediated significant reduction in Tbet protein levels and secretion of the T_H1 cytokine IFN- γ in human polarized T_H1 cells (**C**). Results are presented as mean $\pm$ SEM or individual data points. Data in (Fig 2, A) and (Fig 2, B) represent cumulative results of 2 independent experiments, with $n = 8$ animals per group. Significance is displayed as * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with placebo and # $P < .05$, ## $P < .01$, and ### $P < .001$ compared with control DNzyme ODNg3. bid, Twice daily; e.c., epicutaneous; od, once daily; s.c., subcutaneous.

(Fig 2, B). In addition, human T_H1 -polarized $CD4^+$ T cells (see Methods section and Tables E3 and E4) were transfected with the human Tbet-specific DNzyme htd70. This resulted in a significant reduction in protein levels of Tbet 22 hours after transfection and a decreased generation of the T_H1 cytokine IFN- γ compared with ODNg3-transfected controls (Fig 2, C).

In summary, DNazymes specifically targeting central transcription factors of inflammatory disease-associated T_H -cell

populations improved inflammation-related symptoms in models of cutaneous inflammatory conditions *in vivo* and may thus represent new and promising therapeutic agents for the topical treatment of inflammatory skin diseases.

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METHODS

DNAzymes

10–23 DNAzymes represent a particular class of antisense molecules combining the specificity of antisense molecules with an inherent catalytic activity. They are single-stranded DNA molecules with 2 sequence-specific RNA-binding domains flanking a central catalytic domain that exerts RNA cleavage activity after appropriate binding. We generated target-specific DNAzymes to bind and cleave the central transcription factor for T_H2 and T_H1 differentiation, GATA-3 and Tbet, respectively. In addition, a control DNAzyme was generated containing the same catalytic domain but scrambled binding arms with no known binding specificity (Table E1). Given that DNAzymes are specifically sensitive to degradation from the 3'-end by exonucleases present in body fluids and on the skin surface, DNAzymes applied in the study were stabilized by the addition of an inverted thymidine at the 3'-end. All DNAzymes were chemically synthesized by Biospring GmbH (Frankfurt am Main, Germany). A schematic description of DNAzyme structure and action is given in Fig E1.

DNAzyme formulation

For dermal application, DNAzymes were formulated in a proprietary water-in-oil-in-water formulation specifically designed to protect the DNAzyme and enhance penetration into the skin.^{E1} The emulsion consists of an inner water phase containing the DNAzyme in solution that is emulsified with an oil phase. This intermediate is surrounded by an outer water phase.

Animals

Male BALB/c mice 6 to 7 weeks old (Harlan Winkelmann, Hannover, Germany) and male DO11.10 mice 8 to 13 weeks old (animal facility BMFZ, Philipps University, Marburg, Germany) were maintained under specific pathogen-free conditions. OVA-free diet and water were supplied *ad libitum*. All animal experiments met national and international guidelines and were approved by the responsible local authorities (Regierungspräsidium Gießen, Germany).

Oxazolone-induced dermatitis model

BALB/c mice were sensitized to oxazolone (OXA, Fluka at Sigma Aldrich, St Louis, Mo) by epicutaneous application of 0.2% OXA solution in acetone/olive oil (4:1) on day –3 on the shaved back skin followed by epicutaneous challenge with 0.2% OXA solution on day 0. Control mice were sham-sensitized and sham-challenged with acetone/olive oil solvent (solvent) and sham-treated with placebo emulsion. Kinetics of OXA-induced skin fold thickness compared with sham-induced skin-fold thickness in this model is displayed in Fig E2.

DNAzyme treatment was performed once daily on days –4 to 2 by epicutaneous application of w/o/w emulsions of formulated hgd40 (0.4%, 2%), ODN_{g3} (0.4%, 2%), mtd32 (2%), or no DNAzyme (placebo). Skin swelling responses were measured before challenge (day 0) and once daily after challenge (days 1–3). Sampling for additional *ex vivo* analyses was performed 6 hours after sensitization for analysis of mRNA expression and on day 3 after challenge for immunohistochemistry.

