Aus dem Institut für Laboratoriumsmedizin und Pathobiochemie,

Molekulare Diagnostik

Direktor: Prof. Dr. med. H. Renz

des Fachbereichs Medizin der Philipps-Universität Marburg

in Zusammenarbeit mit der Firma

sterna biologicals

Investigating the efficacy of transcription factor-specific DNAzymes in animal models of inflammatory skin diseases

Inaugural-Dissertation zur Erlangung des Doktorgrades

der Naturwissenschaft (Dr. rer. nat.)

Philipps Universität Marburg

Dem Fachbereich Medizin der Philipps-Universität Marburg

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Marburg, 2016

Angenommen vom Fachbereich Medizin der Philipps- Universität Marburg

am: 09.03.2016

Gedruckt mit Genehmigung des Fachbereiches.

Dekanat: Prof. Dr. H. Schäfer

Referent: PD Dr. H. Garn

Koreferent: Prof.Dr. M. Huber

To the Country that lives in me …

To the beloved Syria …

May Peace and love find their way back to you …

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1 INTRODUCTION

1.1 Skin structure and immune sentinels

The skin is the body's largest organ and the one with the biggest interface with the environment. It provides protection against physical and chemical injuries and acts as the first-line defence against microbial pathogens. Immune surveillance in such an organ is very complex, it requires a network of structural as well as immunological cells and pathways working together in perfect harmony.

The skin can be divided into two different compartments, the epidermis and the dermis with different structural and immune cells present in each compartment (Fig. 1).

Epidermis

The epidermis is the outer layer responsible for the vital functions of the skin. It consists of four layers: In the innermost layer, the stratum basale, epidermal keratinocytes are continuously generated. As they migrate towards the surface of the epidermis, these cells form the stratum spinosum while undergoing terminal differentiation with cell cycle arrest, loss of adherence to the basement membrane and increase in keratin production. The stratum spinosum is followed by the stratum granulosum, with tight junctions and lamellar granules containing extracellular structural proteins, and various antibacterial peptides such as cathelicidin and β-defensin 2 (Oren et al. 2003, Braff et al. 2005). The final and outer layer is the stratum corneum consisting of keratin-rich corneocytes, which among other functions prevent epidermal water loss making them an essential part of the skin barrier.

Immune cells residing in the epidermis consist mostly of Langerhans cells (LCs) while T cells are rare and limited to very few $CD8^+$ T cells that can be found in the stratum spinosum and stratum basale (Nestle et al. 2009).

Keratinocytes

Keratinocytes (KCs) are first in line to encounter foreign and possibly dangerous agents and they play an essential role in promoting skin immune responses. Their inflammasome machinery and ability to express toll-like receptors (TLRs) - including TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR9 enables keratinocytes to recognize pathogen-associated molecular patterns (PAMPs) of microbial origins (Lebre et al. 2006). Keratinocytes also express NOD-like receptors (NLRs) that can recognize danger associated molecular patterns (DAMPs) including toxins and irritants (Nestle et al. 2009).

During skin infections, keratinocytes produce antimicrobial peptides (AMPs) among which are β-defensin and cathelicidins as well as pro-inflammatory cytokines including IL-1β, IL6, IL18, and TNFα (Albanesi C1 2005). Activated keratinocytes attract effector T cells to the skin through the expression of CC-chemokines ligand 20 (CCL20), CXC-chemokine ligand 9 (CXCL9), CXCL10 and CXCL11 (Albanesi C1 2005).

Keratinocytes have also been described to induce functional responses in memory T cells. They can process peptide antigens or exogenous peptides, present it to $CD4^+$ or $CD8^+$ T cells which in turn induce both Th1 and Th2-type cytokine production as well as target cell lysis, respectively (Black et al. 2007).

Langerhans cells

Langerhans cells (LCs) represent a specialized subset of dendritic cells (DCs) that are found in the epidermis. They survey the epidermis for antigens using their extended dendrites and migrate to the lymph nodes upon antigen-mediated activation. Migratory LCs express less E-cadherin, a homotypic adhesion molecule that anchors LCs to neighbouring keratinocytes. On the other hand, they show increased expression of MHC molecules, co-stimulatory molecules such as CD40 as well as CC-chemokine receptor 7 (CCR7), which is essential for LC migration to the skin-draining lymph nodes (Merad et al. 2008). Recently, more attention is being given to the role of LCs in the induction of tolerance to peripheral antigens in the steady state (Steinman et al. 2003, Shklovskaya et al. 2011, van der Aar et al. 2013).

Dermis

The dermis is anatomically more complicated than the epidermis and is the residence for most skin immune cells. It consists of an extracellular matrix formed by fibroblasts that produce collagen, elastin and structural proteoglycans. It also contains a pool of different immune cells including mast cells, macrophages, DCs, T cells and a population of innate lymphoid cells (ILCs) (Emilsson, Breaker 2002).

Dendritic cells

The dermis-resident DCs play a critical role in cell-mediated immunity. DCs engulf antigens, become activated by innate immune mechanisms and exposure to pro-inflammatory cytokines, and undergo maturation as they migrate to draining lymph nodes. Mature DCs have an enhanced capacity of antigen presentation with increased

expression of surface MHC molecules and co-stimulatory molecules like CD80 and CD86 (Pierre et al. 1997), (Schuller et al. 2001). In the lymph nodes, mature DCs come in contact with naïve T cells for antigen presentation resulting in T cell-proliferation, clonal expansion and differentiation into memory/ effector T cells. The generated T cells are antigen-specific, express skin homing receptors and can be rapidly recruited to the site of initial antigen encounter in the skin.

T cells

T cells are strongly present in the skin at numbers two times higher when compared to the blood. In the dermis, T cells, mostly memory $CD4^+$ or $CD8^+$ T cells that express the cutaneous lymphocyte-associated antigen (CLA), preferentially cluster around post capillary venules. Their role in the first-line defence against secondary antigen challenge makes them important effector cells of skin immunity (Romani et al. 2012).

Conventional T cells are the main players in various inflammatory skin diseases. T helper 1 (Th1) cells are present and active during skin infection with intracellular organism and in autoimmune diseases such as psoriasis while Th2-cell responses are associated with allergic skin diseases like atopic dermatitis. The role of other T helper cell subtypes such as Th17 cells and Th22 cells in the pathology of different inflammatory skin diseases has also been the focus of several studies (Weaver et al. 2007, Nograles et al. 2009). Unconventional T cells including γδ T cells, natural killer T cells (NKT) and invariant NKT (iNKT) cells are also found in the skin and are associated with inflammatory diseases and skin carcinogenesis (Nestle et al. 2009).

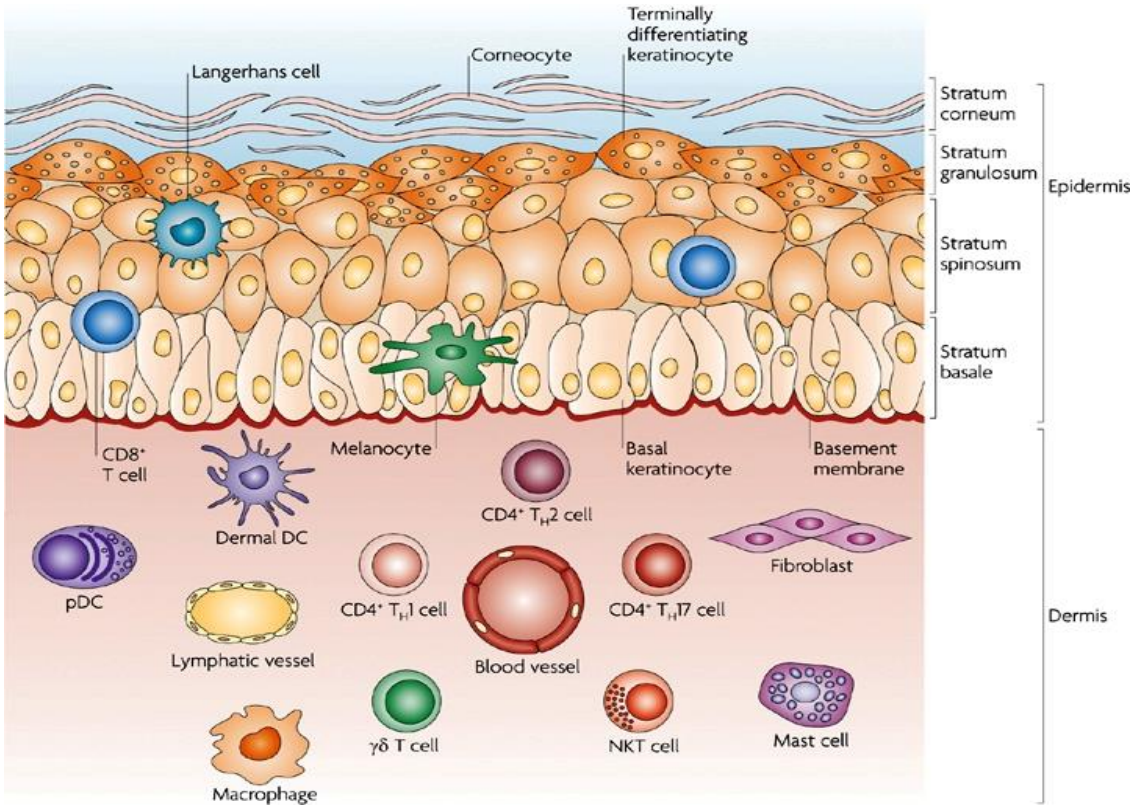

Figure 1. Skin anatomy and cellular effectors (Nestle et al. 2009).

The different layers of the skin give it its functions from providing a physical barrier to maintaining the body temperature and gathering sensory information from the surrounding environment. The cells residing the skin provide the skin with its role in the immune system. The epidermis contains several layers and is responsible for the barrier function of the skin. Immune cells in the epidermis include Langerhans cells and very few $CD8^+$ cytotoxic T cells. The dermis contains many specialized cells including different subtypes of dendritic cells (DCs), T helper cells, macrophages, mast cells and fibroblasts.

1.2 Inflammatory skin diseases

1.2.1 Allergic contact dermatitis

Allergic contact dermatitis (ACD) is a common acute inflammatory skin disease usually caused by contact with chemicals. In Europe about 20% of the general population suffer from contact allergy to at least one allergen (Peiser et al. 2012). Contact allergens are small organic molecules with chemical activity and a molecular weight of ≤ 500 Dalton called “haptens”. They include metal ions and salts such as nickel, chromate fragrances and dyes and are found in a lot of cosmetics, jewellery and at different work places which explains the classification of ACD as a major cause of occupational skin diseases. The risk of developing ACD depends to a large extent on the exposure patterns and habits. For instance, the higher prevalence of ACD in women than in men (Thyssen et al. 2007)

can be attributed to the habit of wearing nickel-containing jewelry rather than genetic factors. However, genetic predisposition cannot be ruled out in ACD, since among the high rate of exposure only a minority actually develops the disease.
Because of their low molecular weight, haptens can pass the stratum corneum and move further to the basal layer without being recognized by the immune system until they covalently attach to self-proteins and become immunogenic. When bound to cytoplasmic proteins, they are presented to $CD8^+$ T cells on MHC class I molecules, whereas binding to extracellular tissue proteins leads to their capture and process by antigen presenting cells (APCs), which present them on MHC class II inducing a $CD4^+$ T cell response (Martins 2011).

Pathophysiology

ACD is identified as a T cell- mediated response to contact allergens (Kimber, Dearman 2002) that occurs in two phases, a sensitization phase and an elicitation phase.

During the sensitization phase, the irritant effect of haptens generates danger signals in the skin inducing innate immune responses through the activation of TLR2/TLR4 (Martin et al. 2011) and the inflammasome (Watanabe et al. 2007). This results in the production of pro-inflammatory cytokines including IL-1β, TNF-α and GM-CSF which support the activation of DCs upon antigen uptake. Activated DCs produce more IL-1β, change their profile of chemokines receptors and migrate to the regional lymphnodes. There, they present the antigen to naïve T lymphocytes and drive their differentiation into effector T cells. These hapten-specific T cells then enter the blood circulation and are preferentially directed to the skin due to their expression of the cutaneous lymphocyte antigen (CLA).

In the elicitation phase and within a couple of hours following a secondary exposure to the same hapten, a variety of immune cells including mast cells, neutrophils as well as hapten-specific and non-specific T cells infiltrate into the challenged skin. Both $CD8^+$ and $CD4^+$ T cells are recruited to the site of inflammation and peak within 24-48 h after challenge. Whether the recruited $CD4^+$ T cells are Th1 or Th2 is highly dependent on the nature of the hapten. Studies in animal models showed infiltration of $CD4^+$ T cells expressing Th1-specific chemokine receptors upon sensitization and challenge with the hapten dinitrofluorobenzene (DNFB) while a greater influx of T cells expressing chemokine receptors consistent with Th2 cells was observed when using the hapten oxazolone (Christensen, Haase 2012). Either way, T cells promote the killing of haptenized cells and produce inflammatory cytokines like IFNγ and IL-4 thus promoting

further cellular infiltration to the site of inflammation and contributing to the characteristic edema (Kaplan et al. 2012).

1.2.2 Atopic dermatitis

Atopic dermatitis (AD) is a chronic, highly pruritic inflammatory skin disease which is often linked with the development of asthma and allergic rhinitis in early childhood. Clinical manifestations of AD include pruritus, cutaneous hyperreactivity, dry skin and recurring eczema, which usually start during early infancy or childhood. These symptoms can sometimes be accompanied by complications like food allergies, asthma, and/or allergic rhinitis during the first years of life, in a process called “atopic march” (Kubo et al. 2012). The prevalence of AD is increasing with currently 10-20% of children and 1-3% of adults affected in developed countries (Larsen 2002). Continuous management of the conditions’ necessities poses an economic burden and recurring symptoms of AD like pruritus and itching creates a high social impact in sense by affect the patient’s quality of life.

The pathogenesis of AD is not clearly understood but it appears to be a result of different genetic, epigenetic and environmental factors. Skin barrier abnormalities such as mutations in the *FLG* gene, which encodes the structural protein fillagrin, are risk factors in the predisposition towards AD (McAleer, Irvine 2013). A disrupted skin barrier leads to transepidermal water loss and renders the skin vulnerable for allergens and infectious agents.

Innate and adaptive immune responses to the penetrating antigens play a major role in the pathogenesis of the disease. Patients with AD were found to be deficient in the production of antimicrobial peptides by keratinocytes (Kuo et al. 2013), making them prone to microbial colonization especially by *Staphylococcus aureus*.

Immune responses in AD

T cell responses in AD can be described as biphasic. Acute lesions are characterized by a Th2 predominant phenotype and increased expression of the Th2 cytokines IL-4, IL-5 and IL-13 while chronic conditions are associated with a rise in the expression of IL-12 and a switch to the Th1 phenotype (Fig. 2).

Exposure to allergen plays an important role in the exacerbation of AD. The immune mechanisms underlying AD are similar to those of asthma. Th2 cytokines IL-4, IL-5 and IL-13 are significantly increased in lesional and non-lesional skin in the acute phase of AD. IL-13 is known to be a major stimulator of inflammation and tissue remodelling at sites of Th2 inflammation (Mu et al. 2014), while IL-4 further promotes Th2 development. In addition, both IL-4 and IL-13 promote isotype switching in B cells to IgE and result in elevated circulating levels of IgE in most AD patients. Characteristic eosinophilia and macrophage infiltration in AD are mediated by IL-5 and GM-CSF, respectively.

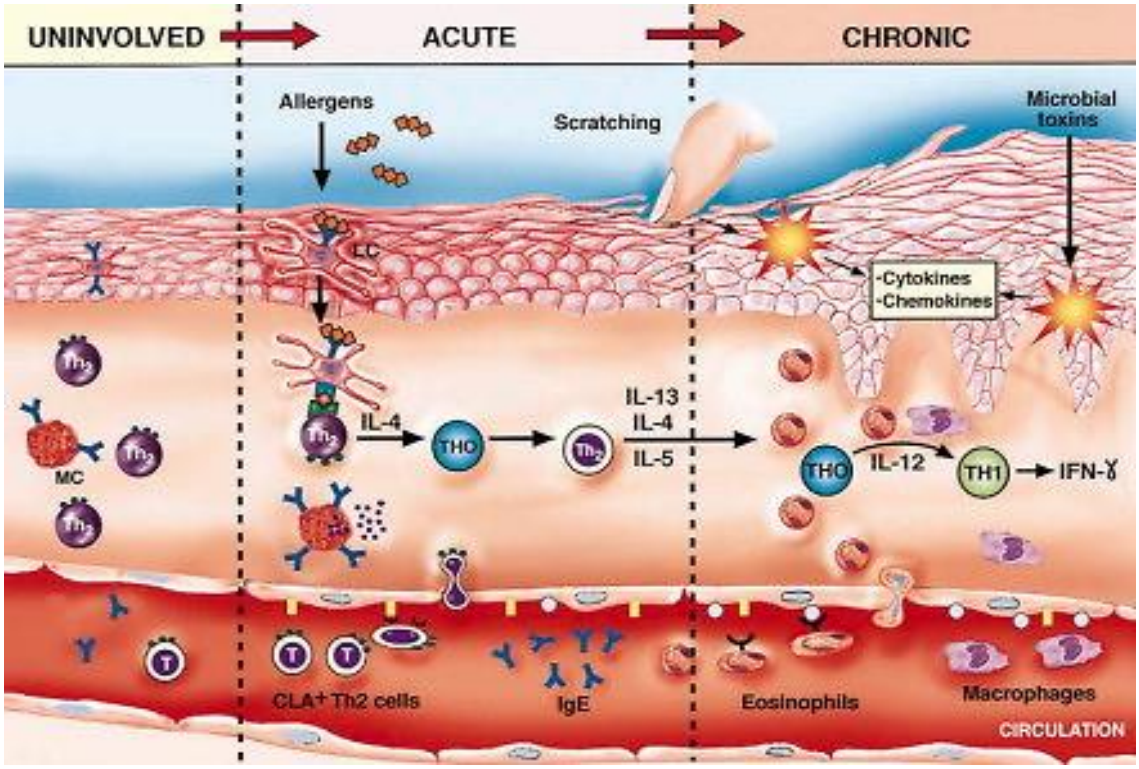

Figure 2. Immunologic pathways in the progression of AD (Leung DY. 2000).

Patients with AD show a systemic Th2 response, elevated IgE levels and eosinophilia with low levels of Th2 cytokines in uninvolved skin. In acute lesions of AD, there is an increased infiltration of Th2 cells and increased expression of Th2 cytokines. The infiltration of macrophages and eosinophils increases the expression of IL-12 and lead to a switch in the immune response towards Th1.

The Th2 phenotype is highly dependent on GATA3, the key regulator in the Th2 differentiation program. Indeed, human studies revealed significantly higher mRNA expression of GATA3 in AD patients compared to healthy non-atopic individuals (Arakawa et al. 2004). GATA3 belongs to the GATA family of transcription factors and is expressed by a variety of immune cells including ILC2, NK cells, NKT cells and both

naïve and committed CD4$^+$ T cells (Tindemans et al. 2014) (Fig. 3). It is also expressed by structural cells such as epithelial cells. In the skin, GATA3 is expressed in the keratinocytes of the epidermis and in the inner root sheath of the hair follicle (Chikh et al. 2007).

Upon triggering of the T cell receptor (TCR) by antigen ligation, GATA3 expression is upregulated through IL-4-STAT6-mediated signalling. In the case of low dose antigen stimulation GATA3 expression is regulated in an IL-4 independent manner (Yamane et al. 2005). The transcription factor GATA3 binds to the promotor at the *Il4*, *Il5* and *Il13* genes and induces their transcription leading to increased Th2- cytokine levels. Increased levels of IL-4 lead to prolonged induction of growth factor indepent-1 (Gfi-1) through IL-4-STAT6 signalling (Zhu et al. 2002), which in turn promotes the expansion of GATA3- expressing cells (Zhu et al. 2006). In addition to driving Th2 polarization and proliferation, GATA3 inhibits the development of Th1 cells by different mechanisms such as inhibiting IL-12Rβ and STAT4 expression and silencing the *Ifng* gene (Wei et al. 2011).

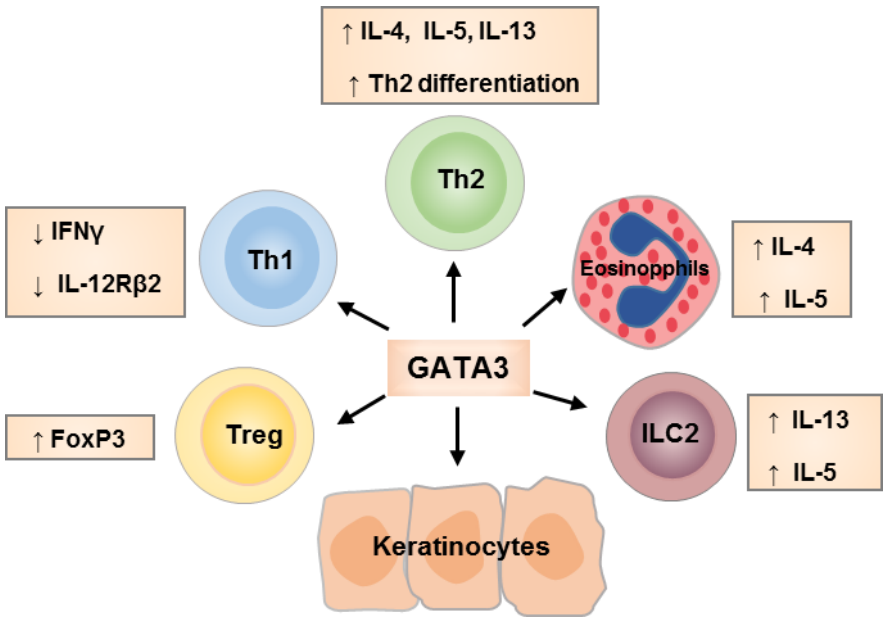

Figure 3. Effects of GATA3 expression in different cells.

Most recent, it has been shown that certain single nucleotide polymorphisms (SNPs) in the GATA3 gene are associated with an increased risk for the development of allergic

sensitisation (Huebner et al.) including allergic eczematous skin diseases like atopic dermatitis (Arshad et al. 2008).

1.2.3 Psoriasis

Psoriasis is a lifelong inflammatory skin disease which affects about 1-2% of adults around the world and is characterised by scaly, erythematous cutaneous plaques. Although it is considered an autoimmune disease, no autoantigens have yet been identified (Kupper 2003).

Environmental factors like stress or trauma trigger psoriasis in genetically predisposed individuals. Keratinocytes initiate the process by producing self-DNA molecules that bind to the antimicrobial peptide LL37 and by releasing anti-inflammatory cytokines to activate DCs and enhance their antigen presenting activity. This results in the recruitment of T cells, mostly Th1 and Th17, to the skin and the production of different cytokines including IFNγ, TNF, IL-17a and IL-22. This cytokine milieu increases keratinocyte proliferation and stimulates the production of pro-inflammatory mediators and AMPs by keratinocytes, which sustains and amplifies chronic inflammatory disease processes (Nestle et al. 2009).

