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RNase 7 downregulates TH2 cytokine production by activated human T-cells

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Abstract

Background:

The antimicrobial peptide (AMP) RNase 7 is constitutively expressed in the epidermis of healthy human skin and has been found to be upregulated in chronic inflammatory skin diseases such as atopic dermatitis and psoriasis. Activated T-cells in lesional skin of patients with atopic dermatitis (AD) and psoriasis (PSO) might be directly exposed to RNase 7. In addition to their antimicrobial activity immunoregulatory functions have been published for several AMPs. In this study we investigated immunoregulatory effects of the antimicrobial peptide RNase 7 on activated T-cells.

Methods:

Isolated human CD3⁺ T-cells were stimulated with RNase 7 and screened for possible effects by mRNA microarray analysis. The results of the mRNA microarray were confirmed in isolated CD4⁺T-cells and in polarised TH2 cells using skin derived native RNase 7 and a recombinant ribonuclease-inactive RNase 7 mutant. Activation of GATA3 was analysed by electrophoretic mobility shift assay.

Results:

Treatment of activated human CD4⁺ T-cells and TH2 cells with RNase 7 selectively reduced the expression of TH2 cytokines (IL-13, IL-4 and IL-5). Experiments with a ribonuclease-inactive recombinant RNase 7 mutant showed that RNase 7 ribonuclease activity is dispensable for the observed regulatory effect. We further demonstrate that CD4⁺T-cells from AD patients revealed a significantly less pronounced downregulation of IL-13 in response to RNase 7 compared to healthy control. Finally, we show that GATA3 activation was diminished upon cultivation of T-cells with RNase7.

Conclusion:

Our data indicate that RNase 7 has immunomodulatory functions on TH2-cells and decreases the production of TH2 cytokines in the skin.

Short title

RNase 7 downregulates TH2 cytokine production

Keywords

RNase 7, TH2 cytokines, atopic dermatitis

Abbreviations

AMP, antimicrobial peptides; AD, atopic dermatitis; PSO, psoriasis

Introduction

The skin is the primary interface between the body and the environment and provides the first line of defence against environmental influences. Keratinocytes are the most abundant cells of the epidermis. In addition to their function as a physical barrier keratinocytes are an important source for antimicrobial peptides which represent a chemical defence system against bacteria, fungi and viruses[1]. One of the major AMP secreted by keratinocytes is RNase 7, a member of the RNase A family [2]. RNase 7 is constitutively expressed in the epidermis of healthy human skin leading to high concentration in the stratum corneum [3]. The Expression can be further upregulated by stimulation with proinflammatory cytokines, by skin injury or in response to diverse bacteria including *Staphylococcus aureus* [2, 4, 5].

Chronic inflammatory skin diseases such as atopic dermatitis (AD) and psoriasis (PSO) are characterized by the infiltration of activated T-cells into the epidermal compartment including the epidermis [6-8]. Several cytokines released by infiltrating T-cells have been shown to be involved in the regulation of AMP production by keratinocytes. While IL-17, IFN γ and IL-22 are strong

inducers of AMP production, TH2 cytokines such as IL-4, IL-10 and IL-13 have been reported to suppress the production of some AMPs in the skin [9-11]. Psoriatic skin is characterized by a high expression of various AMPs [12] while induction of some AMPs is impaired in patients suffering from atopic dermatitis [13]. On the other hand, several AMPs, including RNase 7, are expressed at significantly higher amounts in lesional and non-lesional skin of AD patients compared with healthy controls [4]. In addition to their antimicrobial activity immunoregulatory functions on T-cells have been described for several skin-derived AMPs such as human β -defensins and psoriasin [14-16]. Activated T-cells in lesional skin of patients with AD or psoriasis are presumably exposed to keratinocyte-derived RNase 7. To date no immunoregulatory function has been described for RNase 7. Therefore we investigated the influence of RNase 7 on T-cell cytokine production.

Methods

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) from buffy coats or AD patients were separated by density gradient centrifugation on Pancoll (PAN Biotech, Aidenbach, Germany). T-cells and monocytes were isolated using a negative selection kit (purity was $\geq 97\%$) (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were cultured in IAB medium; Iscove medium (Biochrom, Berlin, Germany) supplemented with 4% human AB serum and stimulated with activating anti-CD3 (1 $\mu\text{g}/\text{ml}$) and anti-CD28 (0.2 $\mu\text{g}/\text{ml}$) antibodies (Sanquin, Amsterdam, Netherlands) for one day before cells were stimulated with native RNase 7 or a ribonuclease inactive RNase 7 mutant (RNase 7M) respectively at the indicated concentrations for the indicated periods. Polarization of TH2 cells was performed as previously described [17]. Briefly, isolated naive CD4⁺ T cells were resuspended in IAB medium, stimulated with activating anti-CD3 (0.5 $\mu\text{g}/\text{mL}$) and anti-CD28 (0.2 $\mu\text{g}/\text{mL}$) antibodies as well as IL-2 (20 ng/mL; TEBU Peprotech, Offenbach, Germany) and were cultured for 10 days in presence of IL-4 (20 ng/mL; R&D Systems) neutralizing anti-IFN γ antibody (10 $\mu\text{g}/\text{mL}$; R&D Systems) and neutralizing anti-IL-12 antibody (0,5 $\mu\text{g}/\text{mL}$; R&D Systems) (TH2 polarizing medium). For the control of proper polarization, intracellular flow-cytometric staining with antibodies to IFN γ , CCR4 and IL-4 (BD Bioscience,

Heidelberg, Germany) was carried out after restimulation with phorbol 12-myristate 13-acetate and ionomycin (Sigma Aldrich, Deisenhofen, Germany) for 6 hours. The polarized TH2 cells were stimulated with activating anti-CD3 (1 µg/ml) and anti-CD28 (0.2 µg/ml) antibodies and RNase 7 or RNase 7M, respectively, at the indicated concentrations for the indicated periods.