OVA-induced dermatitis model

DO11.10 mice were sensitized to the protein antigen OVA (Sigma Aldrich, St Louis, Mo). A total of 75 µg of OVA (grade V) was dissolved in PBS and then emulsified in complete Freund's adjuvant (50 % vol/vol, Sigma Aldrich). A total of 200 µL of the emulsion was injected subcutaneously into the abdomen. Skin inflammation was elicited 7 days later by topical application of 50 µL of 10% OVA dissolved in 10% dimethyl sulfoxide to the shaved back skin. Skin fold thickness was measured before and daily after challenge. Topical treatment with w/o/w emulsions containing 0.4% DNAzymes mtd32 or ODN_{g3} or placebo started a day before challenge with twice-daily applications. Mice were sacrificed for analyses at day 4 after challenge.

Assessment of skin swelling responses – measurement of skin fold thickness

For assessment of skin swelling responses, the skin fold thickness was measured. A skin fold was formed in the middle of the inflammatory site along the head-to-tail axis, and skin fold thickness was measured in the center of the treatment area using a digital caliper (Bochem, Weilburg, Germany). The antigen-induced skin swelling response is expressed as difference to the skin fold thickness at day 0 (=before challenge) for each animal.

Immunohistochemistry

Skin samples of the inflammatory site were fixed in 6% paraformaldehyde immediately after removal, embedded in paraffin wax, and sectioned at a thickness of 5 µm. For immunohistochemical staining, slides were rehydrated through a descending series of alcohol to water. Epitope retrieval was performed by heating the slides in a pressure cooker (Highlights, ZHGmbH, Offenburg, Germany) containing citreous acid buffer (pH 6) at maximum temperature for 5 minutes. The slides were immersed in PBS containing 0.6% hydrogen peroxide at 4°C to block endogenous peroxidase activity. Immunostaining was performed using the appropriate ImmPRESS Polymer Detection Kit (Vector Laboratories, Peterborough, United Kingdom) following the manufacturer's protocol. As primary antibody a polyclonal rabbit antimouse-CD4 antibody (Novus Biologicals, Cambridge Science Park, United Kingdom) diluted 1:400 for incubation at room temperature for 1 hour at 4°C was used. Positive signals were counted by dividing each section into 3 equal parts and randomly choosing an hpf per part. Cell counts per hpf were averaged for all sections of the same treatment group. Hair follicles and upper layers of the epidermis were not included in the area to be evaluated for CD4⁺ T-cell presence.

Quantitative RT-PCR analysis of GATA-3 mRNA expression

Skin samples were taken 6 hours after sensitization, and mRNA was isolated by mincing the skin samples in peqGold TriFast (peqlab, Erlangen, Germany) with subsequent incubation at room temperature. After addition of chloroform, the aqueous phase was collected following centrifugation and RNA was precipitated by isopropanol precipitation. Resulting RNA was further purified using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) with remaining DNA removal according to manufacturer's instruction (RNase-free DNase Set, Qiagen). mRNA was reverse transcribed to obtain first-strand cDNA using the Omniscript kit (Qiagen) and Oligo-dT primers according to standard protocols.

Differences in mRNA expression levels were analyzed by subsequent quantitative PCR using QuantitTectTM SYBRGreen-Kit (Qiagen) and primers for GATA-3 and L32 synthesized by Metabion (Planegg, Germany) as indicated in Table E2. Gene expression was normalized to the housekeeping gene L32, and relative gene expression was calculated to a reference skin sample. The reference was included into each PCR run to compare and normalize all individual runs within 1 experiment and all individual experiments of the same model. Gene expression calculation was conducted using the following equations:

$$\Delta C_{t_{\text{sample}}} = C_{t_{\text{target gene sample}}} - C_{t_{L32 \text{ sample}}}$$