The Th1 response plays a major role in the pathogenesis of psoriasis, with IFNγ – producing Th1 cells infiltrating the skin. These Th1 lymphocytes are responsible for the pathological reactions in psoriatic skin leading to keratinocyte hyperproliferation, small vessel proliferation and neutrophilic infiltration (Ghoreschi et al. 2003). Moreover, psoriatic patients with increased IFNγ serum concentration also showed increased expression of Tbet-mRNA in their PBMCs and a much higher Tbet/GATA3 ratio than in controls (Zhu et al. 2010).

Tbet belongs to the Tbox family of transcription factors and is expressed in and functionally essential for different cells of the innate and adaptive immune system including DCs, NK cells, NKT cells, ILCs, $CD4^+$ and $CD8^+$ T effector cells, B cells, γδ T cells and a subset of T regulatory (T_{Reg}) cells (Lazarevic et al. 2013). However, Tbet is mostly known as the master regulator of the differentiation of Th0 cells to Th1 cells and the induction of the Th1 cytokine IFNγ. Tbet expression is absent in naïve T cells and is first induced via TCR and IFNγR signalling through signal transducer and activator of transcription 1 (STAT1). The second wave of Tbet expression stabilizes the Th1 cell

phenotype and is induced by IL-12, functioning via STAT4 (Lazarevic et al. 2013). Tbet not only controls Th1 differentiation, it also induces the production of IFNγ by directly binding to regulatory elements at the *Ifng* locus, leading to changes in histone modification and enhanced *Ifng* transcription (Kanhere et al. 2012) as well as up-regulation of IL-12Rb2 (Afkarian et al. 2002). Furthermore, it suppresses the Th2 cell lineage commitment by silencing the Il4 gene as well as repressing the binding of GATA3 to *Il5* promoter DNA (Kanhere et al. 2012). These functions of Tbet polarize the immune response towards Th1 making Tbet an important possible target for future therapies in autoimmunity and Th1-mediated diseases.

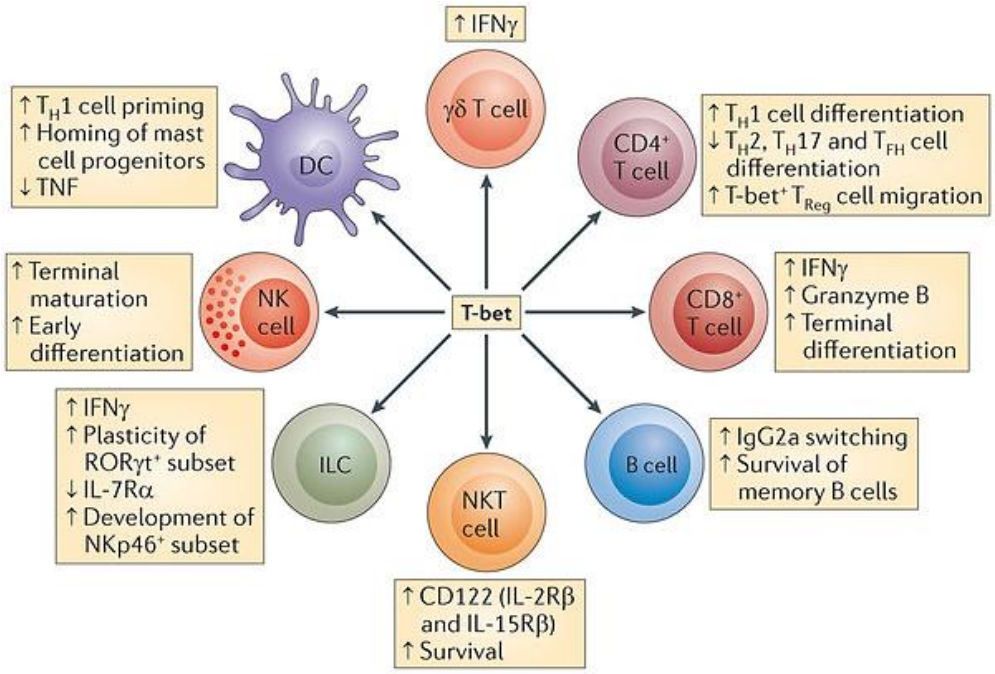

Figure 4. Expression and functions of Tbet in immune cells (Lazarevic et al. 2013).

1.3 Current strategies in the management of inflammatory skin diseases

All of the skin conditions discussed above share symptoms and many aspects of the inflammation and therefore similar approaches are used in their treatment. Identification and elimination of allergic and inflammatory triggers as well as emotional stressors are most important in preventing the flares while application of moisturizers helps control the itch, reduces transepidermal water loss and minimizes the risk of infections in chronic conditions.

Anti-inflammatory and immunosuppressive therapies, including corticosteroids and calcinurine inhibitors, are used to control the inflammatory response resulting from immune system dysfunction in these diseases.

Corticosteroid are the first–line treatment in skin diseases like AD and psoriasis and can range from low-potency topical application as maintenance therapy in chronic conditions to high-potency topical and/or systemic administration in severe cases. They act on a variety of immune cells carrying the glucocorticoid receptors (GR) including DCs, T lymphocytes, eosinophils, mast cells and macrophages. They suppress the transcription of genes encoding pro-inflammatory mediators such as IL-1α and IL-1β and enhance the transcription of anti-inflammatory genes, including inhibitors of nuclear factor-κB (IκBα), IL-1RII, annexin I, IL-10, and secretory leukocyte protease inhibitor (SLPI) (Holgate, Polosa 2008).

Long- term use of topical corticosteroids in chronic conditions, bears the risk of severe side effects including skin atrophy, striae, telangiectasia and acne (Walling HW 2010).

Second-line treatment includes the use of topical calcineurine inhibitors (TCI) that act specifically on T cells. Calcineurine is responsible for the activation of the cytosolic nuclear factor of activated T cells (NFAT). Inactive NFAT cannot enter the nucleus to activate the transcription of cytokine-encoding genes. As a result, the production of inflammatory cytokines (IL-2, IL-4, IL-10 and IFNγ) as well as T cell proliferation is inhibited (Hultsch et al. 2005). Less conventional treatments include the use of tar preparations, UV therapy, and allergen immunotherapy.

In recent years, there has been a growing interest in more specific therapies that target molecules involved in the elicitation of inflammatory skin diseases.

Therapeutic antibodies especially those targeting cytokines that induce the inflammatory response have become an important class of drugs during the past two decades. For instance, several tumour necrosis factor (TNF) antagonists have already been approved for the treatment of plaque psoriasis (Chan, Carter 2010) in addition to the final clinical testing of IL-17A- and IL-23-specific antibodies in the treatment of this disease (Leonardi et al. 2012, Rich et al. 2013, and Papp et al. 2012). Moreover, the clinical effects of a monoclonal antibody that blocks both IL-4 and IL-13 have most recently been tested in patients with moderate to severe AD (Beck et al. 2014). Anti IgE antibodies are also being investigated in the treatment of atopic dermatitis with contradictory reports about

their efficacy especially in patients with elevated IgE serum levels (Vigo et al. 2006, Krathen, Hsu 2005).

Recently, vitamin D deficiency is being increasingly linked to the development of allergic diseases (Muehleisen 2013). In addition, psoriatic patients were reported to have lower levels of vitamin D in comparison to healthy controls (Vähävihu et al. 2010). Therefore, oral vitamin D supplementation is proposed to have beneficial effects in AD by the upregulation of antimicrobial peptides and induction of Tregs (van der Aar et al. 2011).

1.4 New therapeutic approaches using DNAyzme technology

DNAzyme technology depends on the use of a specific family of anti-sense molecules called “DNAzymes” to inhibit the expression of a gene on mRNA level. As they are composed of DNA, DNAzymes are more active than ribozymes and are relatively easy to synthesize and handle, making them suitable promising candidates for therapeutic interventions. The function as well as present and possible future therapeutic applications of these molecules are discussed below.

1.4.1 Molecular structure and mode of action

DNAzymes (Deoxyribozymes) are single stranded, synthetic DNA molecules that comprise a cation-dependent catalytic core. They were first discovered in 1994 by Ronald Breaker and Gerald Joyce who reported that, using an *in vitro* selection technique, they were able to obtain a DNAzyme which catalysed the Pb^{2}-dependent cleavage of an RNA phosphoester in a reaction processed with rapid turnover (Breaker, Joyce 1994). One year later, they generated Mg^{2+}-dependent DNAzymes, which were able to cleave RNA with a high catalytic efficiency of $k_{cat}/K_m \approx 10^9\ M^{-1}*min^{-1}$ in conditions comparable to those of the intracellular environment (Breaker, Joyce 1995, Santoro, Joyce 1997). Further work resulted in the generation of the 10-23 family of DNAzymes, which consists of a catalytic core of 15 nucleotides and two substrate binding arms of variable length and sequence. These binding arms can specifically bind to complementary sequences on RNA through Watson-Crick base pairing. DNAzymes target and cleave mRNA in a Mg^{+2}–dependent manner through de-esterification between unpaired purine and paired pyrimidine residues (Santoro, Joyce 1997, Santoro, Joyce 1998). Cleaved mRNA fails to be translated and is degraded after the dissociation of the DNAzyme. The DNAzyme itself binds to another mRNA molecule and the process is

started again (Fig. 5). The efficiency of DNAzymes in cleaving RNA is comparable to that of protein enzymes (Silverman 2005).

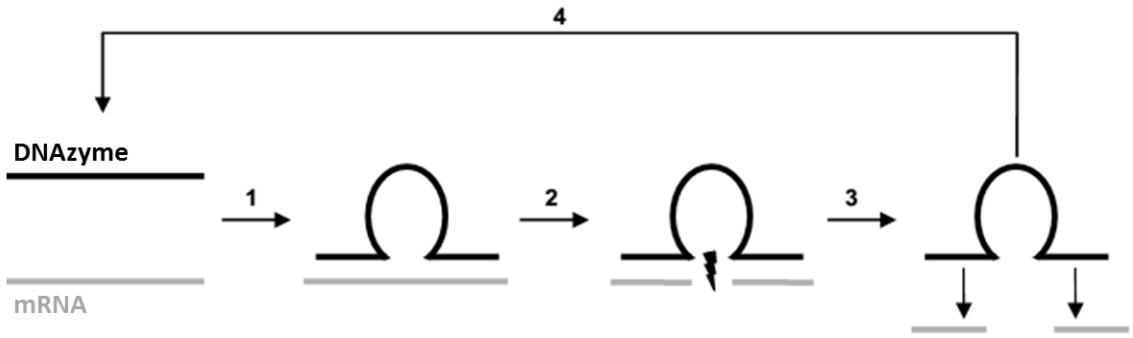

Figure 5. mRNA cleavage by DNAzymes.

1) DNAzymes binding to target mRNA. **2**) Cleaving of mRNA. **3)** Dissociation of DNAzymes and degradation of mRNA-cleavage products. **4)** DNAzyme moves to the next mRNA molecule (Dissertation Tanja Dicke 2009).

In order to use DNAzymes in *in vivo* applications, they need to be stabilized against nucleolytic degradation in body fluids. Several chemical modifications have been used to enhance DNAzyme biostability throughout the years. The most important and commonly used modification is the incorporation of a 3'-3' inverted thymidine at the 3'-end of the DNAzyme. The inversion creates a 5'-end on the 3'-terminus preventing exonuclease degradation and extending the half-life of DNAzymes from ≈70 minutes to over 21 hours in human serum. In addition, the reversion promotes stability of the DNAzyme during cellular uptake (Dass et al. 2002). Another modification is the phosphorothioate linkage replacing one of the non-bridging oxygens by sulphur. Even though this alteration reduces the action of endogenous nucleases, it is associated with toxicity and off-target effects. Locked Nucleic Acids (LNAs) are also used to modify DNAzymes. They comprise a 2′-O 4-C methylene bridge that locks in a C3′-endo conformation, thus increasing the binding affinity and activity of DNAzyems (Vester et al. 2002, Schubert 2003). The DNAzymes used in this study were modified by adding a 3'-3' inverted thymidine at the 3'-end to impart resistance to nuclease degradation.

1.4.2 Transcription factors as immunological targets for DNAzymes in inflammatory skin diseases

Immune mechanisms involved in the pathogenesis of inflammatory skin diseases involve the recruitment of T cell subpopulations to mediate the inflammatory response. The contribution of these subtypes to the development of certain pathologies differs depending on the disease. In atopic dermatitis for example, Th2 cells predominate the acute phase and disrupt the Th1/Th2 balance by increased infiltration and production of Th2 cytokines such as IL-4, IL-5 and IL-13. This Th2 immune response is controlled by the transcription factor GATA3, which regulates the differentiation of Th2 cells and Th2-cytokine production (Fig. 3). In a similar way, Th1 cells play the major role in the immunopathology of psoriasis with increased production of the Th1 cytokine IFNγ. The transcription factor Tbet, is, in this case, the orchestrator of such a response by among other effects controlling the differentiation and function of Th1 cells (Fig. 4) (Lazarevic et al. 2013).

These central roles of GATA3 and Tbet make them interesting and promising targets for DNAzyme-treatment of AD and psoriasis. In fact, DNAzymes targeting GATA3 mRNA have been developed and their therapeutic potentials in murine models of asthma have already been demonstrated successfully (Sel et al. 2008).

1.4.3 Drug delivery systems for dermal application of DNAzymes

Although DNAzymes have been tested as therapeutic tools in different models including asthma and cancer (Sel et al. 2008, Mitchell et al. 2004), the use of these molecules in a dermal application can be challenging. The skin surface is compact with a natural flora of bacteria and fungi (Grice et al. 2008), producing great amounts of DNases and lowering the skin`s PH (Lambers et al. 2006), which can degrade or affect the DNAzymes activity before they even reach their targets. Another difficulty in this type of application is the necessity to transport these large, hydrophilic molecules through the skin compartment to the site of action, where they can encounter their target structure. To overcome these problems, Schmidts et al. developed a water-in-oil-in-water (w/o/w) multiple emulsion in which the DNAzymes with the inverted thymidine, were encapsulated in an inner aqueous phase. This aqueous phase was added to an oil phase and emulsified to obtain a water/oil (w/o) homogeneous emulsion, which was then dispersed in an aqueous phase containing a hydrophilic emulsifier to obtain the final w/o/w emulsion (Schmidts et al. 2011).

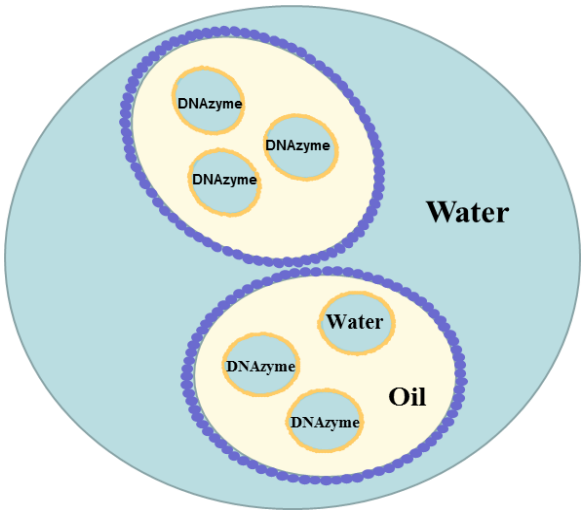

Figure 6. Water –oil- water emulsion.

DNAzymes encapsulated in an inner water phase. These water droplets containing DNAzymes are suspended in an oil phase providing protection and better penetration into the skin. The oil phase itself is suspended in another water phase to obtain a w/o/w emulsion.

This pharmaceutical formulation provided protection of the DNAzyme from degradation and the penetration enhancers added to this formulation facilitated a successful penetration into the skin. The DNAzyme uptake was higher when applying the formulation under gentle massage to help break down the oily membrane of the emulsion and release the DNAzymes from the inner phase (Schmidts et al. 2012).

1.5 Hypothesis and Aims

Inflammatory skin diseases cover a wide range of skin conditions that affect millions around the world. So far the treatment of most of these diseases depends on the use of glucocorticoids and other immune suppressive reagents that, despite their efficacy, have a diversity of side effects with severities ranging from mild to life-threatening and life-altering. The goal of this study was to examine the potentials of DNAzyme-based therapies in inflammatory skin diseases by targeting key transcription factors for the differentiation of T-helper cells.

DNAzymes against GATA3 and Tbet have already been established and GATA3-specific DNAzymes have shown to be effective in the prevention and therapy of experimental models of allergic airway inflammation. The similarities in disease

pathogenesis between asthma and AD, in terms of the underlying Th2 phenotype, raised the question of whether GATA3-DNAzyme treatment will have similar promising effects in AD. Based on the same principle, we wanted to test the therapeutic potentials of Tbet-specific DNAzymes on Th1-mediated inflammatory skin diseases.

The first part of this project was to establish an animal model of allergic skin inflammation with a Th2 predominant phenotype comparable to that observed in AD. Thereafter this model was used to investigate the effects of GATA3-specific DNAzyme treatment on disease symptoms *in vivo* and on cellular and molecular level *ex vivo.*

The second part involved the use of another modified model of inflammatory skin disease with a Th1-predominant phenotype and analysis of possible therapeutic effects of Tbet-specific DNAzymes on disease symptoms *in vivo* and on cellular and molecular level *ex vivo.*

We hypothesized that treatment with GATA3- or Tbet-specific DNAzymes can inhibit the specific Th-polarization towards a Th2 or Th1 –phenotype associated with inflammatory diseases and restore the normal Th1/Th2 balance, which make DNAzymes attractive target-specific therapeutic tools for the therapy of such skin conditions.

2 MATERIALS AND METHODS

2.1 Animals

Animals were kept in a specific pathogen-free environment in optimum temperatures of 20 – 24° C and a semi-natural light cycle of 12:12 hours light: dark.

All experiments were conducted following protocols approved by the local authorities (Regierungspräsidium Giessen). For certain procedures mice were anesthetized by intraperitoneal (i.p.) injection of 200 µl of a Ketamine (76 mg/kg bw)/ Xylazin (10 mg/kg bw) mixture.

Mouse strain	***Age***	***Gender***	***Provider***
BALB/c	*6-8*	*male*	*Charles River*
DO11.10	*8-10*	*male*	*Marburg University*

Material /reagent	***Provider***
Ketamine 50 mg/ml	*Inresa*
Xylazin (Ropmun®) 20 mg/ml	*Bayer*

2.2 Induction of skin inflammation in mice

2.2.1 Oxazolone-induced dermatitis

A combination of mechanical injury and the hapten oxazolone (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one) were used to induce dermatitis in 6-8 week-old male BALB/c mice. The back skin (about 1 x 2 cm) of anesthetized mice was shaved using an electrical shaver followed by a razor. Skin injury was inflicted by tape stripping 5x with an ordinary adhesive tape. Mice were sensitized by epicutaneous application of 0.2% oxazolone (50 µl) dissolved in a 4:1 solution of acetone: olive oil. This first contact with oxazolone leads to the generation of oxazolone-specific T cells, and Th2 cells in particular.

Three days later, skin was tape stripped again and inflammation was elicited by application of 0.2% oxazolone (50 µl) to the shaved skin. To assess the progression of inflammation, skin thickness was measured before challenge and on daily basis afterwards using a digital calliper (see 2.4.1). The control group was sensitized and

challenged with 50 µl acetone: olive oil (4:1). Mice were sacrificed three days after challenge to collect samples for *ex vivo* analysis (Fig. 7A).

Material	***Provider***
Electrical shaver QT4022	*Philips*
Razor	*Wilkinson sword*
Oxazolone	*Sigma-Aldrich*
Olive oil	*Sigma-Aldrich*
Acetone	*Roth*
Adhesive tape	*Schöngene® plast*

2.2.2 OVA / CFA-induced dermatitis

In this model, 8-10 weeks old-male DO11.10 mice were sensitized to the protein antigen, ovalbumin (OVA). 75 µg of OVA (grade V) were dissolved in phosphate buffered saline (PBS) and then emulsified in complete Freund's adjuvant (CFA, 50% vol / vol). 100 µl of the emulsion were subcutaneously (s.c.) injected into the loose skin of each side of the inguinal area (lower left and lower right area of the abdomen). Seven days later, mice were challenged on three consecutive days by epicutaneous application of 50 µl of 20% OVA (dissolved in a 10% dimethyl sulfoxide, DMSO, solution to enhance skin permeability).

A control group was sham sensitized by subcutaneous injection of PBS emulsified in CFA and challenged with 10% DMSO alone. Skin thickness was measured before and then daily after challenge to assess progression of inflammation. Mice were sacrificed four days following challenge and samples were collected for ex vivo analysis (Fig. 7B).

Material	***Provider***
Ovalbumin Grade V	*Sigma-Aldrich*
Complete Freund's adjuvant (CFA)	*Sigma-Aldrich*
Phosphate buffered saline (PBS)	*Sigma-Aldrich*
Dimethyl sulfoxide (DMSO)	*Roth*
Discofix®3-way stopcock	*Braun*

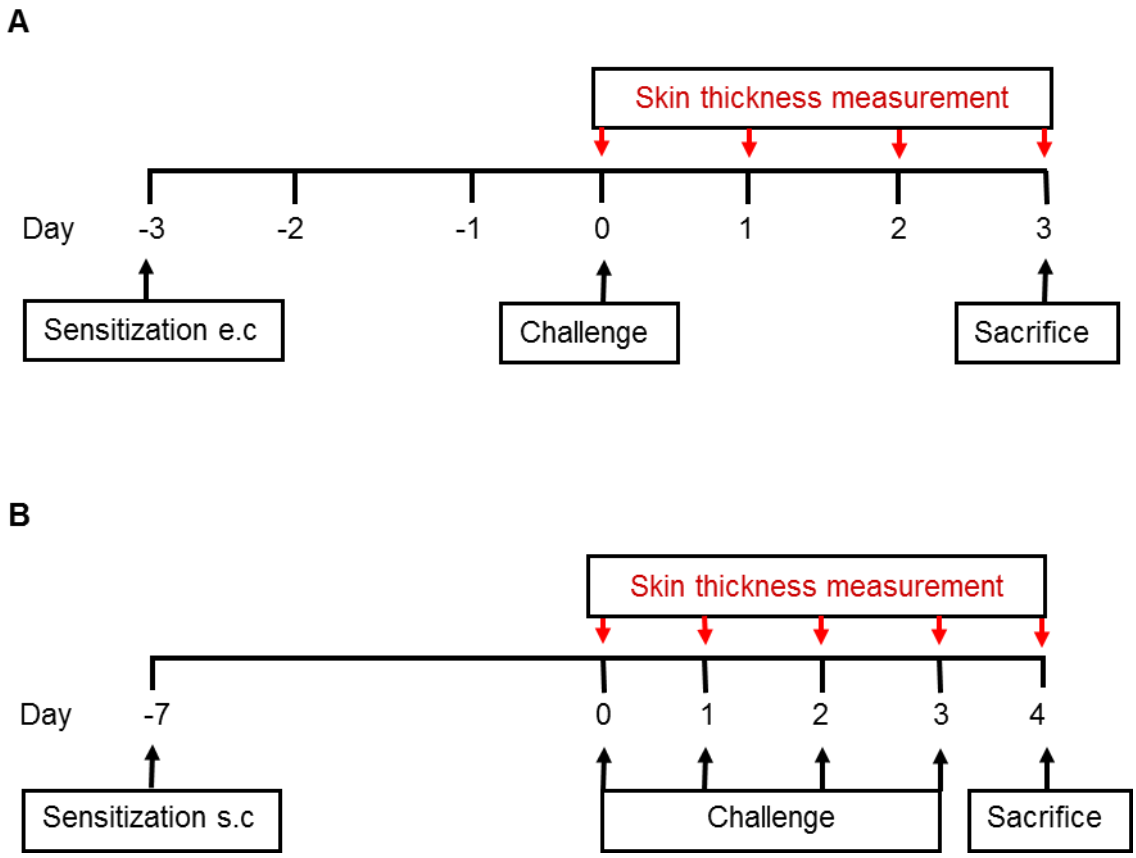

Figure 7. Protocols for induction of skin inflammation.