Flow cytometry

For T-cell proliferation assay isolated CD4⁺ T-cells were stained with CFSE (Carboxyfluorescein succinimidylester) using the Vybrant® CFDA SE Cell Tracer Kit (Invitrogen, Karlsruhe, Germany). CD4⁺ T-cells were stimulated with activating anti-CD3 (1 µg/ml) and anti-CD28 (0.2 µg/ml) antibodies and RNase 7M at the indicated concentrations. Proliferation was analysed by flow cytometry after 5d. To analyse binding of RNase 7 to CD4⁺ T-cells human isolated CD4⁺ T-cells were stimulated with activating anti-CD3 (1 µg/ml) and anti-CD28 (0.2 µg/ml) antibodies for 24h, stimulated with RNase 7 (10µg/ml) for 30 min and washed extensively. Binding of RNase 7 to CD4⁺T-cells was analysed by flow cytometry using an anti-RNase 7 antibody (Atlas, Stockholm, Sweden), in combination with a FITC Goat anti-Mouse antibody. To examine GATA3 expression, polarized TH2 cells were stimulated with activating anti-CD3 (1 µg/ml) and anti-CD28 (0.2 µg/ml) antibodies and RNase 7M at the indicated concentrations for 24h. For intracellular staining cells were fixed, permeabilized with the Cytofix/Cytoperm kit (BD Bioscience) and GATA3 expression was analysed by flow cytometry using an antibody to GATA3 (BD Bioscience). Cell viability was assessed by flow cytometry by staining with Annexin-V or propidium iodide (eBioscience, San Diego, USA) to determine the percentage of viable cells after stimulation with RNase 7M for 24h. Analysis was performed with a FACSCalibur flow cytometer (BD Biosciences).

mRNA microarray analysis

Isolated CD3+ T-cells were stimulated with activating anti-CD3 (1 µg /ml) and anti-CD28 (0.2 µg /ml) antibodies and RNase 7M 10µg/ml for 6h. RNA was extracted from the cells using the High Pure mRNA Isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). The microarray was carried out by the Research Core Unit “Transcriptomics” of Hannover Medical School.

The Whole Human Genome Oligo Microarray Kit 4x44K v2 (G4845A, design ID 026652, Agilent Technologies) was utilized in this study. Microarrays of this design type contain 44495 probes, covering 25940 transcripts.

Synthesis of Cy3- or Cy5-labeled cRNA was performed with the “Quick Amp Labeling kit, two color” (#5190-0444, Agilent Technologies) according to the manufacturer’s recommendations. cRNA fragmentation, hybridization and washing steps were carried-out exactly as recommended in the “Two-Color Microarray-Based Gene Expression Analysis Protocol V5.7” (Agilent). Slides were scanned on the Agilent Micro Array Scanner G2565CA (pixel resolution 5 µm, bit depth 20). Data extraction and processing of raw fluorescence intensity values were performed with the “Feature Extraction Software V10.7.3.1” by using the recommended default extraction protocol file: GE2_107_Sep09.xml.

For evaluation mean ratio of fluorescence intensity for the respective transcripts was calculated. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [18](Edgar et al., 2002) and are accessible through GEO Series accession number GSE94830 from 31.12.2017.

mRNA isolation, reverse transcription and quantitative reverse transcription–polymerase chain reaction

RNA isolation and reverse transcription were performed as previously described [19]by using the High Pure mRNA Isolation kit (Roche Molecular Biochemicals, Mannheim, Germany) and First Strand cDNA Synthesis kit (MBI Fermentas, St Leon-Rot, Germany). For mRNA isolation from human skin the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer’s instructions. The quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed on a LightCycler (Roche

Molecular Biochemicals) using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche Molecular Biochemicals). qRT-PCR for IL-13, IL-4, GATA3, RPS21 and GAPDH was performed with QuantiTect primers according to the manufacturer's protocol (Qiagen, Hilden, Germany). Specific gene expression was normalized to GAPDH gene expression using the Relative Quantification Software (Roche Molecular Biochemicals).

Cytokine detection in supernatants

Cell-free supernatants were analysed by enzyme-linked immunosorbent assay (ELISA): IFN γ , IL-2, IL-4 (Ready-Set-Go kit; eBioscience, Breda, Netherlands), IL-13, IL-17 and IL-5 (R&D Systems) according to the manufacturers manual.

Electrophoretic mobility shift assays

Nuclear extract from TH2 cells was prepared with the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo-Fisher Scientific Inc., Rockford, Illinois, USA) according to the manufacturer's instructions. Electrophoretic mobility shift assay (EMSA) was performed in a 20 μ l binding reaction using the LightShift EMSA Kit (Thermo-Fisher Scientific) containing equal amounts of the nuclear extract for each donor and biotin-labelled GATA3 oligonucleotides (GATA-3 EMSA Kit; Panomics, Santa Clara, USA) as the probe. The reaction mixtures were incubated on ice for 10 min, then biotin-labelled probe was added and the incubation was continued for another 20 min at room temperature. DNA-protein complexes were separated on a 4-20% Precise Protein Gel (Thermo-Fisher Scientific) in 0.5% TBE for 40 min at 100V and were transferred to positively charged nylon membrane (Whatman, Dassel, Germany). Biotin-labelled DNA was detected with the Chemiluminescent Nucleic Acid Detection Kit (Thermo-Fisher Scientific). For control experiments, unlabelled competitor oligonucleotide (GATA-3 EMSA Kit; Panomics) was preincubated with the nuclear extract for 10 min before labelled probe was added. The EMSA was analysed with quantitative densitometry using ImageJ.

Chemotaxis assay

The chemotactic activity of RNase 7 on human monocytes and CD3⁺ T-cells was determined using the microchamber technique ChemoTx System (Neuro Probe, Gaithersburg, MD). A volume of 27 ml of the chemotactic stimulus or control medium was filled in the bottom chamber and covered with a polycarbonate filter with a pore size of 5 μ m. Monocytes or CD3⁺ T-cells were loaded in a volume of 25ml on top of the membrane. After incubation for 3 h at 37 °C, the membrane was carefully removed. The migrated cells present in the lower chamber were lysed with Triton X-100 (0.1% [vol/vol]) in order to quantify them by assessment of NP-specific β -glucuronidase (Sigma-Aldrich). The number of migrated cells in the bottom chamber was calculated, from a standard curve with known cell number.