$$\Delta C_{t_{\text{reference}}} = C_{t_{\text{target gene reference}}} - C_{t_{L32 \text{ reference}}}$$

$$\Delta \Delta C_t = \Delta C_{t_{\text{sample}}} - \Delta C_{t_{\text{reference}}}$$

$$\text{Relative gene expression} = 2^{-\Delta \Delta C_t}$$

$$\text{Relative gene expression (\%)} = (\text{relative gene expression}_{\text{sample}} / \text{mean relative gene expression}_{\text{placebo}}) \times 100$$

Isolation and polarization of naive human CD4⁺ T cells

Naive human CD4⁺ T cells were isolated from whole blood or buffy coats of healthy donors (approved by ethics committee at the Medical University

Hannover, Germany) by Ficoll gradient centrifugation and subsequent enrichment using the naive CD4 T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instruction. Isolated cells were polarized by stimulation with anti-CD3 and anti-CD28 in the presence of IL-2 and T_H1 cytokines and T_H2 cytokines, respectively (see Table E3). After 3 days of culture, anti-CD3 and anti-CD28 were omitted from the polarization media. For the remaining period of polarization (6-7 days), the cells were incubated only with IL-2 and the respective T_H1/T_H2 polarization agents.

Transfection of polarized human T_H cells

After polarization, cells were transfected with 1 µg fluorescein-labeled DNazymes/10⁶ cells, either GATA-3-specific DNzyme hgd40 for T_H2 cells, human Tbet-specific DNzyme htd70 for T_H1 cells or ODNg3 as control, by electroporation using the Amaxa (Human T cell Nucleofactor Kit, Nucleofactor II/2B, Lonza, Basel, Switzerland) system. Transfected cells were replated and incubated for 22 hours, harvested, and analyzed for transcription factor protein expression by fluorescence flow cytometry analysis. For analysis of cytokine expression, transfected cells were restimulated with 1 µg/mL Staphylococcus enterotoxin B for 22 hours and analyzed by using the IFN-γ Secretion Assay (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instruction.

Flow cytometry analysis of transcription factor and cytokine protein expression

For flow cytometry analysis, cells were intracellularly stained for transcription factor protein using the "Transcription Factor Staining Buffer Set" (eBioscience, San Diego, Calif) according to manufacturer's instructions. For antibodies used, see Table E4.

For analysis, gates were set on positively transfected cells as determined by fluorescein positivity and protein expression levels were determined in these cells as geometric mean levels (GeoMean) expressed as difference of the protein-specific GeoMean and the GeoMean of the respective isotype control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. For multiple comparisons, an ANOVA was performed followed by Bonferroni posttest. For comparison of 2 data sets, the Student *t* test was applied. Significance levels are displayed as **P* < .05, ***P* < .01, and ****P* < .001 compared with placebo and #*P* < .05, ##*P* < .01, and ###*P* < .001 compared with control DNzyme ODNg3.

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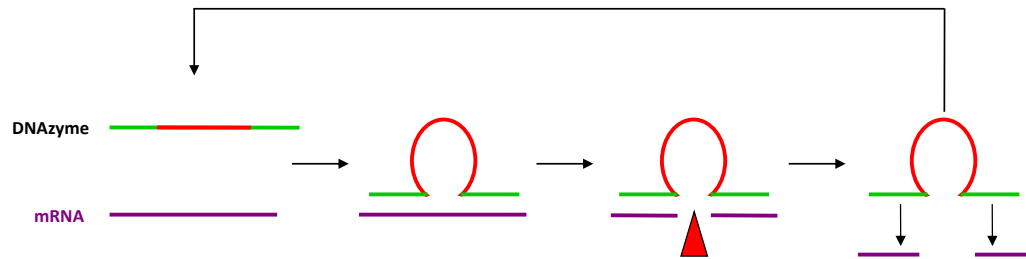


FIG E1. General DNAzyme structure and action. The DNAzyme binds to the complementary mRNA sequence, which is subsequently cleaved by direct catalytic activity of the DNAzyme. The DNAzyme is set free for binding and cleavage of a new mRNA molecule.