(A) Oxazolone-induced dermatitis**, (B)** OVA/CFA-induced dermatitis.

2.3 Treatment protocols for topical application of DNAzymes *in vivo*

2.3.1 DNAzymes and controls used for topical application in vivo

To ensure a better penetration and delivery of DNAzymes through the skin, they were used in a form of a w/o/w multiple emulsions prepared in collaboration with the "Technical hochschule mittelhessn,THM" as described in (1.4.3) and in (Schmidts et al. 2011). Briefly, hgd40 (GATA3-DNAzyme) or td32 (Tbet-DNAzyme) were encapsulated in an inner water phase, which was then emulsified with an oil phase until a homogenized w/o emulsion was obtained. This emulsion was later dispersed in another aqueous phase to achieve the final w/o/w emulsion. Similarly, another w/o/w emulsion containing control DNAzyme named ODNg3, which has an active catalytic domain and a non-specific sequence in the binding arms, was also used in the study. To eliminate any formulation-related effects, both placebo (no active ingredient) and Dexamethasone were also

formulated in w/o/w emulsion.

2.3.2 Prophylactic treatment of oxazolone- induced dermatitis with hgd40

Skin inflammation, was induced in mice using oxazolone as described in paragraph (2.2.1). For the prophylactic treatment, the back skin of mice in each different treatment group (see table 1) was treated by applying and gently massaging 50 µl of the respective emulsion. This process was conducted once daily starting one day before sensitization until the day before termination (Fig. 8A). On challenge day, mice were treated with the emulsion in the morning and challenged later in the afternoon. The skin was wiped with acetone before challenge to eliminate any emulsion residues on the skin surface.

Table 1. Groups in the prophylactic treatment of oxazolone-induced dermatitis

Group	Sensitization	Challenge	Treatment
1	Solvent	Solvent	Placebo
2	0.2% Oxazolone	0.2% Oxazolone	Placebo
3	0.2% Oxazolone	0.2% Oxazolone	0.4% ODNg3
4	0.2% Oxazolone	0.2% Oxazolone	0.4% hgd40
5	0.2% Oxazolone	0.2% Oxazolone	2% hgd40
6	0.2% Oxazolone	0.2% Oxazolone	0.1% Dexamethasone

2.3.3 Semi-therapeutic treatment with hgd40 in oxazolone-induced dermatitis

Inflammation in the skin was elicited as already mentioned in paragraph (2.2.2). For the semi-therapeutic treatment, 50 µl of the respective emulsion were topically applied to the shaved back once daily starting one day before the challenge (day-1) until the day before termination (Fig. 8B). On challenge day, mice were treated with the emulsion in the morning and challenged later in the afternoon to allow time for absorption through the skin. The skin was wiped with acetone before challenge to eliminate any emulsion residues on the skin surface that may interact with oxazolone.

2.3.4 Preventive treatment with a Tbet-specific DNAzyme in OVA/CFA-induced dermatitis

For the induction of skin inflammation, mice were sensitized and challenged as described in paragraph (2.2.2). For the preventive intervention, 50 µl of the respective emulsion were topically applied twice a day with a 12-hour interval. The first treatment started one day before challenge and continued twice/day until 12h before termination

on day 3. Skin thickness was measured on a daily basis to monitor the inflammation progress. Several hours were left between treatment and OVA challenge to allow enough time for proper absorption of the emulsion. In addition, skin was wiped with acetone before challenge to avoid any interaction between the emulsion component and OVA in the challenge process.

Materials	***Provider***
*hgd40 Oligonucleotide 5´to 3´ GTGGATGGAGGCTAGCTACAACGAGTCTTGGAG[dT-rev-Q]**	*Biospring*
*ODNg3 Oligonucleotide 5´to 3´ CCATGTGGAGGCTAGCTACAACGACTGGAATCA[dT-rev-Q]**	*Biospring*
*Td32 Oligonucleotide 5´to 3´ CTCCCGGAAGGCTAGCTACAACGACCTTTGGCA[dT-rev-Q]**	*Biospring*
Dexamethasone	*Sigma-Aldrich*
All w/o/w emulsions	*THM ***

* *[dT-rev-Q]: inverted thymidine on the 3´end.*

***Technische Hochschule Mittelhessen*

Table 2. Groups in the semi-therapeutic treatment of oxazolon-induced dermatitis.

Group	Sensitization	Challenge	Treatment
1	Solvent	Solvent	Placebo
2	0.2% Oxazolone	0.2% Oxazolone	Placebo
3	0.2% Oxazolone	0.2% Oxazolone	0.4% ODNg3
4	0.2% Oxazolone	0.2% Oxazolone	2% ODNg3
5	0.2% Oxazolone	0.2% Oxazolone	0.4% hgd40
6	0.2% Oxazolone	0.2% Oxazolone	2% hgd40
7	0.2% Oxazolone	0.2% Oxazolone	0.1% Dexamethasone

Table 3.Treatment groups in the OVA/CFA model.

Group	Sensitization	Challenge	Treatment
1	OVA/CFA	20% OVA	Placebo
2	OVA/CFA	20% OVA	0.4% ODNg3
3	OVA/CFA	20% OVA	0.4% td32
4	OVA/CFA	20% OVA	0.4% hgd40

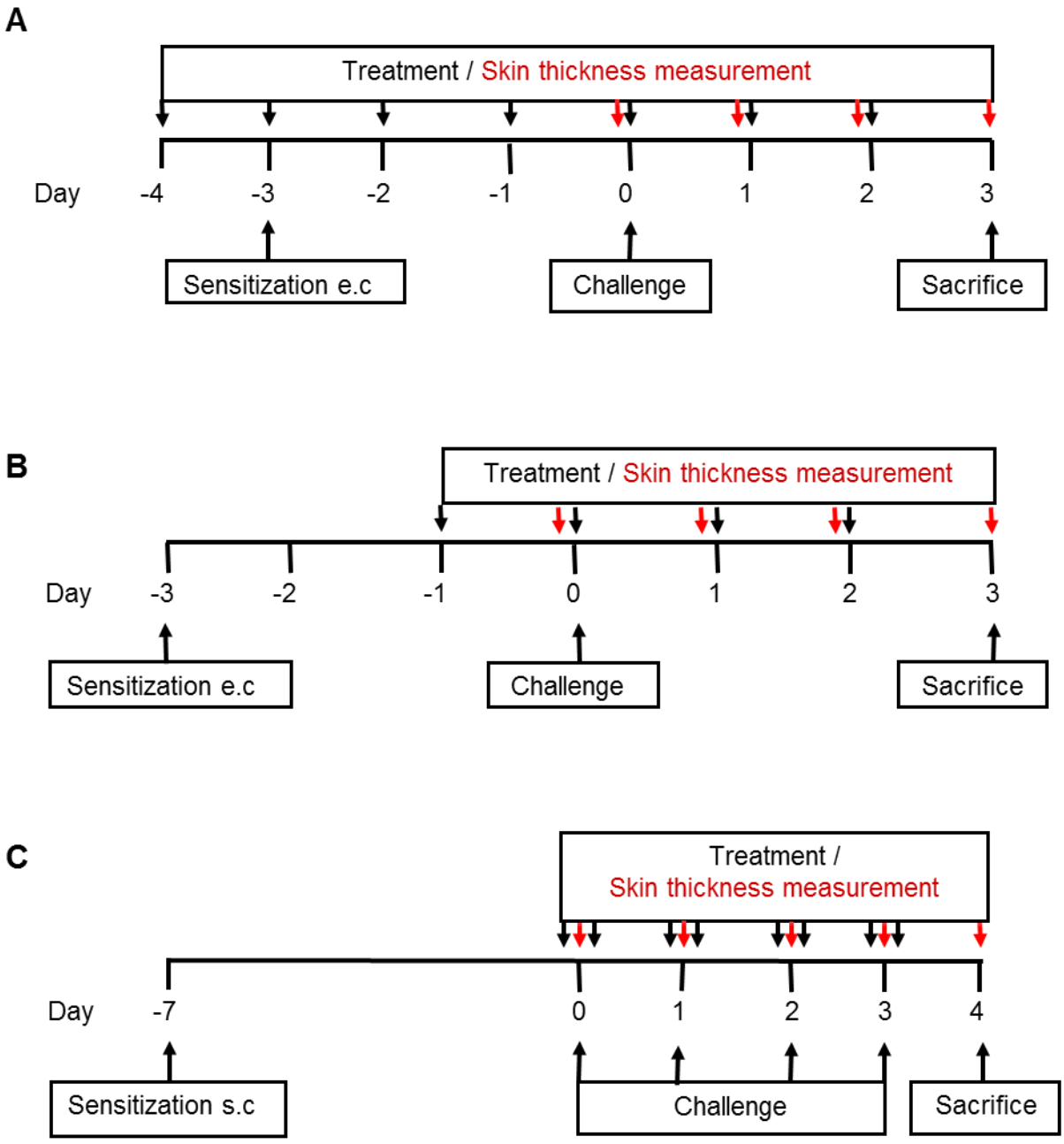

Figure 8. Treatment protocols of skin inflammation with DNAzymes.

(A) Prophylactic treatment protocol of oxazolone-induced dermatitis**. (B)** Semi-therapeutic treatment of oxazolone-induced dermatitis. **(C)** Semi-therapeutic treatment of OVA/ CFA-induced dermatitis.

2.4 *In vivo* assessment of skin inflammation

Skin thickness measurement

The changes in skin permeability during inflammation result in edema formation. This in turn is reflected by characteristic increase in skin thickness which can be measured as an indicator of disease severity. In our animal models skin thickness was measured throughout the experiment to monitor inflammation progression *in vivo*. A skin fold was formed in the middle of the inflamed 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, Germany*) with an accuracy of +/- 0.03 mm and a resolution of 0.01 mm. Skin thickness on the day of challenge day 0 was used as a baseline and deviation of skin thickness to day 0 (skin thickness $_{day\ x}$ – skin thickness $_{day\ 0}$) was calculated in each animal for every day after challenge including termination day. All measurements were conducted on anesthetized mice.

2.5 Animal sacrifice and sample collection

Animals were sacrificed by intraperitoneal injection of 200 µl of 5x anaesthesia solution. Once the animal has stopped breathing and showed no more reflexes (eye lids, paws), the shaved piece of dorsal skin was surgically removed while maintaining orientation of the tissue, anterior to posterior. The obtained skin sample was then cut into three pieces with similar sizes and tissue orientation. One piece was transferred into a cryotube, snap frozen in liquid Nitrogen and stored at -80°C for further processing (see next section on Tissue lysis and RNA isolation). Each of the other two pieces were placed on a histology cassette with epidermis facing up and skin oriented anterior to posterior. One cassette was emerged in 6% Paraformaldehyde and the other in zinc fixative for histological analysis (see section 2.7).

2.6 Gene expression analysis

2.6.1 Tissues lysis and RNA isolation

Frozen skin samples were weighed and ground to fine powder using mortars previously cooled in liquid Nitrogen. The powder was homogenized in 1 ml Trifast lysis buffer/100 mg tissue. This suspension was next passed through a 0.9 mm syringe needle for 4 times to further disrupt the cells and then transferred to 1.5 ml RNase-free tube. After

short incubation (about 5 min) at RT, the suspension was centrifuged at 12000 g for 10 min at 4° C and the supernatant was collected. 200 µl chloroform were vortexed with the supernatant and then incubated for 5 min at RT. Next, the mixture was centrifuged at 12000 g for 5 min at 4° C and the upper aqueous phase containing the RNA was collected. To precipitate the RNA, the aqueous phase was mixed with 0.5 ml isopropanol and incubated for ≥ 15 min either on ice or at 4° C and then centrifuged at 12000 g for 10 min at 4◦ C. After discarding the supernatant, the RNA pellet, having a gel-like appearance, was washed twice with 1 ml 75% ethanol and finally dissolved in 50 µl RNase-free water.

Material	***Provider***
peqGOLD TriFast ™	*Peqlab*
Trichlormethan / Chloroform	*Roth*
2-Propanol / Isopropanol	*Sigma - Aldrich*
Ethanol 100% molecular biology grade	*Sigma - Aldrich*
RNase-free water	*Qiagen*
Needle	*BD*
Cryo-tubes 1.7ml	*Sarstaedt*
Safe seal® tubes 1.5 ml	*Carl Roth*
Micro 200R Centrifuge	*Hettich*

2.6.2 RNA clean-up and on column DNA digestion

To obtain a highly purified RNA, RNA clean-up using RNeasy mini spin columns was performed according to manufacturer's instruction. The volume of the RNA sample was increased to 100 µl using RNA-free water. Next, 350 µl RLT buffer and 250 µl 96% ethanol were added and the whole 700 µl were transferred to RNeasy mini spin columns and centrifuged at 8000 g for 1 min at RT. The high salt content of the RLT buffer allows binding of the total RNA only to the RNeasy silica membrane while ethanol improves the binding conditions. After RNA-binding all other contaminants were washed away by the addition of 350 µl washing buffer RW1 and centrifugation for one minute at 8000 g at RT.

To eliminate any possible contamination with DNA, a DNA digestion was also performed on the RNeasy mini spin columns using the RNase-free DNase set ®. After the first washing steps in RNA clean-up as described above, 80 µl of a mixture of DNase I and

RDD buffer (10 µl DNase I and 70 µl RDD buffer) was added to the spin column membrane and incubated at RT for 15 min. The DNase I was removed during subsequent washing steps with RW1 and RPE buffers and RNA elute was obtained by the addition of 30 µl RNase-free water to the spin column membrane followed by centrifugation for 1 min at 8000 g.

Material	***Provider***
RNeasy® mini kit	*Qiagen*
RNase-Free DNase® Set	*Qiagen*

2.6.3 RNA quantitation and quality assessment

2.6.3.1 RNA quantitation

RNA concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo scientific). 1 µl of the RNA sample was retained in place via surface tension between two fibre optic cables and the absorbance was measured at two wave lengths, 260 nm where the RNA absorbs and 280 nm at which protein and phenol absorb. Based on the 260 / 280 ratio the RNA purity was assessed. A ratio between 1.8- 2 was regarded as “pure” RNA.

2.6.3.2 RNA quality assessment

The overall quality of the RNA was assessed by gel electrophoresis. All equipment, including electrophoresis chamber, gel form and combs were washed with RNase Zap to create an RNase-free working environment. To prepare the samples, a mix of 1 µg RNA, 5 µl RNase-free water and 5 µl RNA-sample buffer was incubated at 65° C for 10 min then placed directly on ice. Samples as well as a RNA ladder were then loaded on a 2% agarose gel containing 5 µl ethidium bromide. The gel was run for an hour at 100 V in Tris Acetate EDTA (TAE) buffer.

For mammalian total RNA, two intensive bands, representing the 28S and the 18S of the ribosomal RNA, but no degradation products should be observed.

Material	*Provider*
2x RNA sample loading dye	*Peqlab*
High range RNA ladder	*Peqlab*
Tris Acetate EDTA (TAE)	*Sigma*
Ethidium bromide solution 0.07%	*AppliChem*
Universal RNase-free Agarose	*Peqlab*
RNase zap	*Ambion*
Fusion FX7^{TM} system	*Peqlab*
Fusion-capt software	*Peqlab*

2.6.4 Reverse transcription

In this procedure the isolated RNA was reverse transcribed to obtain first-strand cDNA using the Omniscript® kit. Oligo dt primers, which hybridize to the poly A tail of mRNA, were added to the reaction to provide a transcription starting point for the polymerases.

The reaction mix was prepared as detailed below and reverse transcription was performed in a 3-step- procedure using a Biometra TRIO-Thermo block. During the first step the reaction mix was incubated for 1 hour at 37◦ C followed by a degradation step of 5 min at 93◦C to stop transcription. In the final step the reaction was cooled to 4◦C.

Reaction mix:

X µl	RNA (500 ng)
2 µl	10x RT-Buffer
2 µl	dNTP
1 µl	Oligo-dt 18 primer
1 µl	Omniscript reverse transcriptase
Y µl	RNase-Free water
20 µl	End volume

Material	*Provider*
Omniscript ® RT Kit	*Qiagen*
Oligo dt 18 Primer	*Metabion*
TRIO-Thermo block	*Biometra*

2.6.5 Real time polymerase chain reaction (RT-PCR)

Expression levels of different genes were analysed using real time quantitative PCR. The cDNA obtained from the reverse transcription was amplified using a pair of primers for the gene of interest and the QuantitTect™ SYBR®Green-Kit (containing a mixture of Taq ploymerases, dNTP, PCR buffer and SYBR Green I dye). The reaction was performed in RotorGene3000 which simultaneously enables quantification of the target gene by detecting fluorescence of the SYBR Green dye I bound to the amplified double strand DNA.

Because the SYBR Green I dye used in the reaction binds to any double stranded DNA including any genomic DNA contaminating the sample, all primers were designed to flank at least one intron. This allows the discrimination between products amplified from cDNA and those amplified from genomic DNA based on the size of products.

In addition, GATA3 primer also designed to flank the region where the DNAzyme catalyses the mRNA, to ensure that no degradation products of the targeted RNA, but only intact non-catalysed RNA is amplified

The PCR reaction was set as shown below:

5 µl QuantitTect™ SYBR®Green master mix
0.25 µl Forward primer (10 pMol)
0.25 µl Reverse primer (10 pMol)
4.5 µl cDNA (0.5 µg)
10 µl end volume

The tubes were then loaded into the RotorGene 3000 and ran for 40 cycles under the following cycling profile:

Activating Polymerases	95°C	15 min	
Denaturation	94°C	15 sec	40 cycles
Annealing	Primer T_m	30 sec	
Extension	72°C	30 sec	

The gene expression was normalized to the housekeeping gene ribosomal protein L32, which was included in each PCR run. Housekeeping genes are usually expressed on

constant levels in pathologic and non-pathologic conditions and are used as internal controls in RT-PCR. The relative mRNA expression was calculated to a reference sample. The reference was generated as follows: The cDNA from one skin sample was used as a template in several different PCR reactions, each performed to amplify a different gene of interest. The different PCR products were then diluted to obtain a specific amount of each gene of interest (reflected by cycle threshold number Ct). The reference was used to compare and normalize all individual runs within one experiment and all individual experiments of the same model. Therefore, it was also included into each PCR run.

Table 4. List of primers used in the RT-PCR.

Gene	*Primer Sequence*	*Tm*	*Product size*
L32	*For: 5'-GCA AGT TCC TGG TCC ACA AT -3'* *Rev: 5'-GGG ATT GGT GAC TCT GAT GG -3*	*58◦ C*	*152 bp*
GATA3	*For: 5'-ACG GAA GAG GTG GAC GTA CT -3'* *Rev: 5'-CGC CAG AGA GGA TGA AG -3'*	*60◦ C*	*297 bp*
IL-4	*For: 5'-TCA ACC CCC AGC TAG TTG -3'* *Rev: 5'-TGT TCT TCG TTG CTG TGA -3'*	*60◦ C*	*177 bp*
IFNγ	*For: 5'-GCT TTG CAG CTC TTC CTC AT -3'* *Rev: 5'-GCA GGA TTT TCA TGT CAC CA -3'*	*58◦ C*	*175 bp*
Tbet	*For: 5'-AGG TGT CTG GGA AGC TGA GA -3´* *Rev: 5`- CCA CAT CCA CAA ACA TCC TG -3*	*60◦ C*	*174 bp*

Relative mRNA expression was calculated using the equations listed below:

$\Delta Ct_{sample} = Ct_{target\ gene\ sample} - Ct_{L32\ sample}$

$\Delta Ct_{reference} = Ct_{target\ gene\ reference} - Ct_{L32\ reference}$

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$

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

Relative gene expression (%) =

Relative gene expression $_{sample}$ / mean relative gene expression $_{placebo}$ x 100

Material	*Provider*
QuantitTect™ SYBR®Green	*Qiagen*
RotorGene™	*Corbett Research*
Primers	*Metabion*

2.7 Histology

Specimens from the sensitized and treated skin area were placed in embedding cassettes with the epidermis facing up. The whole cassettes were immersed in either a Zinc or a 6% paraformaldehyde (PFA) fixative fluid for 24-48 h. Zinc fixation allows preservation of the tissue and cells as they naturally occur and prevents autolysis by coagulating and hence deactivating lysosomal enzymes. PFA, on the other hand, reacts with primary amines on proteins and nucleic acids to form partially-reversible methylene bridges.

After removal from the fixative, samples were washed with water and then dehydrated by transferring the cassettes into a series of alcohol-water solutions (70%, 96% and 100% ethanol).

The alcohol was then replaced with Roticlear® (Xylol substitute) and samples were finally embedded into paraffin blocks. Using a microtome, 5 µm sections were made from the paraffin blocks and mounted onto microscope slides.

Material	*Provider*
Zinc fixative	*BD pharmingen*
6% Paraformaldehyde (PFA)	*Merck*
Ethanol absolute, 96%, 70%	*Otto Fischar*
Roticlear®	*Roth*
Microm HM 355S ™	*Thermo scientific*
Microscope slides	*Thermo scientific*
Cassettes	*Engelbrecht*

2.7.1 Haematoxylin and eosin staining

This staining was used to provide a general overview of the skin structure and of the cellular influx into site of inflammation.

The H&E staining uses two different dyes, haematoxylin which is a combination of the haematin dye and aluminium ions (AL^{3+}) giving it a positive charge. Therefore, haematoxylin binds to the negatively charged chromatin in the nucleus and stains it purple-blue. Eosin, on the other hand, is a negatively charged pink dye that binds to the positively charged structures including the proteins in the cytoplasm and connective tissues.

For staining, paraffin was removed by 20 min incubation in Xylol. The sections were then rehydrated by washes in a series of gradient alcohol (100%, 96%, 70%) and finally water. After the deparaffinization and rehydration, sections were stained in Gill's Haematoxylin No.2 for 10 min. The excess dye was discarded by washing with running water for 10 min followed by washing with distilled water. Next, sections were stained in eosin for 5 min followed by washing with distilled water.

After staining, sections were dehydrated through a gradient alcohol series (70%, 96% and 100%) and cleared in roticlear, then covered with cover slips using the mounting medium Entellan® for adhesion.

Material	***Provider***
Roticlear ®	*Roth*
Ethanol absolute, 96%, 70%	*Otto Fischar*
Gill's Hematoxylin No.2	*Merck*
Eosin G	*Merck*
Entellan®	*Merck*
Cover slips	*Thermo scientific*

2.7.2 Immunohistochemistry

Surface protein markers are used as targets for antibodies to stain certain cells within the tissue section. A specific staining for CD4+ cells and GATA3 in skin sections was performed with the help of Dr. Ulrich Purath.