Immunohistological staining

Three-millimeter punch biopsies from normal skin were fixed in formaldehyde, thereafter they were embedded in paraffin and sections (5 μ m) were prepared. For staining of CD3⁺ T-cells, the fixed sections were treated with Target Retrieval Solution (DakoCytomation, Hamburg, Germany); polyclonal rabbit anti-human CD3⁺ antibody (Dako) 1:100 and as isotype control rabbit IgG (R&D Systems) were used. Staining was performed according to the manufacturer's instructions using the Dako EnVision staining kit.

Antimicrobial peptides

Native human RNase 7 isolated from human skin was isolated as previously described [2]. Purity of native human RNase 7 was verified by reverse phase HPLC and electrospray ionization mass spectrometry analyses. Recombinant RNase 7 and a RNase 7 construct without ribonuclease activity was generated as described before [3] and the cDNA / mutated cDNA was introduced by Agrobacterium transformation into potato plants under control of a sink-specific promotor. Transgenic potato plants were grown in the greenhouse, potato tubers expressing the mutated recombinant RNase7 were harvested. The recombinant proteins were isolated from potato tubers by different

chromatographic steps. Finally, purification by polishing of the proteins was realized by HPLC and the correct mass was verified by ESI-MS-analysis. The purified recombinant proteins produced in potato tubers were subsequently tested against different bacterial strains yielding a bioactive (anti-microbial) molecule with or without RNase activity.

RNase 7 treatment of skin resident T-cells

Normal human skin was cultured in TH2 polarizing medium for 48h. The regulation of GATA3 mRNA was verified by PCR. Afterwards, three-millimeter punch biopsies were cultured in IAB medium with or without RNase 7 (1µg/ml) for 24h. The regulation of IL-13 mRNA expression was analysed by PCR.

Statistical analysis

For statistical evaluation of normal distributed data, the paired t-test (Figure 2c, 3c/d) was used, and for data not normally distributed, the Wilcoxon matched pairs test (Figure S1, 1, 2a/b/d/e/f/g, 3a/b, 4) was used; a P-value below 0.05 was regarded as significant. $P < 0.05$ is depicted with *, $P < 0.01$ with ** and $P < 0.001$ with ***. The program GraphPad Prism version 3.02 (GraphPad Software Inc., San Diego, CA) was used for statistical analysis.

Ethics

The study was approved by the Medical Ethics Committee of the Hannover Medical School and was conducted according to the Declaration of Helsinki Principles. All patients gave their written informed consent.

Results

mRNA microarray analyses identifies T-cell cytokines regulated by RNase 7

To obtain a first overview on possible effects of RNase 7 on human T-cells, mRNA microarray analysis covering 25.940 transcripts was performed. To exclude the influence of the RNase function on RNA quality we stimulated the CD3+ T-cells with a ribonuclease-inactive recombinant RNase 7 mutant (RNase 7M). We found a downregulation of mRNA expression for the TH2 cytokines IL-4, IL-5 and IL-13 in RNase 7M stimulated cells while the mRNA levels of IFN γ and IL-17 were not regulated (Fig.1a). To confirm the results of the mRNA microarray we investigated the effect of RNase 7M on TH2 cytokine mRNA transcription in polarised TH2 cells generated from naive CD4+ T-cells. Dose titration indicated that up to 100 times lower concentrations of RNase 7M than used in the microarray were sufficient to reduce IL-13 mRNA expression (Fig S1). Stimulation of polarised TH2 cells with low concentrations RNase 7M significantly reduced IL-4 and IL-13 mRNA expression (Fig. 1b/c). To evaluate binding of RNase 7 to human T-cells, CD4+ T-cells were isolated and stimulated with RNase 7 for 30 min, washed extensively and binding of RNase 7 was detected by FACS using an α -RNase 7 antibody. RNase 7 showed strong binding to activated CD4+ T-cells (Fig. 1d)

RNase 7M selectively downregulates the secretion of TH2 cytokines

Next we investigate secretion of different T-cell cytokines in cell supernatants of RNase 7M treated T-cells. Stimulation of polarised TH2 cells with RNase 7M significantly reduced the release of IL-13, IL-4 and IL-5 (Fig. 2 a-c). Activated CD4+ T-cells secreted lower amounts of TH2 cytokines, still a significant reduction of IL-5 and IL-13 was detectable after stimulation with RNase 7M (Fig. 2 d/e). Amounts of IL-4 released by activated CD4+ T-cells were too low for evaluation. In concordance with the microarray data IFN γ and IL-17 secretion were not significantly regulated by RNase 7 M in activated CD4+ T-cells (Fig. 2 f/g). To assure that the inhibition of TH2 cytokine production was not due to cell death or enhanced apoptosis in the presence of RNase 7M, we stimulated CD4+ T-cells with increasing amounts of RNase 7M and measured binding of annexin V and uptake of propidium iodide by FACS analysis. We did not detect higher binding of annexin V or propidium iodide uptake in RNase 7M stimulated T-cells compared to control after 2d (supplementary Fig. S1 a/b).

Furthermore we analysed the influence of RNase 7M on T-cell activation. We found that T-cell proliferation was not influenced by RNase 7M stimulation as well as production of IL-2 (supplementary Fig. S1 c/d).

Downregulation of TH2 cytokine production by native RNase 7

To exclude that regulation of TH2 cytokines by recombinant mutated RNase 7 observed in our previous experiments is due to unique properties of the manipulated RNase 7 we repeated our experiments with native, skin-derived RNase 7. Treatment with RNase 7 clearly reduced the release of IL-13, IL-4 and IL-5 by polarised TH2 cells and the release of IL-13 and IL-5 by activated CD4+ T-cells (Fig. 3). Again, amounts of IL-4 released by activated CD4+ T-cells were too low for evaluation.

CD4+ T-cells from AD patients show reduced reactivity to RNase 7M compared to healthy control.

A previous study showed that different AMPs including RNase 7 are upregulated in acute and chronic skin lesions of patients with atopic dermatitis compared to healthy controls [4, 20]. Here we investigated if T-cells from donors with atopic dermatitis show a different reaction to stimulation with RNase 7 with regard to the regulation of TH2 cytokines. We found that stimulation of CD4+ T-cells from AD patients with RNase 7M reduced the production of IL-13 in most donors. However, when compared to healthy donors the downregulation of IL-13 was significantly less pronounced (Fig.4).