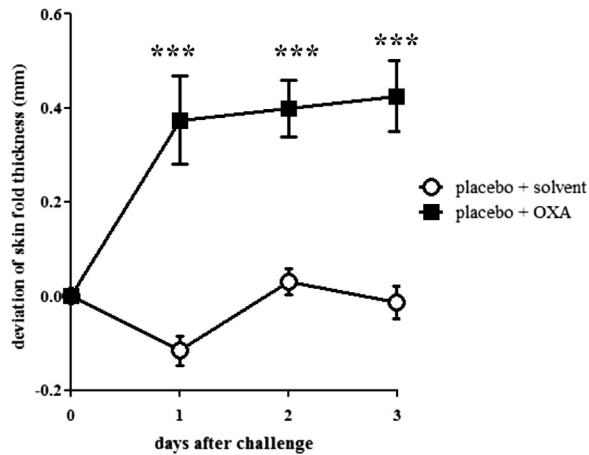


FIG E2. Kinetics of oxazolone-induced skin swelling. The back skin of mice was sensitized and challenged with either solvent or OXA solution. Skin fold thickness was measured at 0, 1, 2, and 3 days and deviation to day 0 was calculated. Data are compiled from 3 independent experiments ($n = 23$ animals per group) and are shown as mean $\pm$ SEM. Significant differences are marked *** $P < .001$ compared with solvent at the same time point.

TABLE E1. DNAzyme sequences

DNAzyme	Specificity	Sequence
hgd40	Human and mouse GATA-3	GTGGATGGAGGCTAGCTACAACGAGTCTTGAG
htd70	Human Tbet	TCACGGCAAGGCTAGCTACAACGAGAACTGGGT
mtd32	Mouse Tbet	CTCCCGGAAGGCTAGCTACAACGACCTTTGGCA
ODNg3	None	CCATGTGGAGGCTAGCTACAACGACTGGAATCA

Underlined sequence = catalytic domain.

TABLE E2. Primer sequences

Primer	Sequence
Oligo-dT primer	5'-TTTTTTTTTTTTTTTTT-3'
L32 forward	5'-GCA AGT TCC TGG TCC ACA AT-3'
L32 reverse	5'-GGG ATT GGT GAC TCT GAT GG-3'
GATA-3 forward	5'-ACG GAA GAG GTG GAC GTA CT-3'
GATA-3 reverse	5'-CGC CAG AGA AGA GGA TGA AG-3'

TABLE E3. Antibodies and cytokines for human T_H-cell polarization

T _H subtype	Antibody/ cytokine	Concentration	Supplier
All	IL-2	20 ng/mL	R&D, Minneapolis, Minn
	Anti-CD3	450 ng/mL	BD Pharmingen, San Jose, Calif
	Anti-CD28	200 ng/mL	BD Pharmingen, San Jose, Calif
T _H 1	IL-12	25 ng/mL	R&D, Minneapolis, Minn
	IL-27	25 ng/mL	eBiosciences, San Diego, Calif
	Anti-IL-4	1 µg/mL	R&D, Minneapolis, Minn
T _H 2	IL-4	20 ng/mL	R&D, Minneapolis, Minn
	Anti-IFN-γ	1 µg/mL	R&D, Minneapolis, Minn

TABLE E4. Antibodies for fluorescence-activated cell sorting analysis

Antibody	Supplier
Alexa Fluor-647 mouse IgG1, κ	BD Pharmingen, San Jose, Calif
Alexa Fluor-647 anti-GATA-3	BD Pharmingen, San Jose, Calif
Alexa Fluor-647 anti-T-bet	Biolegend, San Diego, Calif