Sections were deparaffinised and rehydrated by immersing in roticlear® and a series of gradient alcohol and finally in distilled water as previously described (2.7.1). To make the epitopes available for antibody binding, an antigen retrieval procedure was conducted. For this purpose, slides were immersed in a preheated, citrate-based unmasking retrieval solution (pH= 6.0) and then heated in a pressure cooker for 40 min. When the pressure marker went off, the slides were removed and washed in PBS for 10 min. Next, endogenous peroxidase activity was blocked with a hydrogen peroxidase (H_2O_2) solution (1 ml 30% H_2O_2 in 50 ml PBS) for 15 min in the dark and then washed in PBS for 10 min. Sections were then incubated with horse serum for 30 min to block unspecific binding. After the serum was removed (without washing) sections were stained either for CD4 or GATA3.

For CD4 staining, sections were incubated with the primary antibody (anti CD4 1:400 in PBS) at RT for one hour. The primary antibody was rinsed off with PBS and sections were incubated for 20 min with the ImmPRESS™ reagent containing the secondary antibody (anti-rabbit Ig) coupled with peroxidase. After a washing step with PBS, few drops of DAB were added to the sections. DAB is rapidly oxidized by the highly active peroxidase micro polymers attached to the secondary antibody. This forms a stable brown precipitate to detect the epitope specific antibody binding. Sections were finally washed with tap water, dehydrated in a series of gradient alcohol (70%, 96% and 100%) and roticlear® then mounted in Entellan®.

For GATA3 staining, sections were incubated with the primary antibody (anti GATA3 1:300) for one hour, washed with PBS and then incubated for another 20 min with the ImmPRESS™ anti mouse-Ig complex. The signal was detected by addition of DAB substrate. Sections were finally washed, dehydrated and eventually mounted in Entellan®.

For quantification of positively stained cells, each section was divided into 3 equal parts and a high power field was chosen in each part. Positive signals were counted per high power field and averaged for all sections of the same treatment group. Hair follicles and upper layers of the epidermis were excluded from the quantification.

Material	*Provider*
Rabbit polyclonal anti- CD4	*Novus Biologicals*
Mouse monoclonal anti- GATA3	*BD-Pharmingen*
ImmPRESS Anti-Rabbit Ig Polymer Detection Kit	*Vector labs*
ImmPRESS Anti-mouse Ig Polymer Detection Kit	*Vector labs*
Impact DAB	*Vector labs*
Normal horse serum blocking solution	*Vector labs*
Low PH citrate- based antigen masking solution	*Vector labs*
30% H_2O_2	*Roth*
PBS	*Sigma-Aldrich*
Pressure cooker	*Highlights*

2.8 Statistical analysis

Data analysis and graphing was conducted using the software GraphPad Prism, version 6 ®. In each group the mean value ± standard error of the mean was used in the bar graphs. Statistical differences were determined by student's *t* test (between 2 groups), one-way analysis of variance (ANOVA) (between multiple groups) or two-way analysis of variance (ANOVA) (between multiple groups over multiple time points). A p value of smaller than 0.05 ($p \leq 0.05$) was considered to indicate statistical significance.

3 RESULTS

3.1 Establishment of an oxazolone-induced contact dermatitis model

The first goal of this work was establishing an animal model of allergic skin inflammation with a predominant Th2 immune response. For this reason, we used oxazolone, a hapten known to skew a mixed phenotype with a higher influx of Th2 cells, to induce a subacute prolonged contact hypersensitivity reaction in BALB/c mice as described in section (2.2.1). Briefly, the shaved back skin of mice was tape-stripped 5x, to mimic skin injury caused by scratching in patients with AD, and sensitized by epicutaneous application of oxazolone to induce oxazolone-specific T cells. Three days later, mice were tape stripped and challenged the same way to elicit an allergic skin inflammation (Fig. 7A). Three days following challenge, samples were collected for *ex vivo* analysis.

The release of inflammatory mediators during the allergic inflammatory reaction changes skin permeability. This along with infiltration of inflammatory cells into the site of challenge lead to the formation of the characteristic edema noted in allergic skin inflammation, which is usually reflected by increased thickening in the skin. Thus, daily skin thickness measurements were used as a parameter to monitor disease progression *in vivo.*

As our data clearly shows, in mice sensitized and challenged with oxazolone there was a progressive increase in skin thickness in the days following oxazolone challenge. Skin thickness doubled within the first 24 h after challenge and continued to increase until termination on day 3 (Fig. 9). Such an effect was absent in the control group which was sham sensitized and challenged with solvent only. The skin thickness in this group remained unchanged throughout the experiment with values similar to those measured on the day of challenge. The differences in skin thickness between these two groups were significant during the experiment.

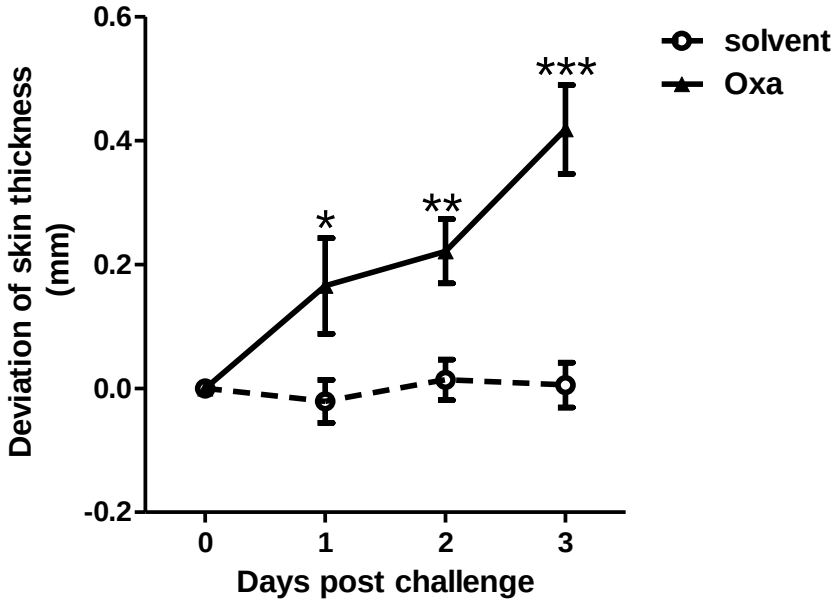

Figure 9. Oxazolone induced a progressive thickening of the skin.

The back skin of mice was tape stripped and mice were sensitized and challenged with either solvent or oxazolone as described in (2.2.1). Skin thickness was measured on days 0, 1, 2, and 3 and deviation to day 0 was calculated. Data are compiled from 4 separate experiments (n= 6-8 per group/experiment) and are shown as mean ± SEM. Significant differences are marked *p<0.05, **p<0.01 and ***p<0.001 compared to solvent at the same time point.

To examine histological changes and cellular influx, skin samples were prepared for histology as described in (2.7) and sections were stained with H&E (2.7.1).

Histological analysis revealed mild epidermal and dermal thickening, edema formation as well as a prominent cellular infiltrate into the dermis in sections of lesional skin samples obtained from oxazolone challenged mice. The inflammatory infiltrate accumulated in the upper part of the dermis and around the small capillaries in the skin. In the solvent group no signs of skin inflammation were noted. There was a minor disruption in the epidermis, probably due to tape stripping, while no prominent changes were observed in the dermis. Moreover, no increase in the dermis thickness nor an accumulation of inflammatory cells at the site of challenge was noted (Fig. 10). These findings support the results obtained from skin thickness measurement.

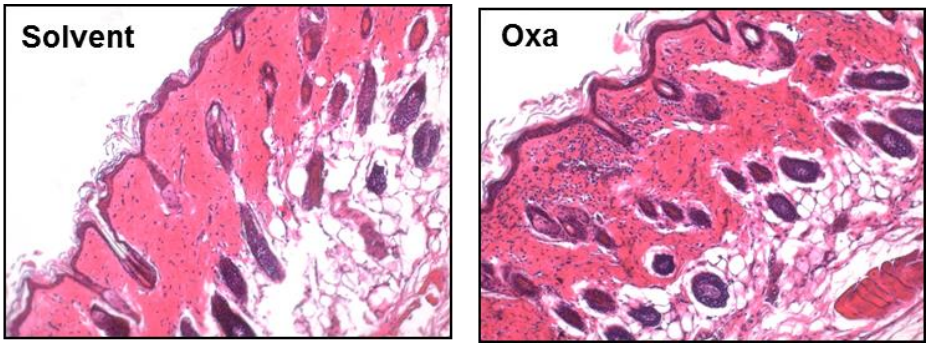

Figure 10. Oxazolone- induced dermatitis was characterized by epidermal thickening and cellular infiltration.

Skin sections from oxazolone or solvent-challenged mice were stained with H&E and examined at a 100X magnification. There was a clear infiltration of cells into the site of inflammation after challenge with oxazolone.

In the literature, oxazolone-induced dermatitis has been described to have a Th1-dominant, a mixed Th1/Th2 or a Th2-dominant phenotype (Dearman et al. 1994, Thomson et al. 1993, Kusumoto et al. 2007). To investigate the phenotype in this model, RNA was isolated from inflamed skin (2.6.1), reverse transcribed (2.6.4) and the relative expression of Th1 and Th2 related transcription factors and major cytokines in the skin was quantified using RT-PCR (2.6.5).

Our data showed that skin injury and oxazolone-challenge resulted in a significant increase in the mRNA expression of GATA3, the main transcription factor in Th2 cell differentiation. In addition, the expression of the Th2 cytokine IL-4 in the skin was also significantly increased in the oxazolone-challenged group (Fig. 11).

No significant changes in the expression of the Th1 cytokine IFNγ or the transcription factor Tbet were detected. There was only a tendency of an increased IFNγ and a decreased Tbet expression.

These results suggest that oxazolone elicited a progressive allergic inflammatory response in the skin with a Th2-predominant phenotype.

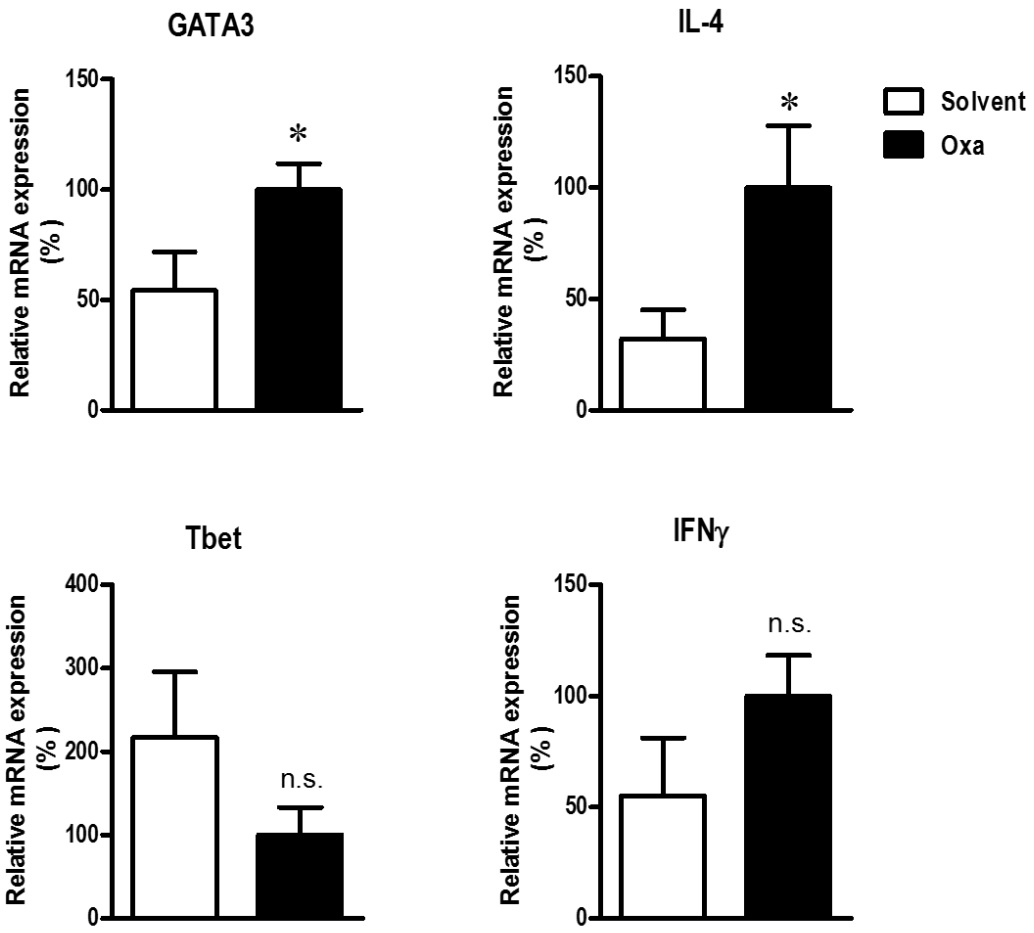

Figure 11. Increased mRNA expression of Th2- related genes in the skin upon oxazolone challenge.

Skin samples were collected on day 3 after challenge and mRNA expression in the skin was quantified using RT-PCR after normalization to the house keeping gene L32 and a reference sample. Data are compiled from two separate experiments (n= 6-8 group/experiment) and are shown as mean ± SEM. Significant differences are marked *p ≤ 0.05 compared to solvent. n.s: not significant.

3.2 Effects of a GATA3-specific DNAzyme on oxazolone-induced dermatitis *in vivo*

In this part of the study, several experiments were conducted using the oxazolone model to test the effects of treatment with the GATA3-specific DNAzyme hgd40 on disease progression. Two preventive approaches were used for treatment. In the prophylactic approach DNAzyme treatment started before the sensitization. For “semi-therapeutic” treatment mice were left untreated during the sensitization phase and first treatment

started 24 h before oxazolone challenge. The GATA3-specific DNAzyme was formulated in a special w/o/w emulsion designed to protect the DNAzyme against degradation and to enhance skin penetration. In addition to the hgd40 treated groups, several treatment control groups were included in these experiments. To discriminate between the placebo and specific DNAzyme effects, a placebo control group treated with the designed emulsion without DNAzyme was analyzed. In addition, another group treated with an emulsion containing the control DNAzyme (ODNg3) was also included in the study to assess the biological specificity of hgd40. This control DNAzyme has an active catalytic domain while the nucleotides in the binding arms are scrambled to create a non-specific sequence. As a positive treatment control, the corticosteroid dexamethasone was used in a similar w/o/w formulation. To evaluate the oxazolone-induced allergic inflammatory response, a control group was sham sensitized and challenged with solvent and treated with placebo. This group was compared directly to the placebo-treated oxazolone-challenged group.

3.2.1 Effects of prophylactic treatment with GATA3-specific DNAzyme hgd40 on disease progression

The efficacy of the GATA3-specific DNAzyme hgd40 was investigated in the oxazolone model. As described earlier, male BALB/c mice were tape-stripped, epicutaneously sensitized with oxazolone and similarly challenged three days later. For the prophylactic approach, mice were first treated one day before the sensitization and the treatment continued once daily until day 2 after challenge (Fig. 8A). 50 µl of the emulsion containing either 0.4% or 2% hgd40 were applied to the skin and gently massaged to help release the active ingredient from the inner phase of the w/o/w emulsion. Control groups were treated with either placebo, ODNg3 or dexamethasone. A detailed description of the different groups can be found in table 1 section 2.3.1.

Throughout the experiment, skin thickness was measured on a daily basis to monitor disease progression and the allergic inflammatory response *in vivo*. At the end of the experiment on day 3 skin samples were collected from sacrificed mice for *ex vivo* analyses.

3.2.1.1 Effects of DNAzyme treatment on skin swelling

Skin measurements showed that one day after oxazolone challenge, skin thickness was significantly increased in all oxazolone-challenged groups compared to the solvent-

challenged group. This increase continued until termination day in the placebo and control DNAzyme (ODNg3)-treated groups. In both hgd40-treated groups showed a significant reduction in skin thickness on days 2 and 3. Mice treated with dexamethasone had a significantly thinner skin upon treatment and throughout the experiment. The skin thickness of the dexamethasone group was even lower than in the solvent group on days 2 and 3 (Fig. 12), indicating possible adverse effects of skin thinning usually associated with topical corticosteroids.

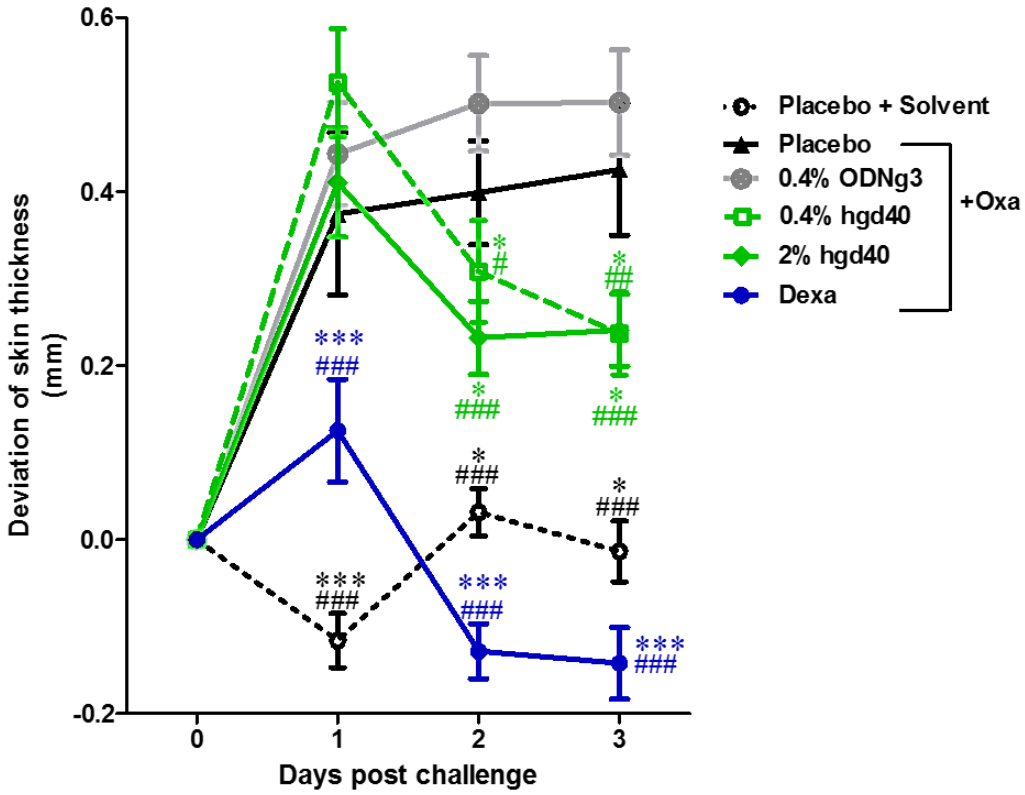

Figure 12. Effects of hgd40 prophylactic treatment on skin thickness in oxazolone-induced dermatitis.

BALB/c mice were sensitized, challenged and treated as mentioned in (2.3.1). Skin thickness was measured on days 0, 1, 2, 3 post challenge and deviation to day 0 was calculated. Data are compiled from 3 different experiments (n= 6-8 per group/ experiment) and are shown as mean ± SEM. Significant differences are marked *p ≤ 0.05, ***p ≤ 0.001 (compared to placebo + Oxa) and #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001 (compared to ODNg3 + Oxa).

In summary, *in vivo* observations revealed significant treatment effects of hgd40 on the manifestation of oxazolone-induced dermatitis reflected in a reduced thickening in the skin after treatment.

3.2.1.2 Effects of DNAzyme treatment on cellular infiltration

Skin swelling associated with contact hypersensitivity is a result of the allergic inflammatory process in the skin including the formation of edema and widening of intracellular spaces in addition to the infiltration of cells into the site of inflammation.

To assess the features of oxazolone-induced dermatitis and changes in the histopathology due to different treatments, skin sections were examined after H&E staining.

Histological analysis showed a characteristic but mild epidermal thickening and a substantial infiltration of inflammatory cells after oxazolone challenge. However, no significant changes in the density of cells infiltrating the dermis were observed neither in the 0.4% nor the 2% hgd40 treated groups compared to placebo or control DNAzyme

ODNg3 groups. In contrast, this cell infiltration was strongly restrained in the dexamethasone group (Fig. 13).

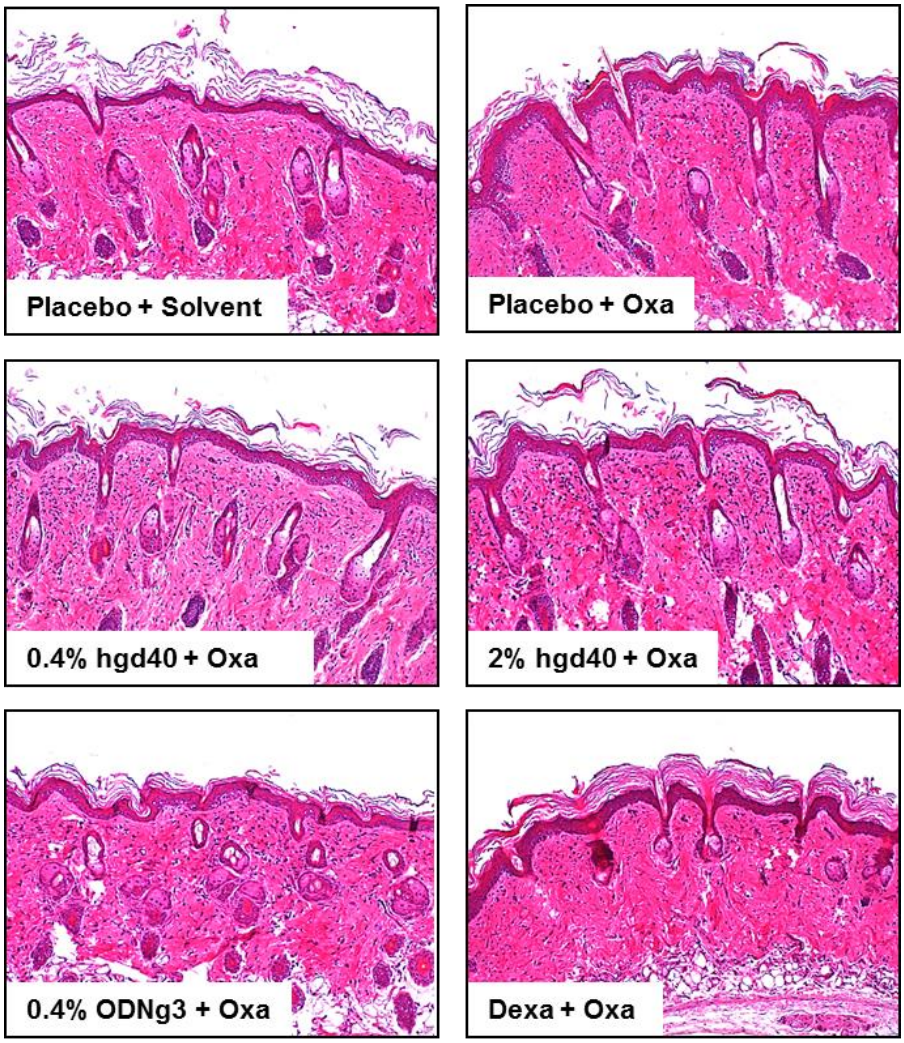

Figure 13. Prophylactic treatment with hgd40 had no significant effect on dermal cell infiltration.