RNase 7 treatment regulates the TH2 cytokine production of skin resident T-cells

T-cells are a normal constituent of human skin (Fig.5a). To investigate the influence of RNase 7 on the TH2 cytokine production of skin resident T-cells we cultured human skin samples from healthy donors in a TH2 polarizing medium. This treatment enhanced the mRNA expression of IL-13 by skin resident T-cells as shown by PCR analysis (Fig.5b). For the experiment punch biopsies from the

TH2 polarized skin were taken and incubated with RNase 7 or medium alone for 24h. PCR analysis revealed that the expression of IL-13 and IL-5 mRNA was reduced in RNase 7 treated skin compared to untreated controls (Fig.5c/d).

Stimulation with RNase 7 leads to reduced GATA3 activation.

Because GATA3 is an important regulator of T-cell development and promotes the expression of TH2 cytokines we assumed that RNase 7 might have an impact on GATA3 expression or activation. Therefore, we stimulated activated TH2 cells with RNase 7M and determined GATA3 expression by PCR and FACS analysis. As shown in Fig. 6 we found no regulation of GATA3 expression on mRNA level. In addition, FACS analysis showed no differences in GATA3 expression between RNase 7M stimulated and non-stimulated TH2 cells. To analyze whether RNase 7 influences GATA 3 activation, EMSA was performed to detect recruitment of GATA3 to the nucleus. Indeed, stimulation of TH2 cells with native RNase 7 reduced the amount of nuclear DNA binding GATA3 compared to positive control (Fig. 6c).

Discussion

Several human members of the RNase A family have been associated with antimicrobial properties [21]. Of note, additional immunoregulatory functions have been described recently for RNase 2 such as chemotaxis maturation and activation of human dendritic cells [22-24]. In the present study, we demonstrate for the first time that the antimicrobial peptide RNase 7 has immunoregulatory functions in human T-cells. RNase 7 is one of the major antimicrobial peptides produced in human skin with the highest concentration in the stratum corneum. Analysis of washing fluids from healthy human skin showed an abundant secretion of up to 3ng/cm² depending on the skin location [3]. The expression of RNase 7 is significantly upregulated in lesional skin of patients with psoriasis and atopic dermatitis. Up to 10 ng/ml RNase 7 have been detected in washing fluids from skin lesions from both groups of patients [4]. The expression of RNase 7 in the epidermis was reported to be considerably higher. While 4-8µg RNase 7/g can be isolated from normal stratum corneum extracts, up to 10-25µg/g RNase 7 are found in psoriatic

scales [25]. In our experiments we observed a clear downregulation of TH2 cytokine production after stimulation of T-cells with 0.1µg/ml RNase 7. Based on the work of Harder et al. we therefore expect that our experimental setup reflects physiological conditions in terms of RNase 7 concentrations used in our experiments.

Most antimicrobial peptides exert their antimicrobial activity by disrupting the microbial cell membrane. This raised the question if AMPs might also show cytotoxic activities against eukaryotic cells. Indeed, cytotoxic activities have been reported for the neutrophil-derived antimicrobial peptides neutrophil α -defensins and LL-37 [26] and RNase 3 [27]. However, our data show that RNase 7 in concentrations up to 10µg/ml exhibited no cytotoxic activity on T-cells and did not influence T-cell proliferation or IL-2 production.

RNase 7 selectively reduced the production of TH2 cytokines in our experimental setup. Furthermore, we found evidence that the regulation of TH2 cytokines was mediated by a reduced GATA3 activation. This indicates that the regulation of T-cell cytokine production is a specific process which might include binding of RNase 7 to a yet unknown receptor.

Several skin-derived AMPs have been shown to act on different cell types, including T-cells, by binding to specific receptors. The human beta-defensins (hBD)-2 and -3 have been described to be chemotactic for a broad spectrum of leukocytes in a CCR6- and CCR2-dependent manner including memory T-cells [16, 28]. Furthermore, a recent work showed that hBD-2 influenced T-cell cytokine production and enhances IFN γ , and IL-10 production and suppresses IL-17 production in T-cells by binding to PTX (pertussis toxin)-sensitive G protein coupled receptors [15]. In addition hBD-2 has been described to enhance the production of TH2 cytokines in activated T-cells [29]. The S100 protein psoriasin was found to be a potent and selective chemotactic inflammatory protein for CD4+ T-cells and neutrophils mediated by the activation of RAGE (receptor for advanced glycation end products) a recently identified receptor for psoriasin [30]. Another keratinocytes derived AMP with chemotactic activity is the cathelicidin LL-37. LL-37 selectively induced chemotaxis of CD4+ T-cells, monocytes and neutrophils by acting on Formyl Peptide Receptor-like 1 (FPRL1) [31]. Interestingly, chemotactic activity for

dendritic cells has been reported for RNase 2, a member of the RNase A family which can be inhibited by pretreatment with pertussis toxin [24]. This led us to the assumption that RNase 7 might act on a G protein coupled receptor. However, we found no chemotactic activity of RNase 7 on human T-cells subsets or monocytes (Supplementary Figure 2) and the regulation of TH2 cytokine production was not recovered after treatment with PTX (data not shown) excluding a G protein coupled receptor as putative receptor for RNase 7. Therefore, the exact mechanism of the observed interaction between RNase 7 and human TH2 cells remains unclear and has to be elucidated in further studies.

It is known that the antimicrobial activity of RNase 7 and RNase 3 is independent from their ribonuclease activity [3, 32]. In contrast, experiments indicate that the ribonuclease activity might be required for the chemotactic activity of RNase 2 [24]. To investigate the role of the RNase activity for the regulation of TH2 cell cytokines we used an inactive RNase 7 mutant. Our results show that the regulatory effect of RNase 7 on TH2 cytokine production does not depend on RNase activity.