Skin samples were collected on day (+3) and sections from different treatment groups were stained with H&E and examined at 100x magnification. All oxazolone-challenged groups showed comparable epidermal thickening and cellular infiltration except the dexamethasone-treated group. Images are representative for 3 separate experiments.

The inflammatory cell infiltrate in ACD is known to be rich in mononuclear cells including, neutrophils (Zollner et al. 2004) and in some models eosinophils have also been reported to infiltrate the inflamed skin (Kondo et al. 1998). Both $CD4^+$ and $CD8^+$ T lymphocytes play a major role in the development of contact hypersensitivity and cannot be distinguished by H&E staining. $CD4^+$ Th2 cells, in particular, are of great interest in this model. They express high levels of GATA3 and are targeted by hgd40 (Turowska unpublished data). Therefore, it was important to quantify $CD4^+$ T cells in the inflamed skin and examine their contribution to the pool of cells infiltrating the skin as well as the effects of hgd40 treatment on their recruitment to the inflamed skin.

Immunohistochemical staining for CD4 was performed on PFA-fixed skin sections using polyclonal rabbit anti-CD4 and a DAB detection system as described in (2.7.2).

Quantification of CD4 cells was achieved by counting positively-stained cells in the dermis and basal layer of the epidermis.

Data indicated only a slight increase in the number of $CD4^+$ T cells infiltrating the dermis after oxazolone challenge (Fig. 14A). Treatment with 0.4% hgd40 reduced the number of infiltrating $CD4^+$ T cells by about 25%. Furthermore, groups treated with a higher concentration of hgd40 showed a more significant decrease with a 30% reduction compared to placebo. Treatment with the control DNAzyme (ODNg3) had almost no effect on the numbers of CD4 cells infiltrating the skin. As expected, dexamethasone treatment had a prominent effect and reduced the numbers by about 48% compared to placebo (Fig. 14B).

3.2.1.3 Effects of DNAzyme treatment on the number of GATA3 expressing cells in the dermis

$CD4^+$ T helper cells are not the only cells expressing GATA3 in the skin. Other cells involved in the allergic inflammatory response including, basophils, eosinophils and keratinocytes also express GATA3 and can be affected by hgd40 treatment.

To examine how GATA3 protein expression in the skin was affected, immunohistochemical staining was performed as previously described (2.7.2).

Epidermal keratinocytes express GATA3 and are the first to come in contact with topically applied therapeutic reagents. The expression of GATA3 in keratinocytes was not altered in inflamed skin compared to normal solvent–challenged skin. It also remained stable after treatment with any of the specific or control DNAzymes as well as dexamethasone (Fig. 15A).

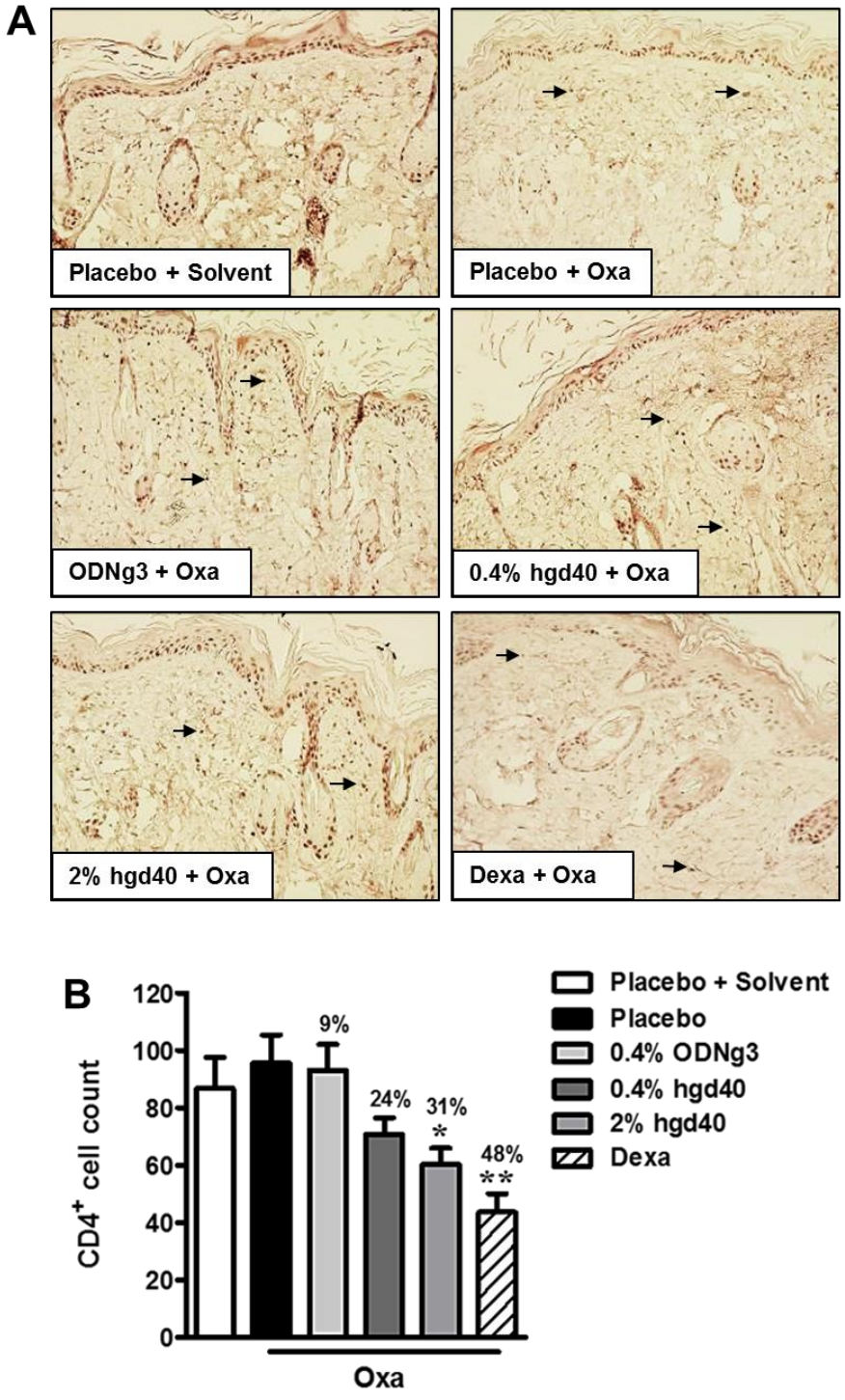

Figure 14. Prophylactic treatment with hgd40 reduced the number of CD4$^+$ cells to baseline level.

(A) Skin sections were stained for CD4 after antigen retrieval and detected using a DAB detection system (2.7.2). Images are taken at a 100x magnification and are representative for 2 independent experiments. **(B)** Graphical representation of CD4 immunohistochemical staining averaged from 3 fields per mouse and 8 mice per group. Data are cumulative from 2 independent experiments are shown as mean ± SEM. Significant differences are marked *p ≤ 0.05 and **p ≤ 0.01 compared to placebo. The numbers above the bars represent the percent of reduction in the number of CD4$^+$ cells compared to the placebo group.

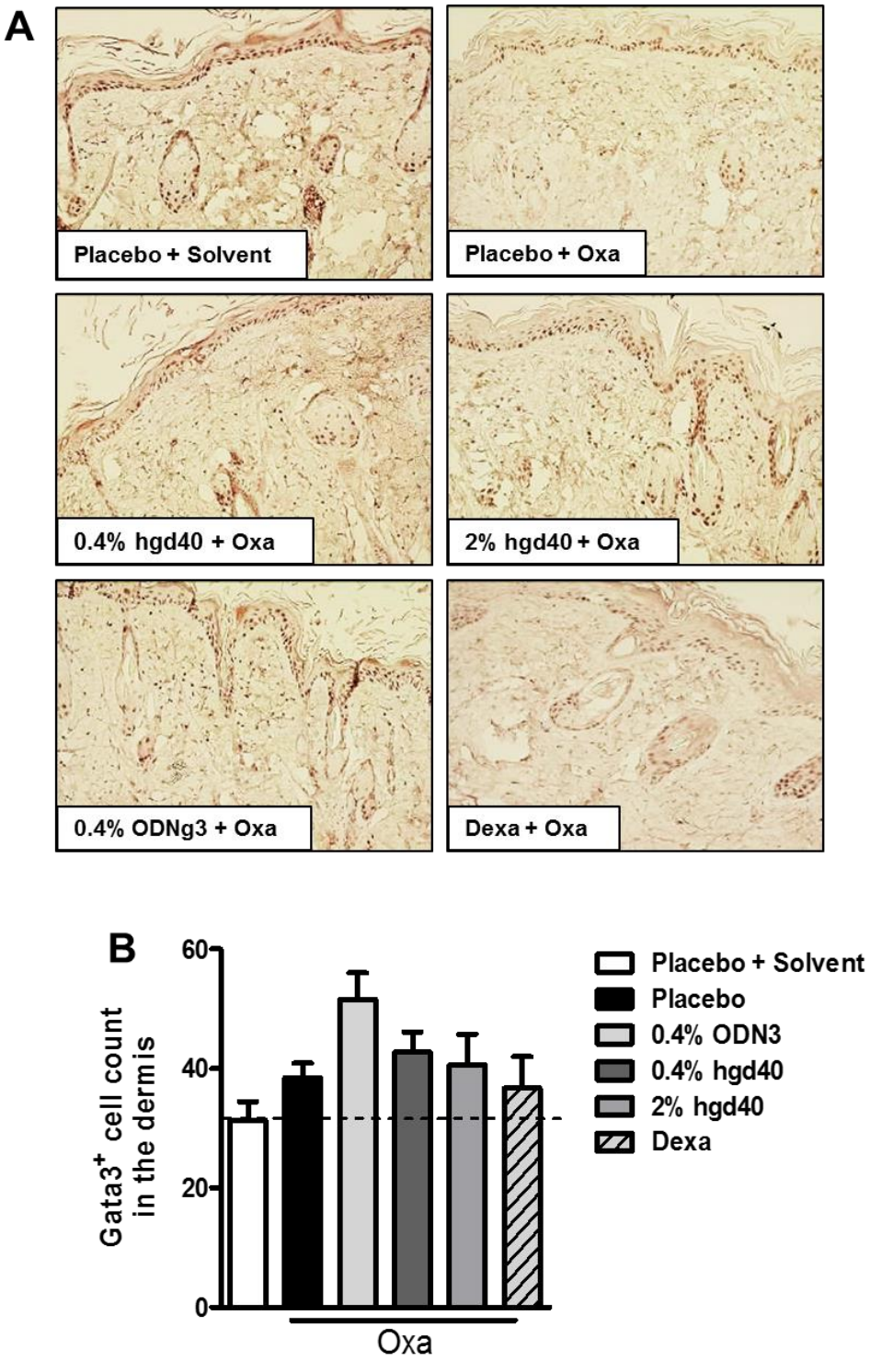

Figure 15. GATA3 protein expression in epidermal keratinocytes and dermal immune cells was not affected after prophylactic treatment with hgd40.
(A) GATA3 immunohistochemical staining was performed as described in (2.7.2) and sections were examined at a 100x magnification. **(B)** Graphical representation of $GATA3^{+}$ cells infiltrating the dermis averaged from stained cells counted in 3 fields of sight per mouse. Data are compiled from 2 separate experiments each with 8 mice per group and are shown as mean ± SEM.

3.2.1.4 Effects of DNAzyme treatment on the allergic phenotype

We showed earlier that skin injury and oxazolone challenge induced an increase in the mRNA expression of the Th2 transcription factor GATA3 and its main cytokine IL-4 in the inflamed skin (Fig. 11). To investigate the effects of different treatments and particularly GATA3-specific DNAzyme on this phenotype, RNA was isolated form skin samples as described in (2.6.1) and RT-PCR for the main Th1 and Th2 transcription factors and cytokines was performed (2.6.5).

There was no detectable increase in the levels of GATA3 mRNA expression three days after oxazolone challenge. The expression was similar in both oxazolone- and solvent-challenged groups. It also remained at the same levels in all different treatment groups, including both GATA3-specifc DNAzymes. However, the expression of IL-4 was significantly induced in response to oxazolone challenge. This increase in the IL-4 mRNA expression was significantly impaired after dexamethasone treatment, while groups treated with either the control DNAzyme or any of the different doses of hgd40 showed no reduction in the levels of IL-4 mRNA.

There was a tendency of increased Tbet expression in the oxazolone-challenged skin upon treatment with the w/o/w emulsion irrespective of the DNAzyme. In the dexamethasone group, however, Tbet expression was downregulated to levels lower than those in the solvent group. Still, all of these changes did not reach statistical significance.

Similarly, the mRNA expression of the main Th1 cytokine, IFNγ, was elevated at this time point following oxazolone challenge. It remained at comparable levels in all treatment groups, except for the dexamethasone group where IFNγ expression was significantly downregulated (Fig. 16).

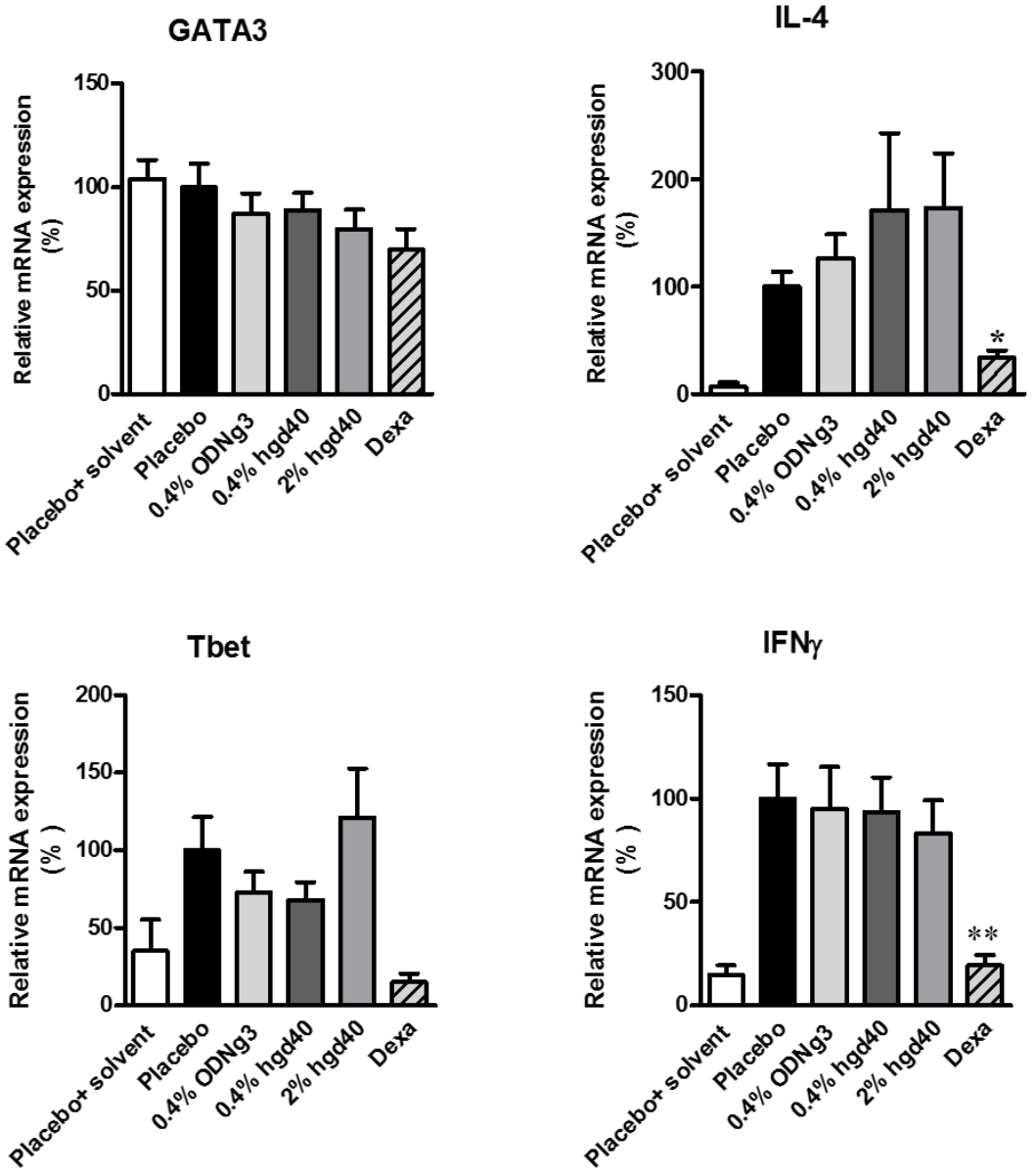

Figure 16. No changes in the levels of GATA3 and IL-4 mRNA expression after prophylactic treatment with hgd40.

RNA was isolated from skin samples collected on day 3 after challenge. mRNA expression was quantified using RT-PCR (2.6.5) after normalization to the house keeping gene L32 and a reference sample. Data are compiled from 3 independent experiments (n=6-8 per group/experiment) and are shown as mean ± SEM. Significant differences are marked *$p \leq 0.05$, **$p \leq 0.01$ (compared to placebo).

GATA3 mRNA is directly targeted by hgd40, and a reduction in its expression is expected in such a specific therapy. Improvement of skin swelling was observed on days 2 and 3 after challenge, yet no reduction in GATA3 mRNA or protein expression was identified

in samples collected three days after challenge. GATA3 is expressed normally in the skin and its levels are usually elevated during the inflammatory process. In these experiments treatment started prior to contact with oxazolone meaning before the induction of an inflammatory response. Since the skin was exposed to GATA3-DNAzyme both before and after challenge, we wanted to investigate if GATA3 was downregulated at time points earlier to those in which the effect on skin swelling was observed. For this reason, mice were sensitized and challenged as described before (see Fig. 8A) and similarly treated in a prophylactic manner with either placebo or GATA3-specific DNAzyme. Skin samples were collected at different time points (6 h and 24 h after sensitization and 24 h, 48 h, 72 h after challenge) and the expression of GATA3-mRNA was quantified with RT-PCR.

Analysis of all sampling time points revealed that differences in the GATA3 mRNA expression were only measurable 6 hours after sensitization. At this time point, GATA3 expression was significantly downregulated in the group receiving hgd40 treatment compared to placebo (Fig. 17).

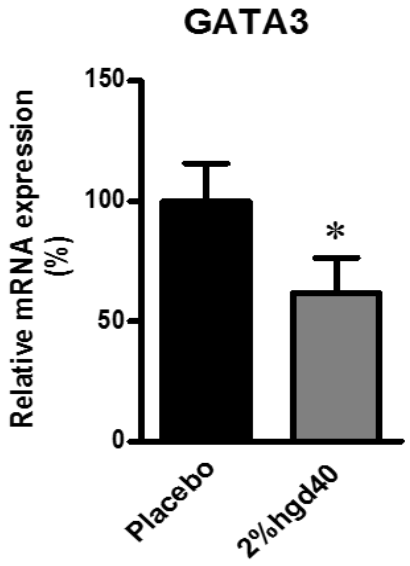

Figure 17. hgd40 reduced GATA3 mRNA expression in the skin early in the sensitization phase.

Mice were sensitized with oxazolone after prophylactic treatment with hgd40 or placebo and skin samples were collected 6 hours after sensitization. mRNA expression was quantified with RT-PCR after normalization to L32 and a reference sample. Data are compiled from 2 separate experiments and are shown as mean ± SEM. Significant differences are marked * $p \leq 0.05$.

3.2.2 Effects of semi-therapeutic treatment with GATA3-specific DNAzyme on oxazolone-induced dermatitis

Semi-therapeutic treatment refers to a type of treatment in which the first topical treatment was performed prior to challenge leaving the sensitization phase unaffected.

BALB/c mice were sensitized by epicutaneous application of oxazolone to the shaved back and challenged three days later with oxazolone at the same site. A control group was sensitized and challenged with solvent only. The first treatment took place 24 h before oxazolone challenge and was continued by single daily applications (Fig. 8B).

Mice were treated by topical application of 50 µl of one of the w/o/w emulsion containing either 0.4% or 2% hgd40 (GATA3- specific DNAzyme), 0.4% or 2% ODNg3 (control DNAzyme), dexamethasone or no active ingredient (placebo). A detailed description of the different groups included in the experiments can be found in table 2 section (2.3.2).

3.2.2.1 Effects of DNAzyme treatment on skin swelling

As mentioned earlier, inflamed skin becomes swollen and skin thickness measurement is used to determine disease progression. There was an acute increase of skin thickness in the first 24 h following oxazolone challenge. This increase in skin thickness remained on days 2 and 3 in the placebo group and to a comparable extent in the ODNg3 groups independent of the dose. The skin was less swollen in the group treated with 0.4% hgd40 after oxazolone challenge and was significantly reduced on day 3 compared to placebo but not to 0.4% ODNg3.

In the 2% hgd40 treated group, there was a reduction in skin thickness on days 2 and 3 following the acute increase observed on day 1. This decrease was significant compared to both placebo and the 2% ODNg3. Dexamethasone, on the other hand, not only prohibited oxazolone-induced skin swelling but also caused thinning of the skin with measurements showing values lower than those in the solvent-challenged group (Fig. 18).

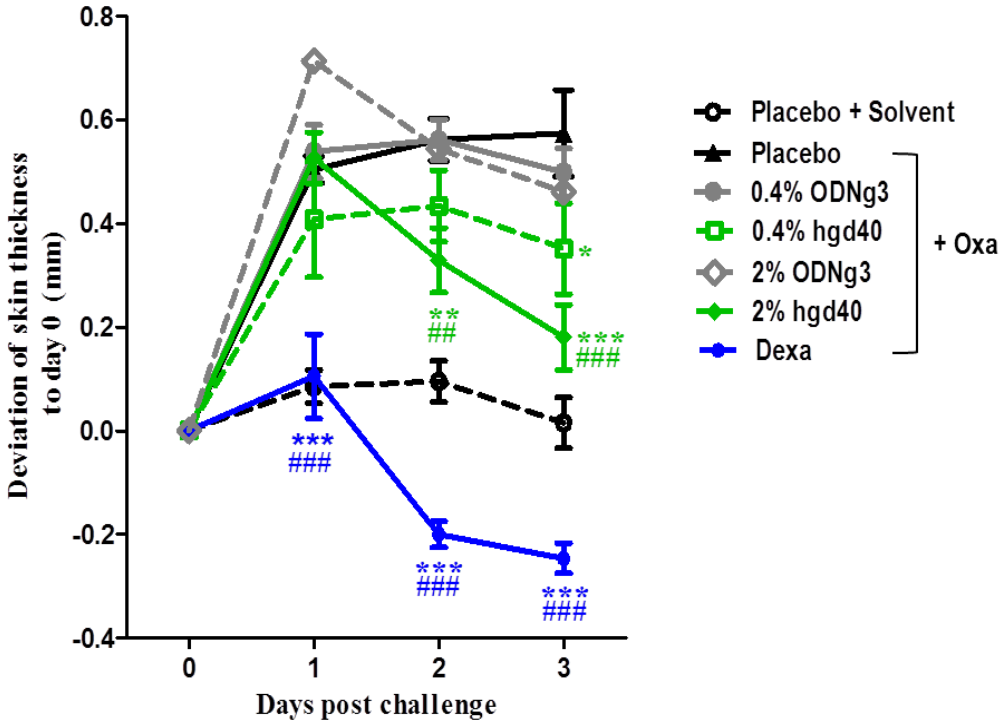

Figure 18. hgd40 reduced skin thickness in a dose dependent manner after semi-therapeutic treatment.