Our data indicate that RNase 7 can influence the TH2 cytokine production of skin infiltrating T-cells. RNase 7 has also been shown to be upregulated in acute skin lesions from patients with atopic dermatitis which are characterised by a TH2 cytokine milieu [20] [4]. Interestingly, compared to healthy donors, CD4+ T-cells from AD patients revealed a significantly less pronounced downregulation of IL-13 in response to RNase 7. Activated CD4+ T-cells from patients with atopic dermatitis showed a general higher expression of IL-13 (mean = 2182pg/ml AD vs. 1553pg/ml healthy). However, we observed no relation between the basic level of IL13 production and intensity of the reduction in both groups. Therefore, a potential dysregulation of the RNase-7-activated signal transduction pathway or a disturbed expression of a putative RNase 7 receptor might be the reason for the less pronounced regulation of IL-13 production in T-cells from AD patients. The relatively lower expression of RNase 7 in skin lesions of AD patients, compared to patients with psoriasis, and the observed reduced responsiveness of CD4+ T-cells from AD patients to RNase 7 stimulation might contribute to the pathogenesis of atopic dermatitis. To understand the role of RNase 7 as an immunomodulator, its mode of action

and its importance in chronic inflammatory skin disease further studies are needed.

Conflict of interest:

M. Kleine is the head of a company which produced R7 and R7M for commercial reasons. All other authors state no conflict of interest

Acknowledgement:

Microarray raw data used or referred to in this publication were generated by the Research Core Unit Transcriptomics of Hannover Medical School.

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Author contributions

VK is the primary author and conducted data collection, analysis, interpretation of the data, and writing the first draft of the manuscript. VK performed the experiments analyzing GATA3 expression and the GATA3 EMSA. LB performed the experiments analyzing the regulation of IL-13 and IL-4 by RNase 7. SW performed the experiments analyzing the regulation of IL-5 by RNase 7.

SW and LB and JH made contributions to the interpretation of the data. JH provided the native RNase 7. KH and MK provided the recombinant RNase 7 and RNase 7 mutant. JH, TW, MK and KH made significant and substantial contributions to the conception, design, and interpretation of the data. All authors reviewed, revised, and approved the manuscript for publication.

1. Harder, J. and J.M. Schroder, *Antimicrobial peptides in human skin*. Chem Immunol Allergy, 2005. **86**: p. 22-41.
2. Harder, J. and J.M. Schroder, *RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin*. J Biol Chem, 2002. **277**(48): p. 46779-84.
3. Koten, B., et al., *RNase 7 contributes to the cutaneous defense against Enterococcus faecium*. PLoS One, 2009. **4**(7): p. e6424.

- Accepted Article
4. Harder, J., et al., *Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury*. J Invest Dermatol, 2010. **130**(5): p. 1355-64.
 5. Simanski, M., et al., *RNase 7 protects healthy skin from Staphylococcus aureus colonization*. J Invest Dermatol, 2010. **130**(12): p. 2836-8.
 6. Werfel, T., *The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis*. J Invest Dermatol, 2009. **129**(8): p. 1878-91.
 7. Prinz, J.C., *The role of T cells in psoriasis*. J Eur Acad Dermatol Venereol, 2003. **17**(3): p. 257-70.
 8. Trautmann, A., et al., *T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis*. J Clin Invest, 2000. **106**(1): p. 25-35.
 9. Nomura, I., et al., *Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes*. Journal of immunology, 2003. **171**(6): p. 3262-9.
 10. Howell, M.D., et al., *Mechanism of HBD-3 deficiency in atopic dermatitis*. Clinical immunology, 2006. **121**(3): p. 332-8.
 11. Simanski, M., et al., *IL-17A and IFN-gamma synergistically induce RNase 7 expression via STAT3 in primary keratinocytes*. PLoS One, 2013. **8**(3): p. e59531.
 12. Morizane, S. and R.L. Gallo, *Antimicrobial peptides in the pathogenesis of psoriasis*. J Dermatol, 2012. **39**(3): p. 225-30.
 13. Ong, P.Y., et al., *Endogenous antimicrobial peptides and skin infections in atopic dermatitis*. N Engl J Med, 2002. **347**(15): p. 1151-60.
 14. Jinquan, T., et al., *Psoriasin: a novel chemotactic protein*. J Invest Dermatol, 1996. **107**(1): p. 5-10.
 15. Kanda, N., et al., *Human beta-defensin-2 enhances IFN-gamma and IL-10 production and suppresses IL-17 production in T cells*. Journal of leukocyte biology, 2011. **89**(6): p. 935-44.
 16. Yang, D., et al., *Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6*. Science, 1999. **286**(5439): p. 525-8.
 17. Gutzmer, R., et al., *The histamine H4 receptor is functionally expressed on T(H)2 cells*. J Allergy Clin Immunol, 2009. **123**(3): p. 619-25.
 18. Edgar, R., M. Domrachev, and A.E. Lash, *Gene Expression Omnibus: NCBI gene expression and hybridization array data repository*. Nucleic Acids Res, 2002. **30**(1): p. 207-10.
 19. Wittmann, M., et al., *Cell-to-cell contact between activated CD4+ T lymphocytes and unprimed monocytes interferes with a TH1 response*. J Allergy Clin Immunol, 2004. **114**(4): p. 965-73.
 20. Gambichler, T., et al., *Differential mRNA expression of antimicrobial peptides and proteins in atopic dermatitis as compared to psoriasis vulgaris and healthy skin*. Int Arch Allergy Immunol, 2008. **147**(1): p. 17-24.

- Accepted Article
21. Gupta, S.K., et al., *The mammalian secreted RNases: mechanisms of action in host defence*. *Innate Immun*, 2013. **19**(1): p. 86-97.
 22. Yang, D., et al., *Human ribonuclease A superfamily members, eosinophil-derived neurotoxin and pancreatic ribonuclease, induce dendritic cell maturation and activation*. *J Immunol*, 2004. **173**(10): p. 6134-42.
 23. Yang, D., et al., *Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances Th2 immune responses*. *J Exp Med*, 2008. **205**(1): p. 79-90.
 24. Yang, D., et al., *Eosinophil-derived neurotoxin (EDN), an antimicrobial protein with chemotactic activities for dendritic cells*. *Blood*, 2003. **102**(9): p. 3396-403.
 25. Harder, J. and J.M. Schroder, *Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins*. *J Leukoc Biol*, 2005. **77**(4): p. 476-86.
 26. Aarbiou, J., et al., *Mechanisms of cell death induced by the neutrophil antimicrobial peptides alpha-defensins and LL-37*. *Inflamm Res*, 2006. **55**(3): p. 119-27.
 27. Navarro, S., et al., *The cytotoxicity of eosinophil cationic protein/ribonuclease 3 on eukaryotic cell lines takes place through its aggregation on the cell membrane*. *Cell Mol Life Sci*, 2008. **65**(2): p. 324-37.
 28. Rohrl, J., et al., *Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2*. *Journal of immunology*, 2010. **184**(12): p. 6688-94.
 29. Kanda, N. and S. Watanabe, *Increased serum human beta-defensin-2 levels in atopic dermatitis: relationship to IL-22 and oncostatin M*. *Immunobiology*, 2012. **217**(4): p. 436-45.
 30. Wolf, R., et al., *Chemotactic activity of S100A7 (Psoriasin) is mediated by the receptor for advanced glycation end products and potentiates inflammation with highly homologous but functionally distinct S100A15*. *Journal of immunology*, 2008. **181**(2): p. 1499-506.
 31. De, Y., et al., *LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPR1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells*. *J Exp Med*, 2000. **192**(7): p. 1069-74.
 32. Zhang, J., K.D. Dyer, and H.F. Rosenberg, *Human RNase 7: a new cationic ribonuclease of the RNase A superfamily*. *Nucleic Acids Res*, 2003. **31**(2): p. 602-7.