Mice were sensitized and challenged with oxazolone and treated once daily starting on day -1 with different emulsions (2.3.2). Skin thickness was measured on days 0, 1, 2, and 3 and deviation to day 0 was calculated. Data are compiled from 2 separate experiments each with 8 mice per group. Significant differences are marked *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (compared to placebo), ##$p \leq 0.01$ and ###$p \leq 0.001$(compared to ODNg3).

3.2.2.2 Effects of DNAzyme treatment on cellular infiltration

Examination of skin sections stained with H&E revealed the characteristic dermal infiltrate upon oxazolone challenge. This infiltrate has been described in the literature to consist of monocytes, neutrophils and eosinophils (Zhang, Tinkle 2000, Kondo et al. 1998). The density of the cellular infiltrate was comparable in all oxazolone-challenged groups with no visible treatment-caused suppression of cellular influx in any of the DNAzyme-treated groups. In the group treated with dexamethasone this influx was slightly reduced (Fig. 19).

Quantification of $CD4^+$ cells in the skin after oxazolone challenge and semi-therapeutic treatment using immunohistochemical staining showed no differences between the different treatment groups (data not shown).

Similarly, immunohistochemical analysis of GATA3 in the skin sections was similar to that observed in the prophylactic treatment with no significant differences in the numbers of $GATA3^+$ cells infiltrating the dermis (data not shown).

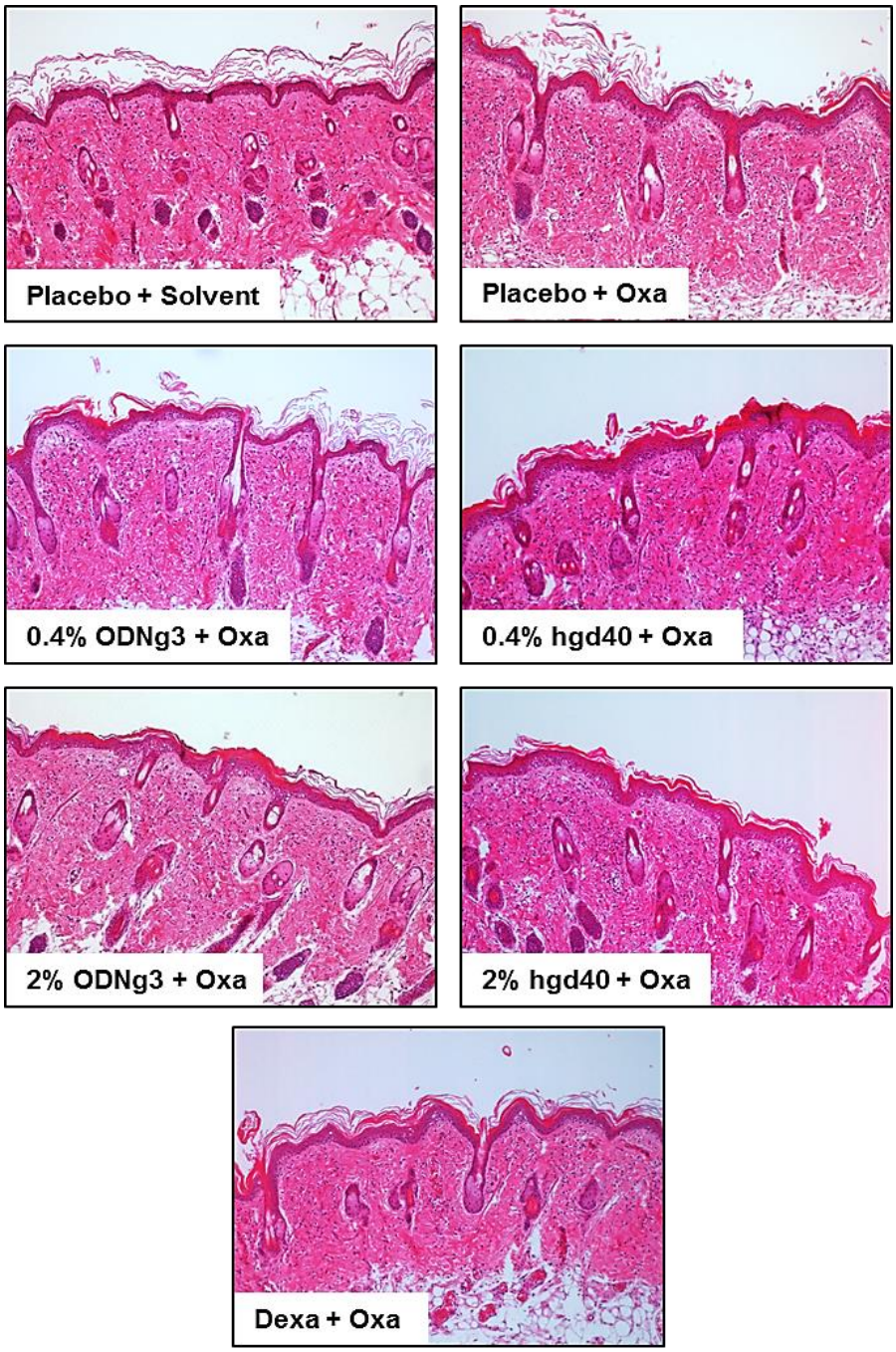

Figure 19. hgd40 treatment did not alter the density of cellular infiltrate in the dermis.

Skin sample were collected on day 3 and skin sections were stained with H&E and examined at 100x magnification.

3.2.2.3 Effects of DNAzyme treatment on mRNA expression

The changes in the mRNA expression of the Th2 phenotype profile was analysed using quantitative RT-PCR. Similarly, to the findings after prophylactic treatment, there was no detectable induction of GATA3 expression in the skin 72 h after challenge with oxazolone and subsequentially no changes in these levels after hgd40 treatment. Yet, in the dexamethasone-treated group, the levels of GATA3 expression were significantly lower than those measured in the solvent group.

IL-4 expression, although significantly upregulated after oxazolone challenge, was also reduced after dexamethasone treatment but not by any of the hgd40 treatments.

Moreover, the expression of both Tbet and IFNγ was elevated after oxazolone challenge. This was evident in all groups including the negative controls (placebo and ODNg3). In the dexamethasone group, the levels of Tbet and IFNγ were only slightly reduced compared to placebo (Fig. 20).

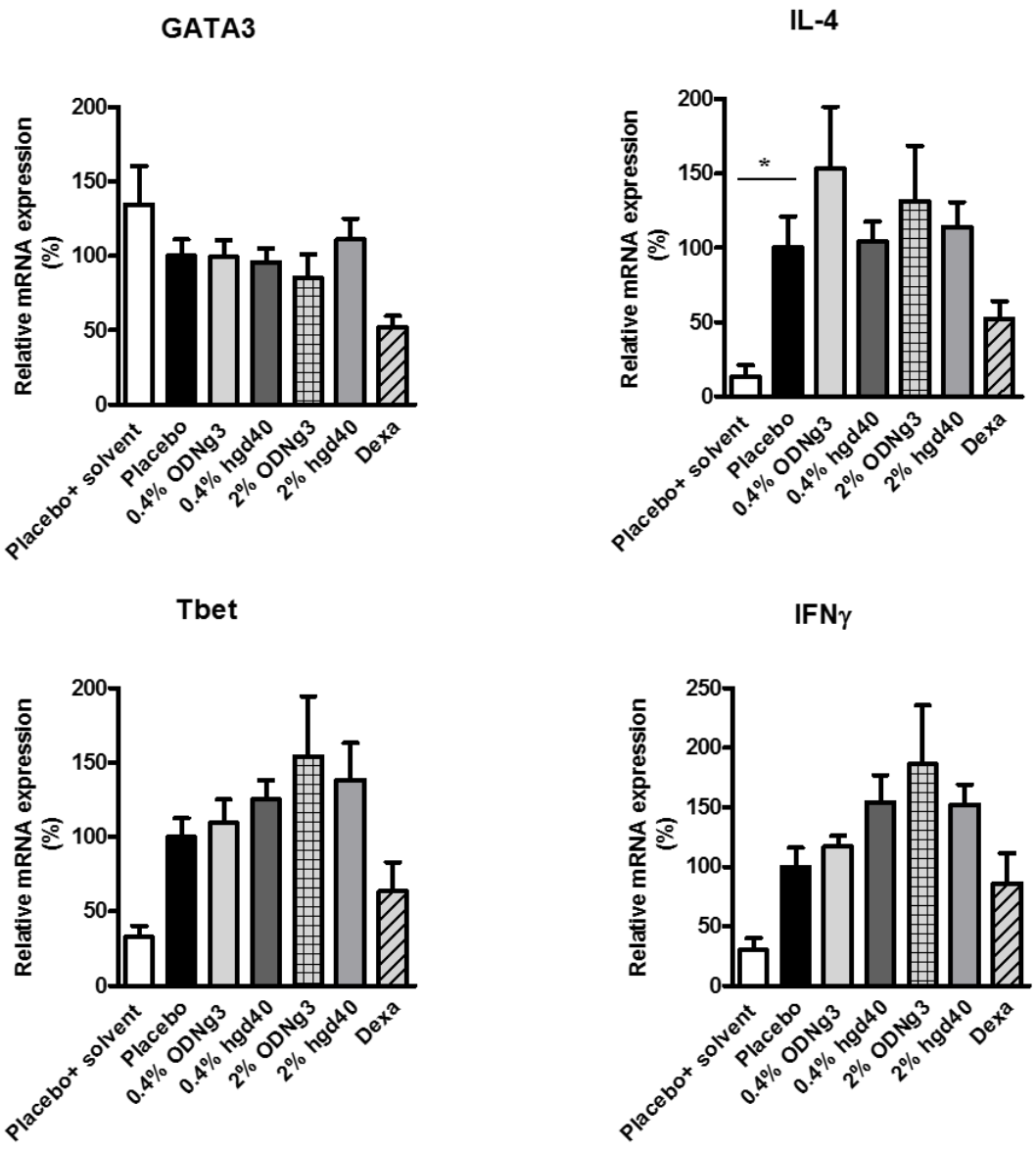

Figure 20. Semi-therapeutic treatment with hgd40 did not affect the mRNA expression of the main Th- associated transcription factors and cytokines.

mRNA expression was quantified using RT-PCR normalized to the house keeping gene L32 and a reference sample. Data are compiled from 2 separate experiments, each with 8 mice per group, and are shown as mean ± SEM. Significant differences are marked *p ≤ 0.05.

3.3 Effects of Tbet-specific DNAzyme (td32) in an animal model of OVA/CFA-induced dermatitis

The goal of this part of the project was to investigate the effects of a Tbet-specific DNAzyme treatment on the progression of a Th1-driven skin inflammation.

Previously established murine models of skin inflammations combined OVA and the adjuvant complete Freund's adjuvant (CFA) for sensitization to skew a Th1 immune response after challenge with OVA (Cho et al. 2001). This published model was used as a base for this study with some additional alterations in the mouse strain as well as the challenge process.

In this study and as described in (2.2.2), OVA-TCR transgenic mice (DO11.10) were subcutaneously sensitized with OVA emulsified with CFA and one week later challenged on three consecutive days by epicutaneous application of OVA to the shaved back skin (Fig. 7B). 90% of the $CD4^+$ cells in the DO11.10 mice carry the OVA-specific T cell receptor and rapidly react to OVA upon challenge. The Complete Freund's adjuvant drives the CD4 inflammatory response towards a Th1 phenotype thus providing a suitable model for targeting Tbet, the major transcription factor of Th1 driven immune responses.

Tbet-specific DNAzyme, named td32, have been established and was formulated in a w/o/w emulsion that provides protection and delivery of these molecules after topical application. In the following experiments, the effects of preventive treatment with Tbet-specific DNAzyme was assessed in comparison to either placebo, a control DNAzyme (ODNg3) with non-specific binding arms or a GATA3-specific DNAzyme.

Skin inflammation was elicited as described earlier and mice were treated by topical application of 50 µl of either one of the different emulsions twice a day, starting one day before challenge (2.3.3). The emulsions were topically applied to the back skin with 12h intervals, and gently massaged to help release the active ingredient from the inner phase. Table 3 in section (2.3.3) highlights the different treatment groups included in the experiments discussed below.

Skin inflammation was assessed *in vivo* by daily measurements of skin thickness while samples for *ex vivo* analysis were collected from euthanized mice 4 days after the first OVA challenge.

3.3.1 Effects of Tbet-specific DNAzyme treatment on skin swelling

During each experiment, the swelling resulting from the inflammatory reaction was monitored by daily measurements of skin thickness. Cumulative data of several experiments showed an acute increase in skin thickness in the two days after the first OVA-challenge that pesisted until termination day. Compared to placebo, treatment with the murine Tbet-specific DNAzyme td32 seemed to slightly decrease the acute reaction within the first 24 h. It also continuously and significantly reduced skin swelling by days 3 and 4 (Fig. 21A).

To investigate whether these results were due to specific targeting of Tbet or were induced by unspecific immune alterations caused by application of synthetic deoxyoligonucleotides, the effect detected in the Tbet DNAzyme group was compared to that of the control DNAzyme (ODNg3). As expected, the reduction in skin thickness was only observed after treatment with Tbet DNAzyme and not with the control DNAzyme ODNg3. The differences between these two groups were significant throughout the experiment following OVA-challenge (Fig. 21B).

The immune response in the OVA/CFA model is highly skewed towards Th1 with an expected minimum contribution of Th2 cells. To verify the specificity of both the model as a Th1-dominant and the Tbet DNAzyme treatment, another group treated with hgd40 was analysed in parallel to the placebo and td32-treated groups in one of the experiments. Again, OVA challenge induced a strong immune reaction that was reflected by the acute increase of skin thickness, while no improvement was noted after hgd40 or placebo treatment, Tbet DNAzyme treatment, on the other hand, significantly reduced skin thickness 4 days after the first challenge with OVA (Fig. 21C).

In the previous experiments, a dexamethasone treated group was also included. However, it was used in the commercially available formula, not the w/o/w formula, which was not tolerated and caused a damage to the skin of mice. For this reason, this group was excluded from all analyses.

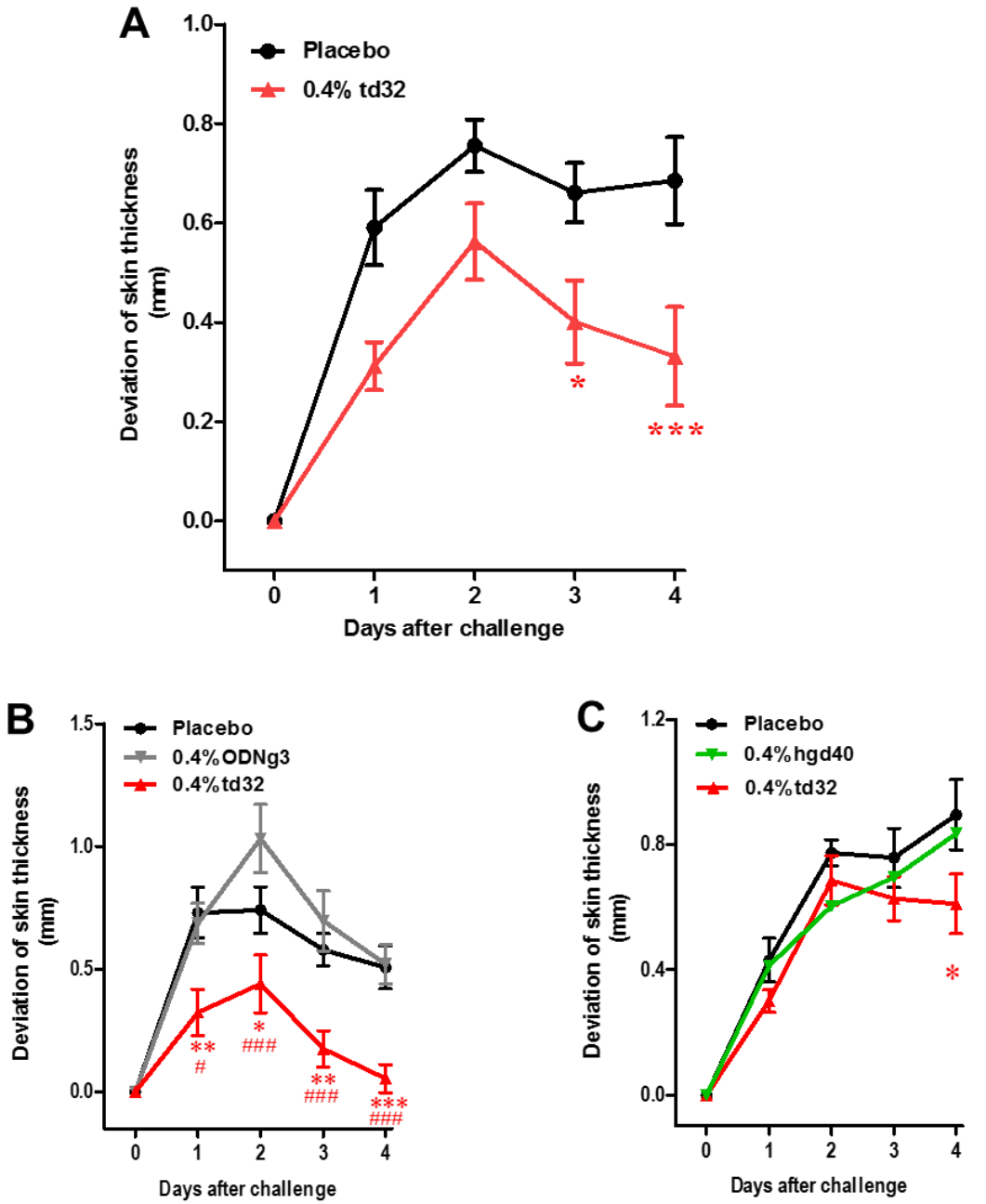

Figure 21. td32 treatment reduced OVA-induced skin swelling.

OVA-TCR tg mice were sensitized with OVA/CFA (s.c.) and seven days later challenged on the back skin with OVA on three consecutive days (2.2.2). Treatment started 12 h before challenge and continued twice daily until day 3 (2.3.3). Skin thickness was measured on a daily basis and differences to day 0 were calculated. Data shown are either combined from 2 independent experiments (A) or represent single experiments with n=6 (B and C). Significant differences are marked, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (compared to placebo), and #p ≤ 0.05, ###p ≤ 0.001(compared to ODNg3).

3.3.2 Effects of the Tbet DNAzyme on inflammatory cell influx

The site of inflammation was optically characterized by the formation of psoriatic plaques. Moreover, examination of skin sections stained with H&E revealed all features of a strong inflammatory response characterized by substantial epidermal hyperproliferation, edema formation and a dense infiltration of immune cells into the site of challenge (Fig. 22).

The density of cells infiltrating the dermis was comparable between the different treatment groups. Moreover, no prominent reduction in the epidermal or dermal hyperproliferation was noted in the Tbet-DNAzyme–treated group compared to any of the other controls.

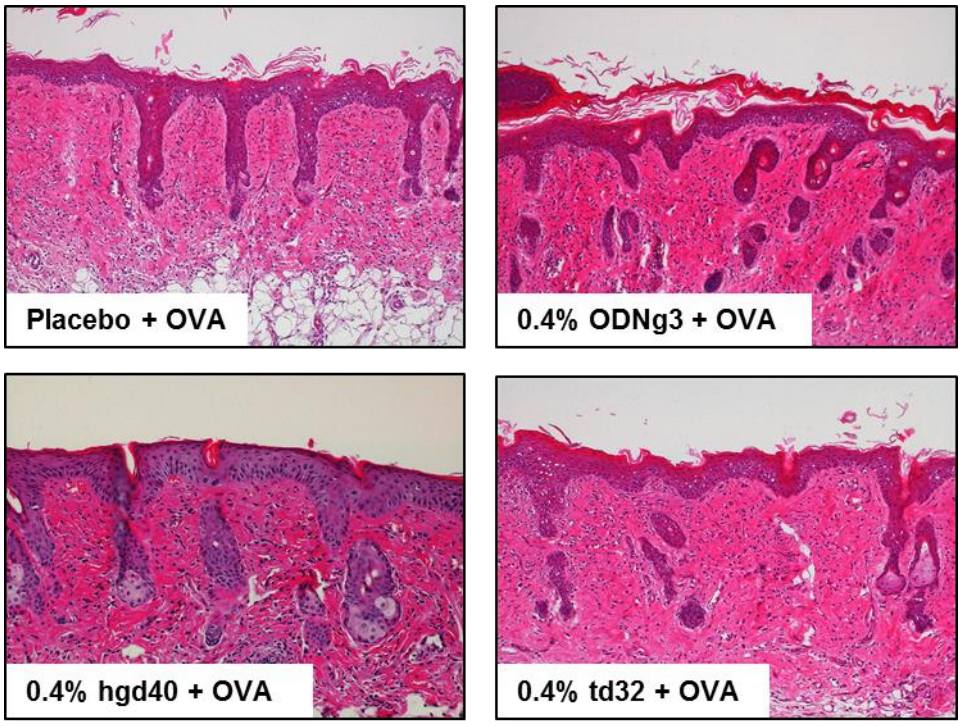

Figure 22. The density of infiltrating cells in response to OVA challenge remained unaffected by DNAzyme treatment.

OVA-TCR tg mice were sensitized with OVA/CFA (s.c.) and seven days later challenged on the back skin with OVA on three consecutive days (2.2.2). Treatment started 12 h before challenge and continued twice daily until day 3 (2.3.3). Skin samples were collected on day 4 and sections were analysed after H&E staining at a 100x magnification.

3.3.3 Effects of preventive treatment on Tbet and IFNγ mRNA expression

Unlike GATA3, Tbet is not expressed by structural cells but mainly in innate and adaptive immune cells including DCs, ILC1 (including NK cells) as well as in $CD8^+$ and Th1 $CD4^+$ cells. All of these cells, particularly Th1 and cytotoxic CD8 cells, contribute to the inflammatory response in OVA/CFA-induced dermatitis and might be targeted by Tbet-specific DNAzyme treatment. In addition, Tbet is a major regulator of IFNγ production and any interference with Tbet may affect the level of IFNγ as well. To investigate the changes in both Tbet and IFNγ expression in the skin, RNA was isolated from skin samples of the different treatment groups, reverse transcribed (2.6.4) and analysed by RT-PCR as described in (2.6.5). The levels of Tbet mRNA were comparable in the groups treated with placebo, the control DNAzyme ODNg3 and Tbet-DNAzyme with no significant differences detectable. IFNγ expression, on the other hand was lower in both DNAzyme groups compared to placebo with a more prominent reduction in the td32-treated group (Fig. 23)

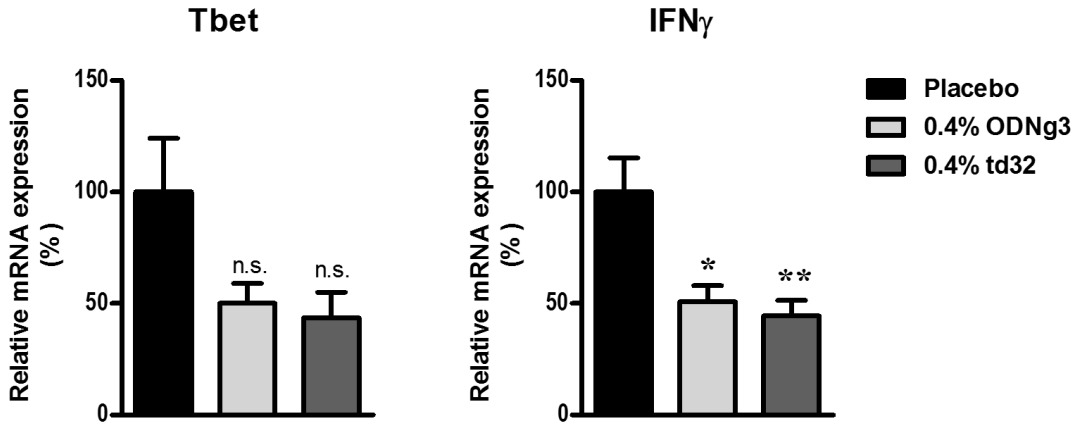

Figure 23. Effects of preventive treatment with Tbet-specific DNAzyme on Th1-related genes.

OVA-TCR tg mice were sensitized with OVA/CFA and challenged with OVA as described in (2.2.2). Emulsions were applied to the skin twice daily starting on day -1 (2.3.3). RNA was isolated from skin samples collected on day 4 and mRNA expression was quantified using RT-PCR and normalized to the house keeping gene L32 and a reference sample as described in (2.6.5). Data are shown as mean ± SEM with n= 6 and significant differences are marked *$p \leq 0.05$, **$p \leq 0.01$ compared to placebo.

Similar tendencies were observed when the GATA3-specific DNAzyme was used as control instead of ODNg3. Preventive treatment with any of the emulsions had no effect on the mRNA expression of Tbet and only a very little effect on the Th1 cytokine IFNγ (Fig. 24)

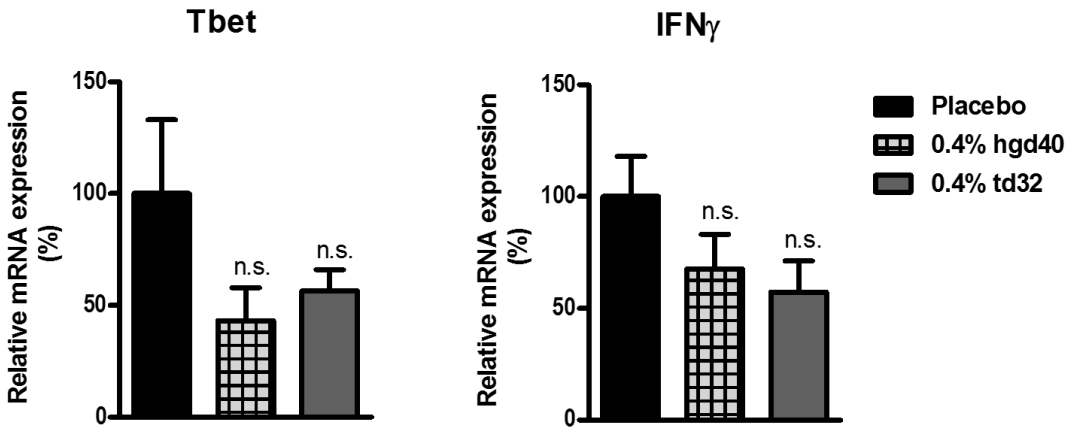

Figure 24. Effects of preventive treatment with a Tbet-specific DNAzyme (td32) and a GATA3-specific DNAzyme (hgd40) on Th1-related genes.

OVA-TCR tg mice were sensitized with OVA/CFA and challenged with OVA as described in (2.2.2). Emulsions were applied to the skin twice daily starting on day -1 (2.3.3). RNA was isolated from skin samples collected on day 4 and mRNA expression was quantified using RT-PCR and normalized to the house keeping gene L32 and reference sample (2.6.5). Data are shown as mean ± SEM with n= 6.

Although Tbet DNAzyme-treatment significantly and specifically reduced skin swelling compared to placebo and control DNAzymes, Tbet expression was not affected when measured at 96h after the first challenge. The slight reduction in IFNγ expression after DNAzyme treatment (both Tbet and the control DNAzyme ODNg3) can be neglected since the differences in the ΔCt values were minimal (0.5-1.5 cycle) and within the range of inner assay variance. This combined with low sample numbers indicates the low physiological relevance of the changes detected. Treatment with the GATA3-specific DNAzyme had no effect on skin swelling or the expression of Th1-related genes Tbet and IFNγ in the OVA/CFA model.

4 DISCUSSION

Atopic dermatitis and psoriasis vulgaris are common chronic, progressive inflammatory skin diseases that have been described as polar Th2 verses Th1 diseases (Fonacier et al. 2010). The transcription factor GATA3 is an essential factor in allergic inflammation through regulating the expression of IL-4, IL-5 and Il-13 and subsequently IgE class switching and eosinophilia, all features of AD (Fiset et al. 2006). GATA3 expression is increased in AD and decreased in psoriasis (Arakawa et al. 2004, Rácz et al. 2011). Tbet, on the other hand, controls the Th1 migratory program and IFNγ production and is upregulated in psoriatic skin lesions and PBMCs isolated from psoriatic patients (Odell, Cook 2013, Zhang et al. 2014). GATA3 and Tbet play a central role in the development and maintenance of AD and psoriasis, respectively. For this reason, targeting these two transcription factors on mRNA level by specific DNAzymes may provide a new therapeutic approach for both diseases. This is particularly important since current treatment regimens are mostly dependent on Glucocorticoids, which are associated with a wide range of side effects especially in case of long-term use (Schoepe et al. 2006, Schäcke et al. 2002).

The DNAzymes used in this work have been previously tested *in vitro* and showed no off target effects (Dicke et al. 2012) . The efficacy of GATA3-specific DNAzyme (hgd40) was intensively studied both *in vitro* and in animal models of allergic airway inflammation in earlier work (Sel et al. 2008, Turowska et al. 2013).

In the current study, we were able to (1) develop an animal model of oxazolone-induced dermatitis with a predominant Th2 phenotype (2) show an improvement in the clinical symptoms of oxazolone-induced dermatitis after prophylactic and semi-therapeutic topical treatment with a GATA3-specific DNAzyme (hgd40) (3) develop an animal model of OVA-induced dermatitis with a predominant Th1 phenotype (4) show a reduction in the clinical manifestations of skin inflammation by topical treatment with a Tbet-specific DNAzyme in an animal model of OVA/CFA dermatitis.

4.1 Induction of a predominant Th2 allergic skin inflammation by skin injury and oxazolone

The immune response in AD is characterized by a Th2 cell-dominant inflammatory infiltration, increased expression of Th2 cytokines and elevated levels of IgE. Several animal models that show certain features of AD have been developed. These include

the use of either mutant mice, such as the Nc/Nga that spontaneously develop AD-like skin lesions (Aioi et al. 2001), or transgenic mice overexpressing certain cytokines like IL-4 or TSLP (Yoo 2005). However, all these artificial systems do not reflect the complexity of AD which is the result of different genetic and environmental factors rather than a defect in a single gene.

In a more generalized model, tape stripping and repeated epicutaneous challenge with the protein allergen OVA was used to elicit an AD-like skin inflammation (Spergel et al. 1999). In this published model, skin sensitization was achieved by the placement of OVA on a patch, which was then secured to the skin for a week. In other models, multiple challenges with haptens like TNCB and oxazolone were used to induce a chronic Th2-like hypersensitivity reaction in the skin (Matsumoto et al. 2004, Man et al. 2007). Despite the importance of these models for the understanding of AD- pathogenesis, they are less convenient for evaluating potential topical therapies. For instance, the placement of the patch on the skin in the OVA model hinders topical application at this site. Even if the patch were to be temporary removed for topical treatment, the prolonged sensitization/challenge required for this model raises the risk of an interaction between treatment agents and the antigen. It could inhibit or alters antigen penetration into the skin creating misleading results. On the other hand, the extremely severe inflammation caused by repeated hapten challenge makes any topical treatment more likely to be insufficient.

In this study, we were able to establish an acute animal model for allergic skin inflammation with a prominent Th2 phenotype by combining skin injury and a single oxazolone challenge.

Haptens are commonly used in allergic contact dermatitis (or contact hypersensitivity) models. Most haptens like TNCB and DNFB induce a Th1 contact hypersensitivity response after a single challenge. Yet, there are contradictory data about the phenotype induced by a single oxazolone challenge. While some reported a Th1-dominant response (Webb et al. 1998), others described a mixed Th1/Th2 phenotype (Thomson et al. 1993). More recently, it was shown that $CD4^+$ T cells infiltrating the skin after oxazolone challenge express the chemokine receptor CCR4, which is expressed on Th2 cells (Kusumoto et al. 2007).

Our data revealed a significant upregulation in the mRNA expression of the Th2 transcription factor GATA3 and main Th2 cytokine IL-4 in tape stripped-oxazolone-challenged skin. Similar observations were reported following the application of picryl

chloride (also known as TNCB) to tape stripped skin (Kondo et al. 1998). Recently Oyoshi et al. (Oyoshi et al. 2010) showed that mechanical injury drives skin dendritic cells to polarize T cells towards Th2 through the upregulation of TSLP expression by keratinocytes. This may explain the Th2-dominant response in our oxazolone model, where skin injury was inflicted by tape stripping before antigen application. Despite the predominant Th2 phenotype, the prominent increase in IFNγ expression, although not significant, suggests that Th1 cells and/or other IFNγ-producing cells also contribute to the inflammatory response.

Local cutaneous inflammation was characterized by edema formation, which was reflected in a progressive thickening of the skin throughout the experiment, mild epidermal hyperplasia and cellular infiltration.

The cellular infiltrate in CHS has been widely described in the literature. In a single oxazolone-challenge model this infiltrate was found to contain mostly mast cells and T lymphocytes (Man et al. 2007), while eosinophils have been reported to infiltrate the skin after hapten challenge in barrier disrupted skin (Kondo et al. 1998). The irritant effect resulting from mechanical injury as well as the chemical irritation by oxazolone in our model can drive the accumulation of neutrophils in the dermis, which in turn increases the levels of IFNγ in the skin. Neutrophils are recruited to the skin 24 h after skin injury and were reported to have a critical role in the Th2-driven allergic inflammation in the OVA model of AD (Oyoshi et al. 2012).

The animal model presented in this study is a mixed contact hypersensitivity reaction with a Th2 predominant phenotype and bears many clinical, immunological and histological similarities to AD. Its reproducibility and rapidity makes it a useful tool in evaluating both the pathogenic mechanisms and potential topical therapies such as the GATA3-specific DNAzyme treatment.

4.2 Effects of GATA3-specific DNAzymes on oxazolone-induced dermatitis

Intensive work has been done to study the effects and safety of the GATA3-specific DNAzyme (hgd40) in experimental models of allergic airway inflammation (Sel et al. 2008, Fuhst et al. 2012, Turowska et al. 2013). Findings from those studies suggest a promising potential for the GATA3-specific DNAzyme as future therapy. The similarities between asthma and AD in terms of immunopathogenesis and phenotype make it

interesting to investigate whether these DNAzymes might also be effective in AD-like skin conditions and thus may show comparable results in an animal model of oxazolone-induced dermatitis.

The data presented in this study show improvement in the clinical outcomes of oxazolone-induced skin inflammation following topical treatment with hgd40. The preventive approaches used included two different regimens: a “prophylactic” in which the treatment was introduced before sensitization and challenge, and a “semi therapeutic” where treatment started before challenge and did not interfere with the sensitization phase.

In both preventive setups, hgd40 treatment resulted in a significant reduction of skin thickness by days 2 and 3 post challenge but had no or very little effect on skin swelling within the first 24 h following challenge.

During the sensitization phase, oxazolone-specific T cells are generated and some of these cells reside in the skin and can rapidly react to a second encounter with oxazolone. However, the irritant effect of oxazolone first activates innate immune mechanisms and leads to the accumulation of innate immune cells including mast cells, macrophages and neutrophils in the skin. These cells along with keratinocytes secrete pro-inflammatory cytokines (IL-1α, IL-1β, and TNFα) that attract more cells and change vascular permeability to eventually result in the acute skin swelling measured at 24 h post challenge. We found that among all treatment groups it was only dexamethasone that suppressed this acute innate response at 24 h. This may be expected since almost all of the cells present in the skin express the GC receptor and prophylactic treatment with dexamethasone would suppress the transcription of pro-inflammatory genes in these cells and reduce the inflammatory process. GATA3 is also expressed by keratinocytes and some of the innate immune cells (like NK cells and ILC2) but is most prominent in T cells which are first recruited to the skin 24 h after challenge. The strong innate immune response overpowers the role of GATA3 in the inflammatory process during the first 24 h post challenge and can explain why the hgd40 treatment did not inhibit the primary irritant skin swelling.

Nevertheless, GATA3-specific DNAzyme treatment was sufficient to reduce skin swelling by days 2 and 3 following provocation with oxazolone, an effect that was both specific and dose dependent. Based on skin thickness measurements, topical application of 0.4% and 2% hgd40 improved the skin swelling while no effect was noted after treatment with similar concentrations of the control DNAyzme or placebo. Moreover, it was evident

that in both preventive treatments the improvement was more prominent when the higher dose 2% of the GATA3-specific DNAyzme (hgd40) was used.

Synthetic deoxyoligonucleotides can stimulate an innate immune response either through the activation of TLR9, which recognizes the CpG motifs in ssDNA (Wagner, Bauer 2006) or through DNA sensing receptors that recognize free cytosolic DNA molecules (Hornung, Latz 2010). This activation of the innate immune system can eventually result in a Th1-biased immune response that counter-regulates the development of Th2 allergic reactions. Both hgd40 and ODNg3 used in this study are single stranded DNA molecules with CpG motifs in the catalytic domain of their sequence and it could be argued that they trigger an innate immune stimulation. However, in our study the improvement in the manifestation of oxazolone-induced dermatitis was achieved after treatment with GATA3-specific DNAzyme but not the control DNAzyme (ODNg3). This supports the concept of a target-specific mode of action of the DNAzyme by specific-down regulation of the target molecule, in this case GATA3, in the inflamed skin. Even more, potential TLR9-dependent and -independent cell activation by DNAzymes were investigated by Dicke et al. using different cell lines and primary cells of the innate immune system (Dicke et al. 2012). The findings of this study revealed no innate immune-related off target effects of hgd40.

Despite the effect of both preventive treatments with hgd40 on skin swelling on day 3, we did not detect a reduction in the level of GATA3 mRNA expression at this time point.

In normal, intact skin GATA3 is expressed by structural cells of the epidermis and in the hair follicles (Guzman Strong et al. 2006, Kaufman et al. 2003) in addition to some of the skin resident immune cells. Our initial findings revealed that 72 h after challenge with oxazolone, GATA3 expression was upregulated. However, when combined with treatment with any of the emulsions, we did not detect any changes in the GATA3 expression 72 h following oxazolone challenge. One possible explanation is that the application of emulsion somehow affected the sensitization or provocation process. Oxazolone is a chemical sensitizer with electrophilic properties. Once it penetrates the skin, it can react with skin proteins and form an immunogenic complex. Several factors can influence the reaction between an electrophile and an amino acid side chain including the 3D structure of the protein and the amino acids' degree of ionization, which in turn is influenced by the pH of the microenvironment (Divkovic et al. 2005). Since we apply the emulsion shortly before sensitization and/or challenge, the ingredients of the emulsion that penetrated the skin might interact with the hapten and modulate its binding to skin proteins resulting in an altered immune response. In our model, we see a

tendency towards an increased expression of Tbet after oxazolone challenge only when combined with treatment which suggests a change in the immune response and an increased participation of the Th1 phenotype.

If the Th2 phenotype is weaker, detection of small differences in GATA3 expression in the cells contributing to the inflammation, might be more difficult. It might also be overpowered by the high GATA3 expression in resident skin cells. The DNAzyme used in these experiments was designed to specifically target GATA3 mRNA and inhibit its translation to protein and therefore hinder the downstream effects of GATA3 in allergic reactions. Theoretically, a DNAzyme binds to its target RNA, cleaves it and then de-attaches and moves to another RNA molecule. The exact turnover or percent of reduction in the mRNA within each cell is not known. Previous data and data obtained from clinical studies of GATA3-specific DNAzyme-treatment of asthma shows that a reduction of 50-70% of the mRNA is sufficient to improve the clinical outcomes (unpublished data, properties of sterna biological). In the skin model in hand, we see an effect on the manifestation of disease but no measurable downregulation of GATA3 on mRNA levels. If these *in vivo* effects resulted from a 50% reduction in mRNA this would be reflected by a one cycle difference in RT-PCR, which makes it very challenging to achieve any significant differences with the group sizes used

When we stained for GATA3 expressing cells in the skin it became obvious that even in the solvent group GATA3 was highly expressed both in the epidermis and dermis. Similarly to what we see on mRNA level, the number of cells expressing GATA3 in the dermis was also comparable between solvent- and oxazolone-challenged skin. None of the treatments seemed to significantly affect the numbers of $GATA3^+$ cells infiltrating the dermis. Taking into account the 50-70% reduction by hgd40 treatment it would also be very challenging to differentiate the intensity of GATA3 expression between cells since those cells would still be producing some levels of GATA3 protein and would still stain positive for GATA3. The detection protocol cannot discriminate between high or low GATA3 expression. Thus the limitation of the detection methods which only discriminates between “expression” and “no expression” might account for the absence of treatment effects detected upon analysis of $GATA3^+$ cells in the dermis.

In addition, even with the dexamethasone treatment no changes of GATA3 levels were detected, neither on mRNA level nor on the protein expression in the cells. This is in contrast to what is known about glucocorticoids and their effect on inhibiting both RNA and protein expression of GATA3 (Liberman et al. 2009). These result support the

hypothesis that it might indeed be a matter of method sensitivity that makes GATA3 changes difficult to assess.

The glucocorticoid dexamethasone suppresses immune mechanisms by binding to the GCR and subsequently negatively or positively regulates pro- or anti-inflammatory genes, respectively. This was evident in the effects dexamethasone had not only on skin thickness, but also on IL-4 and IFNγ expression.

IL-4 is a major Th2 cytokine and is crucial for inducing GATA3 expression in a STAT6-dependent manner. GATA3, in turn, activates the transcription of IL-4 resulting in a positive feedback loop. The initial source of IL-4 is not exactly clear but several cells are known to produce IL-4 including both memory and naïve $CD4^+$ T cell, and some innate immune cells like basophils, mast cells and NKT cells (Godfrey, Kronenberg 2004). In the oxazolone-challenged groups, IL-4 expression was upregulated but remained unaffected by hgd40 treatment while it was significantly downregulated after treatment with dexamethasone. We know that GATA3 controls IL-4 gene expression in T cells (mainly Th2 cells) and other cells, however, it has been proposed that different mechanisms may be involved in IL-4 production in different cells especially those that do not express GATA3 such as mast cells (Weiss, Brown 2001). Moreover, it was obvious that the cellular infiltration was considerably less in the dexamethasone group, which would also be reflected on the mRNA expression levels of different factors including IL-4 and IFNγ that are usually expressed by different cells infiltrating the skin.

Following oxazolone challenge different immune cells are recruited to the site of inflammation. Several studies have shown neutrophils to be the predominant cells infiltrating the skin 24 h after challenge (Zhang, Tinkle 2000). While $CD4^+$ T cells, although increased after 24 h, were reported to reach their peak in the inflamed skin at 48-72 h. This is consistent with the occurrence of reduction in skin swelling in the hgd40-treated groups.

For this reason, we performed immunohistochemical staining of $CD4^+$ cells in the skin and evaluated the changes in the numbers of $CD4^+$ cells infiltrating the skin between different groups. We observed comparable numbers of $CD4^+$ cells in the skin of both oxazolone-and solvent-challenged groups. Although these groups were differentially sensitized and challenged, they both received similar disruption of the skin barrier by tape stripping. Mechanical injury of the skin activates the innate immune system and leads to the release of cytokines and chemokines which in turn increases the skin permeability. Moreover, chemokines work together with an array of adhesion molecules

to recruit leucocytes to the skin (Homey et al. 2006). Among these cells might be a variety of T lymphocytes including committed and naïve T helper cells that may remain present for several weeks after recruitment. Another cell type that contributes to immune responses in the skin is the $CD4^+$ NKT cell. These cells express the TSLP-receptor and might be activated and recruited to the skin in response to TSLP (He, Geha 2010), which is produced in response to barrier disruption. However, whether these $CD4^+$ cells detected in the skin in our model are the result of unspecific innate immune activation of the epithelium or specific phenotypes of the T helper subsets remains open for future investigations. Nevertheless, based on the cytokine profile in the oxazolone-challenged groups, the $CD4^+$ T cells in these groups are more likely to be a mix of both Th1 and Th2 cells.

Th2 cell, in particular, are of great interest in this model. The differentiation, proliferation and cytokine secretion function of Th2 cells is controlled by GATA3 and these cells represent direct targets for GATA3-specific DNAzyme treatment.

We found that in the prophylactic model, the number of $CD4^+$ cells that infiltrated the skin of hgd40-treated groups was less than in the control DNAzyme or placebo groups. This decrease was also more prominent when a higher dose of hgd40 was used. However, we did not find a similar effect on $CD4^+$ cells when animals were treated with either of the two doses of hgd40 in a semi-therapeutic manner. These results suggest that interfering with GATA3 in the sensitization phase modulates the inflammatory process and later affects the infiltration of $CD4^+$ cells into the skin. This might be an effect on the hapten and its binding to skin protein or an effect on early immune events and cytokines in the microenvironment that would later influence the participation of certain cells.

Based on these findings we investigated the changes in GATA3 expression in the prophylactic treatment model at earlier time points both after sensitization and challenge. Consistent with what we speculated, it was early after sensitization - at only 6h - that a down-regulation of GATA3 mRNA level was detected in the hgd40-treated group but not in the placebo group. At this early time point, generation of oxazolone-specific T cells is not to be expected. Yet, other cells expressing GATA3 such as skin resident T cells and innate immune cells that are recruited to the skin could be the ones affected. Recently, innate lymphoid cells (ILCs) have been identified as a group of cells bearing the phenotypical features of lymphoid cells without carrying the antigen specific receptor of adaptive immune cells. Group 2 of the ILCs produce Th2 cytokines including IL-5 and IL-13 and their differentiation and function is mainly controlled by GATA3 (Hoyler et al. 2012, Mjösberg et al. 2012). These cells reside in healthy human and murine skin and

are enriched in AD skin lesions (Kim et al. 2013). In this work of Kim et al. the researchers found group 2 ILCs (ILC2) to be crucial for the development of skin inflammation in murine models of AD. These group2 ILC responses in the skin were highly depended on TSLP-TSLPR interaction. We know from the previous work of Oyoshi et al. (Oyoshi et al. 2010) that tape stripping results in elevated levels of TSLP in the skin. Therefore, it is very likely that the early downregulation of GATA3 mRNA in our model is the result of .targeting GATA3 in these particular early-responsive group 2 ILCs.