Legends to figures

Figure 1: a) anti-CD3/ CD28 activated human CD3⁺ T-cells from four donors were stimulated with a ribonuclease inactive RNase 7 mutant (R7M) 10µg/ml for 6h. Pooled mRNA was subjected to mRNA microarray analysis. Mean ratios for the different cytokines are shown. Polarised TH2 cells were stimulated with the indicated concentrations of RNase 7M (R7M) for 6h. mRNA expression of TH2 cytokines (b) IL-13 (n=8) and (c) IL-4 (n=6) and (d) IL-5 (n=4) was analysed by PCR and normalized to the housekeeping gene GAPDH. e) Isolated human CD4⁺ T-cells were stimulated with anti-CD3/CD28 to activate T-cells for 24h. Afterwards T-cells were incubated with recombinant RNase 7 (R7) for 30 min and binding of R7 to T-cells was detected by staining with an anti-RNase 7 antibody by FACS analysis (n=2). Bars indicate the median values

Figure 2: Polarised, TH2 cells (a, n=21/ b, n= 9/ c, n=6) and human CD4⁺ T-cells (d, n=22/ e, n=20/ f, n=6/ g, n=12) were stimulated with anti-CD3/ CD28 and RNase 7M (R7M) for 3d. Supernatants were harvested and the indicated cytokines were measured by ELISA. Bars indicate the median values (a/b/d/e/f/g) or mean values (c).

Figure 3: Polarised TH2 cells (a, n=8/ b, n=9/ c, n=5) and human CD4⁺ T-cells (d, n=7/ e, n=3) were stimulated with anti-CD3/ CD28 and skin derived native RNase 7 (R7) for 3d. Supernatants were harvested and the indicated cytokines were measured by ELISA. Bars indicate the median values (a/b/e) or the mean values (c/d).

Figure 4: Anti-CD3/CD28 activated human CD4⁺ T-cells from AD patients and healthy donors were stimulated with RNase 7M (R7M) for 3d. Supernatants were harvested and IL-13 production was measured by ELISA. Downregulation of IL-13 was normalized to unstimulated control (n=10). Bars indicate the median values.

Figure 5: (a) Immunohistological staining of CD3+ T-cells in normal human skin (black arrows, objective 10x). (b) Normal human skin was cultured in medium (control) or TH2 polarizing medium for 48h. Expression of IL-13 mRNA was analysed by PCR and normalized to the housekeeping gene RPS21. (c/d) human skin was cultured in TH2 polarizing medium for 48h to enhance TH2 cytokine mRNA expression. Afterwards punch biopsies were taken and incubated with native RNase 7 (R7) or were left untreated (n.s.) for 24h. IL-13 and IL-5 mRNA expression was analysed by PCR and normalized to CD4 (n=3).

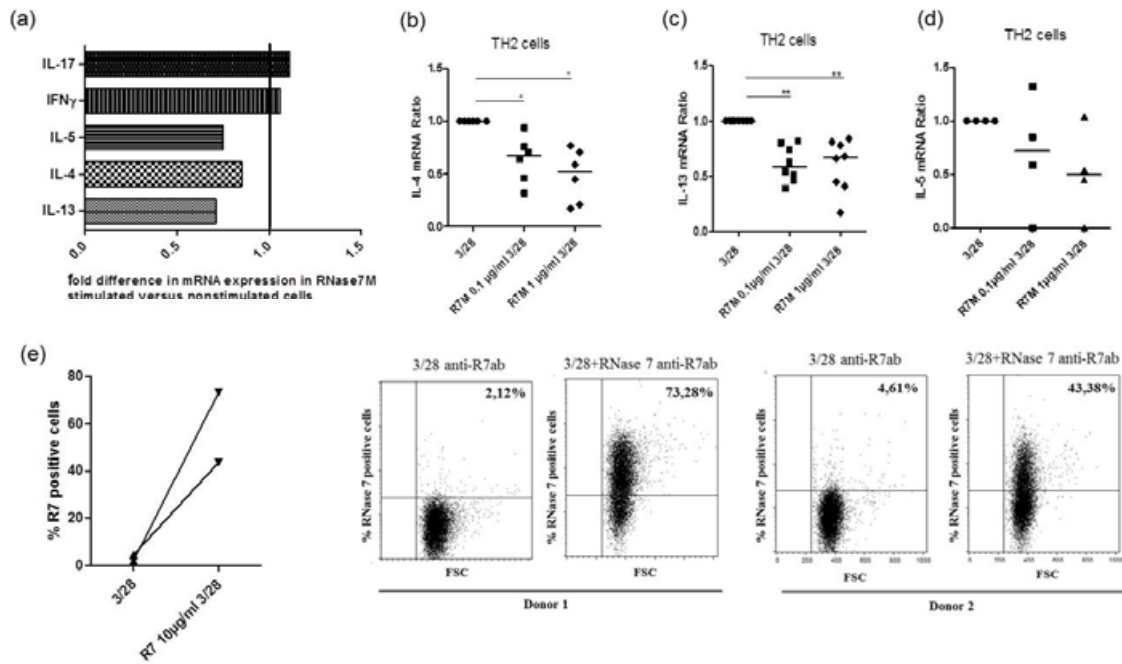
Figure 6: (a) anti-CD3/CD28 activated TH2 cells were stimulated with RNase 7M for 6h. GATA3 mRNA expression was measured by PCR (n=7). (b) anti-CD3/CD28 activated TH2 cells were stimulated with RNase 7M for 24h. GATA3 expression was measured by FACS analysis (n=4). A representative experiment out of four experiments is shown. (c) anti-CD3/CD28 activated TH2 cells were stimulated with skin derived RNase 7 for 24h. Nuclear extracts were harvested and GATA3 binding to a GATA3 specific oligonucleotide compared to non-stimulated control (ns) was analyzed by EMSA. Where indicated control oligonucleotide (cold probe) was added (n=3). The EMSA was analysed with quantitative densitometry (n=3).

Supplementary Figure S1: Polarised TH2 cells were stimulated with the indicated concentrations of RNase 7 mutant (R7M) and anti-CD3/ CD28 for 6h. The mRNA expression of IL-13 was analysed by PCR and normalized to the housekeeping gene GAPDH (n=4). Bars indicate the median values.

Supplementary Figure S2: Anti-CD3/ CD28 activated human CD4+ T-cells were stimulated with R7M for 24h. Viability was analysed by annexin V staining (a, n=3) and propidium iodide staining (b, n=4). (c) anti-CD3/ CD28 activated human CD4+ T-cells were stained with CFSE and stimulated with R7M. Proliferation was analysed by FACS after 5d (n=11). (d) anti-CD3/ CD28 activated human CD4+ T-cells were stimulated with R7M for 3d. Supernatants were harvested and IL-2 production was measured by ELISA (n=6). Bars indicate the median values (a/c) or mean values (b/d).

Supplementary Figure S3: Anti-CD3/ CD28 activated human CD3⁺ T-cells (a) and human monocytes (b) were used for a chemotaxis assay with RNase 7 mutant (R7M) 1 μ g/ml as chemoattractant for 3 h (n=4).

Fig. 1: regulation of T-cell cytokines by RNase 7



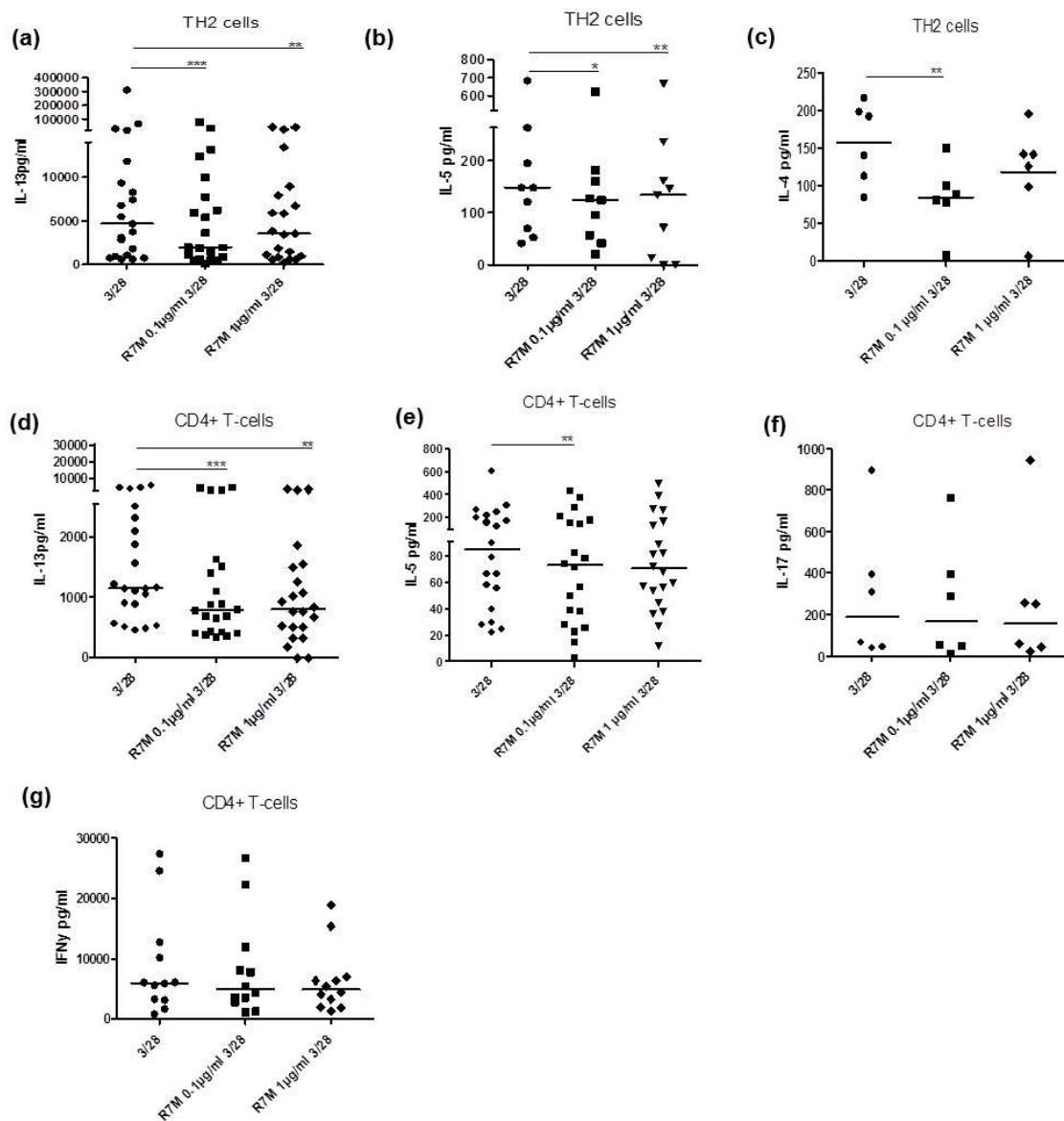
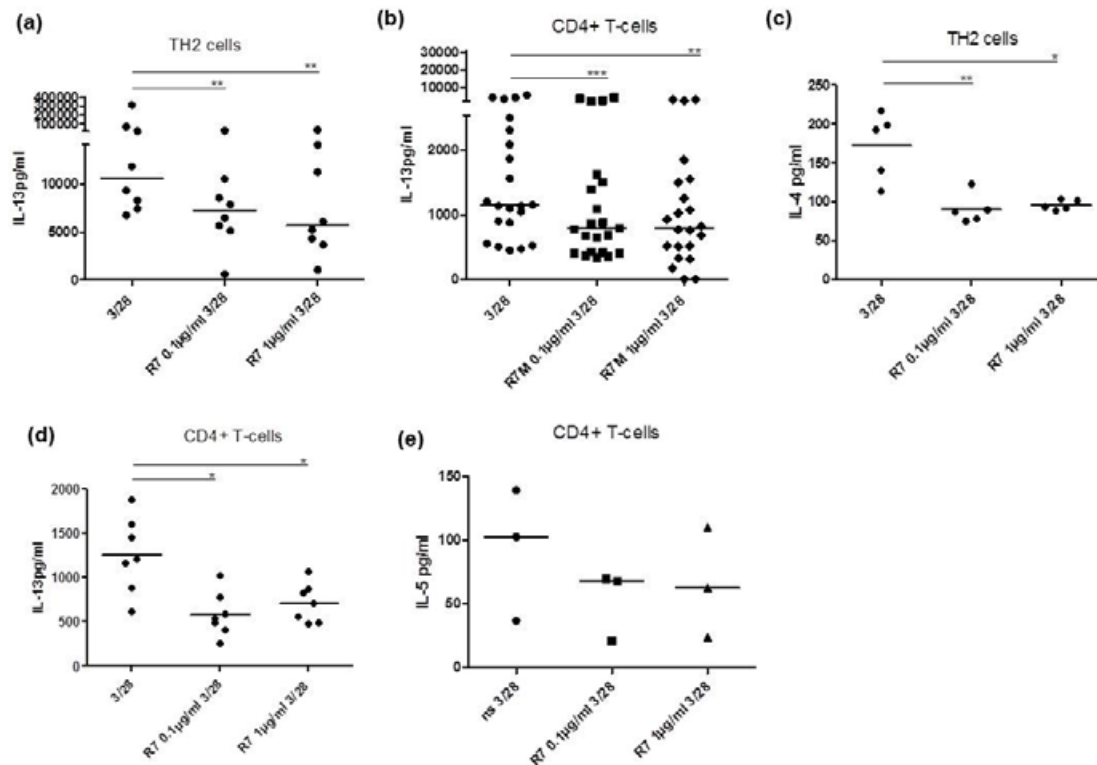