The exact mechanism by which ILC2 promote AD-inflammation is not yet known but ILC2 derived IL-5 and IL-13 may be directly or indirectly responsible for a series of events leading to the inflammatory response in AD. As mentioned earlier, GATA3 is important for the function of ILC2 and their cytokine production. If the expression of GATA3 in these cells was affected by GATA3-specific DNAzyme treatment, this would change the cytokine milieu and affect other innate and later adaptive immune reactions and result in a modulation of the inflammation which might account for the reduction of skin swelling. However, at this stage more studies are still needed to understand the mechanisms by which ILCs contribute to both innate and adaptive immunity.

Our results clearly show a treatment effect on the manifestation of oxazolone-induced dermatitis after GATA3-specific DNAzyme treatment in both preventive approaches. This effect was reflected by a significant and dose-dependent reduction in skin swelling in the GATA3-specific- but not the control-DNAzyme-treated group. However, a down-regulation of the targeted mRNA was only detectable early in the sensitization phase after the prophylactic treatment. We believe that once the inflammatory process starts with different cells recruited to the skin and high levels of cytokines being produced, including IL-4 which up-regulates GATA3 expression, changes in the levels of GATA3 become more difficult to detect. In addition, this early down-regulation of GATA3 may have created an altered cytokine- and maybe even chemokine milieu. This in turn would have affected different aspects of the inflammatory process including the infiltration of CD4 cells. These changes may also involve alterations in the expression pattern of adhesion molecules, vascular permeability and edema formation or other mechanisms involved in the clearance of inflammation. However, analysis of whether or not GATA3 or one of its downstream targets can influence any of these events is beyond the scope of this project and open for future studies.

Based on the findings of this study, we believe that hgd40 may represent a promising therapeutic agent for topical application in Th2-driven inflammatory skin diseases.

4.3 Effects of Tbet-specific DNAzyme treatment *in vivo*

Tbet, the major regulator of differentiation and function of Th1 cells, represents an important therapeutic target in autoimmune and Th1-driven diseases. Therefore, a specific DNAzyme targeting Tbet mRNA was designed and formulated in a W/O/W emulsion suitable for topical application *in vivo*.

The aim of this part of the project was to analyse the ability of this DNAzyme to interfere with disease progression when applied in Th1-predominant skin inflammation. We chose an animal model of OVA/CFA-induced dermatitis to test the effects of Tbet-specific DNAzyme *in vivo*. The combination of CFA as an adjuvant with the protein antigen OVA for sensitization has been described to allow a preferential differentiation towards Th1 immune responses (Shibaki, Katz 2002). This could be due to the mycobacterial products in the CFA, which create a cytokine milieu more suitable for Th1 cell differentiation. Similar murine models have been used to study a Th1-/IFNγ- induced skin inflammation (Cho et al. 2001).

In this model we see a strong inflammation in response to repeated cutaneous OVA challenge characterized by a significant increase in skin swelling and formation of psoriatic plaques. The inflammatory reaction in the skin was also characterized by a dense influx of inflammatory cells consisting mostly of mononuclear cells and neutrophils. Following semi-therapeutic topical treatment with the Tbet-specific DNAzyme td32, a significant reduction in skin swelling was evident. No reduction was detected when skin was treated with either ODNg3 or placebo which suggests specific targeting of Tbet by td32. Moreover, our findings also revealed no effects of topical treatment with hgd40 on skin swelling in the OVA/CFA model, which further supports the previously mentioned reports of the favoured Th1 phenotype in such models. It also emphasizes our hypothesis that the improvement in skin swelling is the result of specific targeting of Tbet.

Unlike what was detected for the GATA3-DNAzyme treatment in the oxazolone model, preventive treatment with the Tbet-DNAzyme reduced the severity of disease progression including the acute phase following first challenge. This may be due to the fact that many of the immune cells involved in the first response (including NK cells, ILC1, γδ T cells) express Tbet (Lazarevic et al. 2013) and thus might be affected by Tbet-DNAzyme treatment. At a later phase of disease progression, we believe that adaptive immune cells including both $CD4^+$ Th1cells and CD8+ CTLs become the predominant cells and can be directly affected by Tbet-DNAzyme treatment.

However, and despite the significant effect on skin swelling, we were unable to detect a down-regulation in the mRNA of Tbet at the endpoint in these experiments. These results are consistent with our findings in the oxazolone model after treatment with the GATA3-DNAzyme. It is possible that the changes on mRNA level of Tbet appear at an earlier time point as it is the case in the oxazolone model. Even more, the effects of such an early change in Tbet and perhaps cytokine environment may have led to a series of events that eventually resulted in the improvement of clinical manifestation of skin inflammation we observed after treatment.

There was a slight, yet significant, reduction in IFNγ expression in the Tbet-specific DNAzyme- which unexpectedly was also detected for the control DNAzyme ODNg3-treated group but to a lesser extent. While these effects on IFNγ seem to correlate with those on skin swelling in the Tbet DNAzyme group, they are inconclusive for the ODNg3 group especially as they were not associated with any improvement in this group. Unspecific effects of deoxyoligonucletides would involve an induction of IFNγ rather than a suppression. Moreover, previous studies showed no off-target effects of ODNg (Dicke et al. 2012). The ODNg3 DNAzyme possesses the active catalytic domain with unspecific binding arms of scrambled sequence. They showed no effect on IFNγ in the oxazolone model or on the symptomatic outcome of either model. Therefore it is also very unlikely that this effect is a result of these DNAzyme binding to IFNγ-mRNA or the mRNA of IFNγ-regulating molecules. The effect on IFNγ expression is most likely an artificial effect especially since the actual differences in the raw data of the qPCR regarding the delta Ct values are minimal within the range of inner assay variance. This combined with low sample numbers points to the low physiological relevance of the changes detected.

In this part of the study we were able to show efficacy of Tbet-specific DNAzyme treatment on OVA-induced dermatitis *in vivo* but no down-regulation of Tbet 96h after the last challenge. The similarities of these findings with those of the GATA3-DNAzyme in the oxazolone model point to possibly similar modes of action in each of the models. Yet, many aspects of the exact chain of events leading to these final results remain unclear and might be a matter for future investigations.

4.4 Pros and Cons of DNAzyme-based therapy

During the past decade, our understanding of disease pathogenesis has improved remarkably. The explosion in the genomic information has led to discoveries of disease-

causing proteins and pathways and opened the door for new, gene-specific therapies such as DNAzymes.

The use of DNAzymes as therapeutic tools in different diseases including cancer and allergic diseases have grown in recent years and in some cases progressed to clinical trials (Khachigian 2004, Badros et al. 2005, Cai et al. 2012 and Krug et al. 2015).

Generally, several criteria can determine the success of DNAzyme therapy including the choice of the target gene and the ability of the DNAzyme to cleave its target mRNA.

In this study, the targeted molecules GATA3 and Tbet, are main orchestrators of the T helper cell mediated immune responses in each of the respective models. In addition both hgd40 and td32 have been shown to cleave their targets in previous *in vitro* studies (Sel et al. 2008 and unpublished data).

Nevertheless, *in vivo* application of DNAzymes bares other challenges starting with protection of the DNAzymes from degradation and a successful delivery to the site of action. This issue was addressed through a combination of chemical alterations to the DNAzyme structure and a drug delivery system that provided both protection and enhanced skin penetration (Schmidts et al. 2012). Although the distribution of DNAzymes after topical application to the skin was beyond the scope of this study, the effective reduction in skin swelling seen exclusively in the specific DNAzyme, and not the control DNAzyme, suggests a successful delivery. Similar effective treatment has been reported for the intranasaly applied GATA3-specific DNAzyme in murine models of allergic airway inflammation (Sel et al. 2008). Later, biodistribution studies involving inhalative exposure of the GATA3-specific DNAzyme hgd40 showed efficient delivery of fluorescently labelled hgd40 into the lung tissues (Turowska et al. 2013). Comparable studies in the skin are planned, but are expected to be challenging due to the high autofluorescence of the skin.

Although the field of DNAzyme therapy is expanding, the use of DNAzymes in skin or airway diseases has the advantage of a topical drug delivery to the target tissue by epicutaneous emulsions or inhaled aerosols. Topical application also bares lower risk of toxicity compared to systemic administration of such antisense molecules. The previously referred to work by Turowska et al. described a rapid availability and elimination of hgd40 from the plasma following pulmonary administration. Even more, toxicological analysis of these molecules in rats and dogs revealed no hgd40-related respiratory, cardiovascular or CNS effects (Turowska et al. 2013). In general, drug

application through the cutaneous root is considered safer and the concentrations of the drugs reaching the blood after once or twice daily applications are expected to be minimal. Moreover, the data provided from previous studies as well as the one at hand indicate no off-target effects of theses DNAzymes by activation of innate immune responses via TLR9 of DNA-sensing molecules.

The GATA3-specific DNAzyme hgd40 used in this study has already moved to clinical trials (phase I and IIa for asthma bronchiale and atopic dermatitis). Most recent published data reported a safe and well tolerability of inhaled GATA3-specific DNAzymes in phase I clinical trials (Homburg et al. 2015). Furthermore, data obtained from a randomized, double blind clinical trial showed significant reduction in both early and late allergic response in asthmatic patients after treatment with GATA3-specific DNAzyme (Krug et al. 2015).

Despite the efficacy and safety profile available so far for this transcription factor-specific DNAzyme, there is still a long way for such therapies to reach the bedside. However, the results of this study support the previous findings on successful reduction of Th2-driven allergic inflammation through the down-regulation of GATA3. It also shows promising results for the Tbet-specific DNAzyme in Th1-driven skin inflammation. These findings open a new possibility for a novel DNAzyme-based therapy in the treatment of Th-mediated skin diseases.

5 SUMMARY

Inflammatory skin diseases cover a wide range of skin conditions that cause dry, itchy, scaly skin and affects millions around the world. Current therapies are mostly symptomatic and often associated with a wide range of side effects. Therefore, there is a growing need for a more specific therapy targeting key molecules in the pathogenesis of such diseases. Investigations revealed a central role for different T helper subsets in the immunopathology of these skin diseases. Atopic dermatitis (Th2), contact dermatitis (Th1) and Psoriasis (Th1, Th17), are associated with one or more of these Th-phenotypes. The differentiation and activation of T helper subtypes is regulated by different transcription factors. This study focused on two specific transcription factors GATA3 and Tbet, which regulate the differentiation and activation of Th2 and Th1, respectively. The levels of these transcription factors were found to be elevated in diseases with the respective Th phenotype, which made them interesting targets for DNAzyme-based therapy. In order to test the efficacy of such transcription factor-specific DNAzymes, two different animal models of inflammatory allergic skin diseases were established. In the first model skin injury and the hapten oxazolone were used to elicit a Th2-dominant contact hypersensitivity with features similar to those of atopic dermatitis. In the second model, Ovalbumin was used to induce a Th1-dominated inflammation in OVA-specific T cell receptor- transgenic mice after systemic sensitization with OVA/CFA.

Effects of topical preventive treatment with GATA3-specific DNAzyme (hgd40) were investigated in the oxazolone model. The DNAzymes were formulated in w/o/w emulsion for protection against degradation and an enhanced skin penetration. Compared to placebo and control non-specific DNAzyme ODNg3, prophylactic treatment with hgd40 significantly reduced skin swelling. It also resulted in lower numbers of $CD4^+$ cells infiltrating the dermis. This was associated with a downregulation of GATA3 mRNA expression in the skin early in the sensitization phase. Similar reduction in skin swelling was also observed after a semi-therapeutic treatment, in which hgd40 was first applied 24 h prior to challenge but not during sensitization.

Using the OVA/CFA model, effects of the Tbet-specific DNAzyme td32 treatment were evaluated. Data indicated a significant reduction in skin swelling following prophylactic treatment with emulsions containing td32. No such effect was observed in the control DNAzyme ODNg3 or placebo groups. In addition, no influence on the progression of skin swelling in this Th1-skewed skin inflammation after treatment with hgd40 was detected, which further supports the hypothesis of specific targeting of Tbet.

In summary, this study shows that treatment with DNAzymes, targeting Th1- and Th2-specific transcription factors Tbet and GATA3 improved inflammatory symptoms *in vivo*. These results pose the DNAzyme as promising tools for future topical treatment of inflammatory skin diseases.

6 ZUSAMMENFASSUNG

Entzündliche Erkrankungen der Haut sind durch trockene, juckende und schuppende Veränderungen des betroffenen Organs gekennzeichnet. Derzeitige Therapien behandeln meist nur die Symptome und sind häufig mit einem großen Spektrum an Nebenwirkungen verbunden. Es gibt daher einen wachsenden Bedarf an spezifischeren Therapieoptionen, welche Schlüsselmoleküle in der Immunopathologie dieser Krankheiten adressieren. Verschiedene Untergruppen von T-Helferzellen spielen eine zentrale Rolle bei der Entwicklung dieser Hauterkrankungen. Die Differenzierung und Aktivierung dieser T-Helferzell (TH)-Subtypen wird durch verschiedene Transkriptionsfaktoren reguliert. In dieser Arbeit liegt der Fokus auf dem TH2- bzw TH1-induzierenden Transkriptionsfaktor GATA3 bzw Tbet, Die atopische Dermatitis (TH2) sowie die Kontaktdermatitis und Psoriasis (TH1) werden mit einem der beiden TH-Zell-Phänotypen assoziiert. In den jeweiligen Erkrankungen konnten erhöhte Mengen des entsprechenden Transkriptionsfaktors nachgewiesen werden, was diese Moleküle zu einer interessanten Zielstruktur für eine DNAzym- Therapie macht. Um die Effizienz von Transkriptionsfaktor-spezifischen DNAzymen zu testen, wurden zwei verschiedene Tiermodelle für entzündliche Hauterkrankungen etabliert. Im ersten Modell wurde durch mechanische Schädigung der Haut und dem Hapten Oxazolon eine TH2-dominierte Kontakthypersensitivität ausgelöst, deren Eigenschaften der Atopischen Dermatitis entsprechen. Im zweiten Modell wurde in OVA-T-Zell-Rezeptor-transgenen Mäusen, nach OVA/CFA Sensibilisierung und anschließender lokaler OVA-Applikation eine TH1-dominierte Entzündung ausgelöst.

Die Effekte einer präventiven lokalen Behandlung mit dem GATA3-spezifischen DNAzym hgd40, wurde im Oxazolon-Modell untersucht. Zum Schutz und zur Penetrationsförderung des Moleküls, wurde dieses in eine w/o/w Emulsion eingebracht. Im Vergleich zum Placebo und dem unspezifischen DNAzym ODNg3 zeigte die prophylaktische Behandlung mit hgd40 eine signifikante Reduktion der Hautschwellung. Ebenso konnte eine geringere Anzahl an infiltrierenden $CD4^+$ Zellen in die Dermis beobachtet werden. Dieser Beobachtung ging in der frühen Sensibilisierungsphase eine niedrigere GATA3 mRNA Expression in der Haut voraus. Eine ähnliche Abnahme der Hautschwellung wurde nach einer semi-therapeutischen Behandlung beobachtet, in der hgd40 erst 24 Stunden vor Provokation aber nicht vor der Sensibilisierung appliziert wurde.

Die Effekte der Behandlung mit dem Tbet-spezifischen DNAzym td32 wurden im OVA/CFA-Modell evaluiert. Die Daten zeigen eine geringere Hautschwellung nach prophylaktischer Behandlung mit einer td32-Emulsion, während keine Effekte nach

Behandlung mit dem Kontroll-DNAzym ODNg3 oder dem Placebo zu beobachten waren. Zusätzlich konnte gezeigt werden, dass die Behandlung mit hgd40 keinen Einfluss auf die Entwicklung der Hautschwellung in dieser TH1-dominierten Entzündung hat. Dies unterstützt die Hypothese der spezifischen Interaktion des DNAzyms mit Tbet.

Zusammenfassendkonnte gezeigt werden, dass durch die Behandlung mit DNAzymen, gerichtet gegen die TH2- und TH1- spezifischen Transkriptionsfaktoren GATA3 und Tbet eine Verbesserung der Entzündungssymptome *in vivo* erreicht werden konnte. Diese Ergebnisse zeigen, dass DNAzyme als vielversprechendes Instrument für die zukünftige lokale Behandlung entzündlicher Hautkrankheiten darstellen können.

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8 LIST OF ABBREVIATIONS

A	Adenine
ACD	Allergic contact dermatitis
AD	Atopic dermatitis
AMPs	Antimicrobial peptides
APCs	Antigen- presenting cells
Bp	Base pair
C	Cytosine
CCL	CC- chemokines ligand
CCR	CC- chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CHS	Contact hypersensitivity
CLA	Cutaneous lymphocyte- associated antigen
CpG	Cytosine-guanine-dinucleotide motive
CXCL	CXC- chemokine ligand
DAMPs	Danger -associated molecular patterns
DCs	Dendritic cells
Dexa	Dexamethasone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNAzymes	Deoxyribozymes
DNFB	Dinitrofluorobenzene
dNTP	Deoxynucleoside triphosphate
Fig.	Figure
G	Guanine
GATA3	GATA binding protein 3
Gfi-1	Growth factor independent -1
GM-CSF	Granulocytes macrophages- colony stimulating factor
GR	Glucocorticoid receptors
H&E	Hematoxylin and eosin
i.p.	intraperitoneal
IFNγ	Interferon gamma
IFNγR	Interferon gamma receptor
IgE	Immunoglobulin type E

IHC	Immunohistochemistry
IL-	Interleukin-
IL-12Rβ2	IL-12 receptor beta 2
ILCs	Innate lymphoid cells
iNKT	Invariant natural killer T cell
IκBα	Inhibitors of nuclear factor κB
KCs	Keratinocytes
LCs	Langerhans cells
LNAs	Locked nucleic acids
MHC	Major histocompatibility complex
mRNA	messenger RNA
NFAT	nuclear factor of activated T cells
NKT	Natural killer T cell
NLAs	Locked nucleic acids
NLRs	NOD-like receptors
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
Oxa	Oxazolone
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Real time PCR
s.c.	subcutaneous
SLPI	Secretory leukocyte protease inhibitor
SNPs	Single nucleotide polymorphisms
ssDNA	Single stranded DNA
STAT	Signal transducer and activator of transcription
T	Thymine
TAE	Tris acetate EDTA
Tbet	T box expressed in T cells
TCI	Topical calcineurine inhibitors
TCR	T cell receptor
Th	T helper

TLRs	Toll- like receptors
TNF	Tumour necrosis factor
TNFα	Tumour necrosis factor alpha
Tregs	T regulatory cells
TSLP	Thymic stromal lymphopoietin
UV	Ultra violet
w/o	Water in oil
w/o/w	Water in oil in water

9 LIST OF FIGURES AND TABLES

9.1 Figures

9.2 Tables

10 PUBLICATIONS AND CONGRESS CONTRIBUTIONS

Ulrich Purath*, **Rouba Ibrahim***, Jana Zeitvogel, Harald Renz, Frank Runkel, Thomas Schmidts, Dorota Dobler, Thomas Werfel, Anke Müller, Holger Garn. Efficacy of T cell transcription factor-specific DNAzymes in murine skin inflammation models. **J Allergy Clin Immunol** (Paper accepted).

Published Abstracts and congress contributions

Ibrahim R, Purath U, Turowska A, Homburg U, Runkel F, Schmidts T, Dobler D; Renz H, Garn H; Mueller A "Effects of interference with GATA-3 expression by target-specific DNAzyme treatment on disease progression in a subacute oxazolone-induced mouse model of atopic dermatitis" **Clin Transl Allergy**. 2015; 5 (Suppl 1): O21.

Purath U, **Ibahim R**, Turowska A, Homburg U, Runkel F, Schmidts T, Dobler D; Renz H Müller A, Garn H; "Therapeutic efficacy of a GATA3-specific DNAzyme in an experimental model of allergic skin disease" **Allergo J** 2013- Frühjahrstagung der Deutschen Gesellschaft für Allergologie und Klinischer Immunologie,Mainz 2013.

11 LIST OF ACADEMIC TEACHERS

My academic teachers include the Professors and Privatdozents from:

Philipps University Marburg : Bauer, Garn, Renz, Steinhoff.

Damascus University: Abu-khamis, Al-Assaf, Al-Fahoum, Al-khayyat, Al-Kubaili, Al-kuwatli, Al-Lahham, Al-Madi, Al-Mardini, l-Saleh, Al-Shammaa, Al-Zain, Al-Zhouri , Am-Ali; Amouri, Amren; Barkil; Diab, Haddad, Haider, Hasan, Hasan-Aga, Herbali, Maarouf, Munen, Natouf, Salami, Yousef.

12 CURRICULUM VITAE

Personal information

Name:	Rouba Ibrahim
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Place of Birth:	Damascus, Syria

Academic qualifications

2003 - 2008	Faculty of Pharmacy Damascus university, (B.sc in pharmacy and pharmaceutical chemistry)
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Work experience

10.2009 – 08.2010	Pharmaceutical sales represent at Al fares Pharmaceuticals, Damascus, Syria.
09.2008 – 07.2009	Pharmaceutical sales represent at the national company for pharmaceutical industry, Damascus, Syria.

Memberships

- Junior member of the European academy for allergy and clinical immunology (EAACI).
- Member of the German society for Immunology ''Deutsche Geselschaft für Immunologie'' (DGfI).
- Member of the Marburg university research academy (MARA).
- Member of the Syrian Pharmacists' union.

Scholarships and awards

- DAAD Scholarship holder for PhD studies.
- Winner of the best poster presentation in the "Assembly of Pharmacy Faculties in Syrian universities" held in Damascus, April 2008.

…….. .....

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13 EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel :

“Investigating the efficacy of transcription factor-specific DNAzyme in animal models of inflammatory skin diseases”

im Institut für Laboratoriumsmedizin und Pathobichemie, Molekular Diagnistik unter Leitung von Prof. Dr. med. Harald Renz ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Teile dieser Arbeit sind in bei dem *Journal of Allergy and Clinical Immunology* eingereicht.

....

(Ort, Datum) (Rouba Ibrahim)

14 ACKNOWLEDGMENT

- Prof. Dr. med Harald Renz for giving me the chance to pursue my PhD degree in his department and for all the support he provided during the past years.

- PD. Dr. Garn for the scientific supervision and guidance throughout my PhD.

- Deutsche Akademische Austausch Dienst (DAAD) for financing my PhD studies. It would have not been possible without your support.

- Dr. Anke Müller for the direct supervision and mentoring during the past three years and for the correction of the thesis.

- Dr. Ulrich Purath for the advice and help.

- Marcel Schimmel for the help with preparing the samples.

- Katharina Reichenbach, Ayse Kilic and Özge Sungur for their feedback.

- All my colleagues and friends for making this experience much more valuable and fun. Thank you: Ayse, Chrysanthi, Katrin.S, Katharina, Katrin F, Daniel, Hani, Sophia, Alex, Morad, Hazar for all the help and laughs.

- Prof. Dr. med. Lucas Greiner and his family for their hospitality, encouragement and support throughout the past years.

- Finally, to my family who never stopped believing in me and were always there for me despite the long distance. Mom and Dad, I am so blessed to have you as parents. Thank you for all the love, dedication, and support and for making it possible for me to live my dream. My sisters: Yasar and Lama, you are my safe haven and my backbone.