Fig.3: Downregulation of TH2 cytokines by native RNase 7



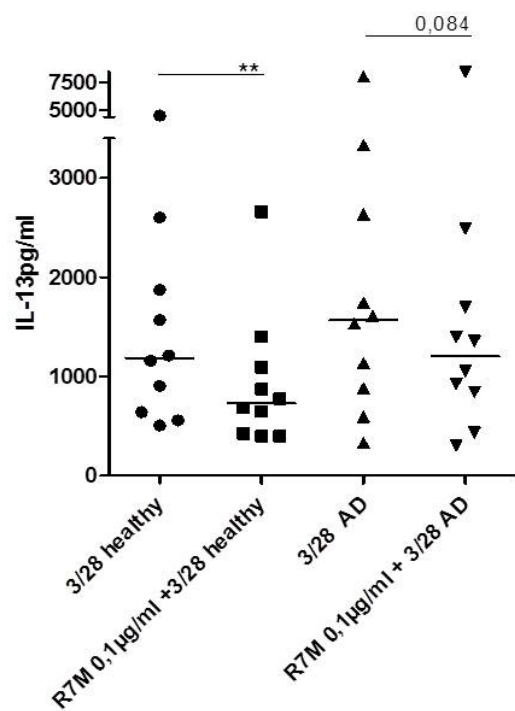


Fig.5: RNase 7 treatment regulates the TH2 cytokine production of skin resident T-cells

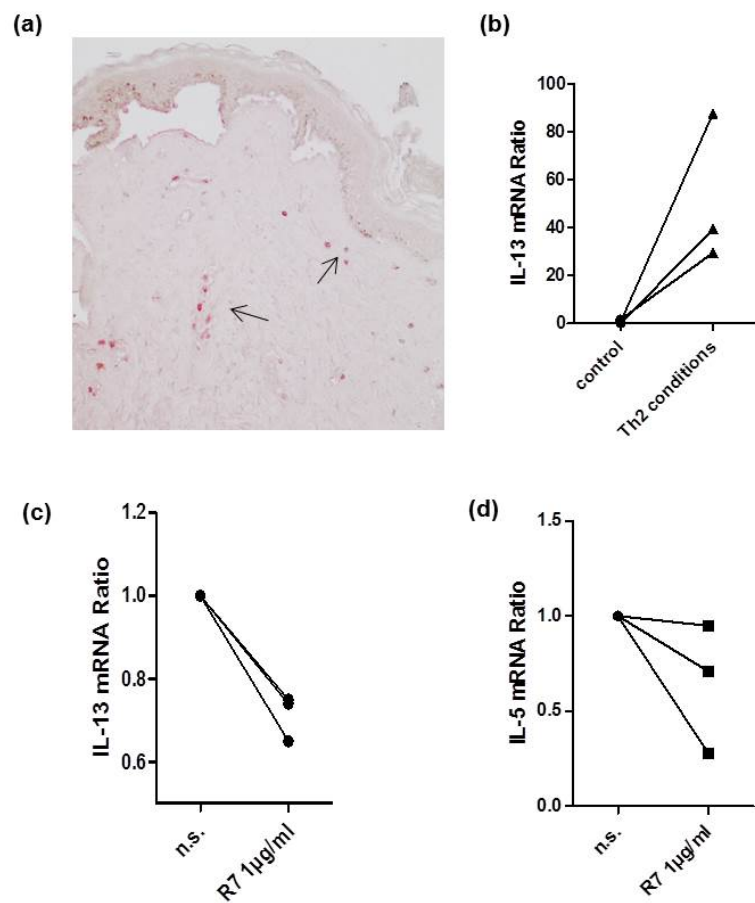


Fig.6: Stimulation with RNase 7 leads to reduced GATA3 activation.

