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microRNA-146a is linked to the production of IgE in mice but not in atopic dermatitis patients

To the Editor,

Atopic dermatitis (AD) is a chronic and complex inflammatory skin disease characterized by pruritus and eczema. Its incidence has increased rapidly during the last 50 years and nowadays it affects up to 20% of children and 5% of adults in developed countries.^{1,2} According to genetic and molecular studies, AD manifestation is associated with skin barrier weakness and susceptibility to sensitization of the immune system to environmental and/or intrinsic factors acting in the skin. Although AD is usually treated as a singular condition, it is

mechanistically a very complex disease and at least two AD subtypes can be distinguished: allergic type (also named as extrinsic), affecting approximately 75% of patients, is characterized by a strong type-2-cell-mediated immune response and remarkably high levels of serum immunoglobulin E (IgE), whereas nonallergic type (also named as intrinsic) is characterized by normal IgE levels.^{1,3} All processes contributing to the development of AD, including innate and adaptive immune responses, can also be influenced by microRNAs.⁴ Among other immune-regulatory microRNAs (miRNAs), miR-146a has been shown to

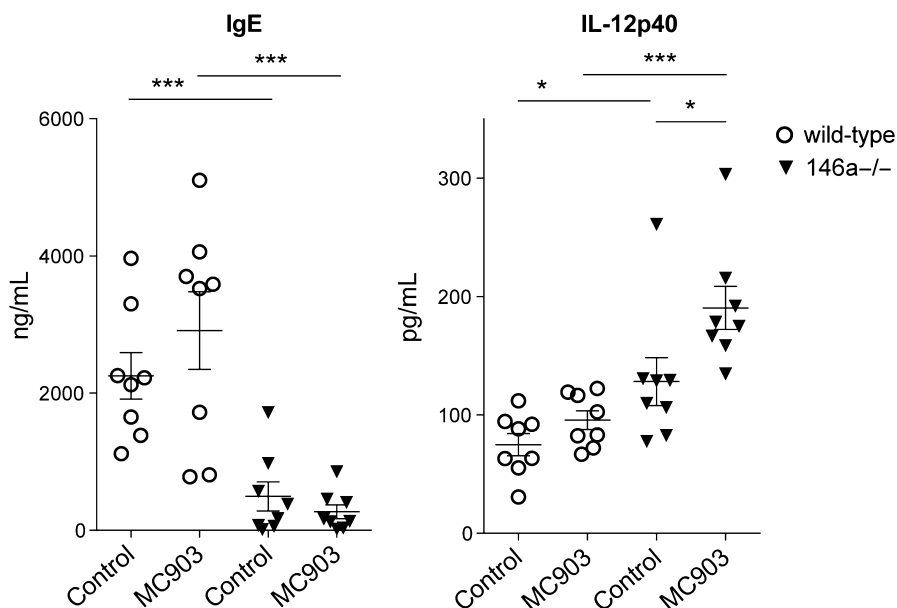


FIGURE 1 Decreased serum IgE levels and increased serum IL-12p40 levels in miR146a^{-/-} mice. Serum IgE and IL-12p40 levels in MC903 and control treated wild-type and miR146a^{-/-} mice. Results from one of two independent experiments are shown. Data are presented as mean with SEM, unpaired student *t* test, **P* < 0.05, ****P* < 0.001

impact various inflammatory processes and miR-146a-deficient mice spontaneously develop autoimmunity at 6–8 months of age.⁵ T cells lacking of miR-146a lead stronger Th1/Th17-type immune responses in mice,⁶ overexpression of miR-146a at organism level induces class switch in B cells and overproduction of IgE in mice⁷ and in a mouse model of AD, we have previously described more severe skin inflammation in miR-146a-deficient mice without the effect on type-2-cell-mediated immune responses in the skin.⁸ However, whether miR-146a has influence on the production of IgE and to the formation of immunological AD phenotype has not been studied before. In the current report, we examined whether there is an association between miR-146a, IgE and cytokine levels using serum samples from miR-146a^{-/-}

and wild-type mice with AD-like skin inflammation induced by cal-cipotriol hydrate (MC903) that we have described earlier⁸ as well as human serum samples from AD patients (See Supplementary Methods section).

Results presented in Figure 1 demonstrate decreased serum IgE levels and increased serum IL-12p40 levels in miR-146a^{-/-} mice compared with wild-type mice ($P < 0.001$ and $P < 0.05$, respectively), which were highly significant comparing MC903-treated mice ($P < 0.001$). IL-4 and IL-13 were almost nondetectable (data not shown) and we did not observe any differences on other detectable cytokines or chemokines, such as IL-5, IL-6, IL-17, CCL5, CXCL1 and granulocyte-colony stimulating factor (G-CSF) between miR-146a^{-/-} mice and wild-type mice, and

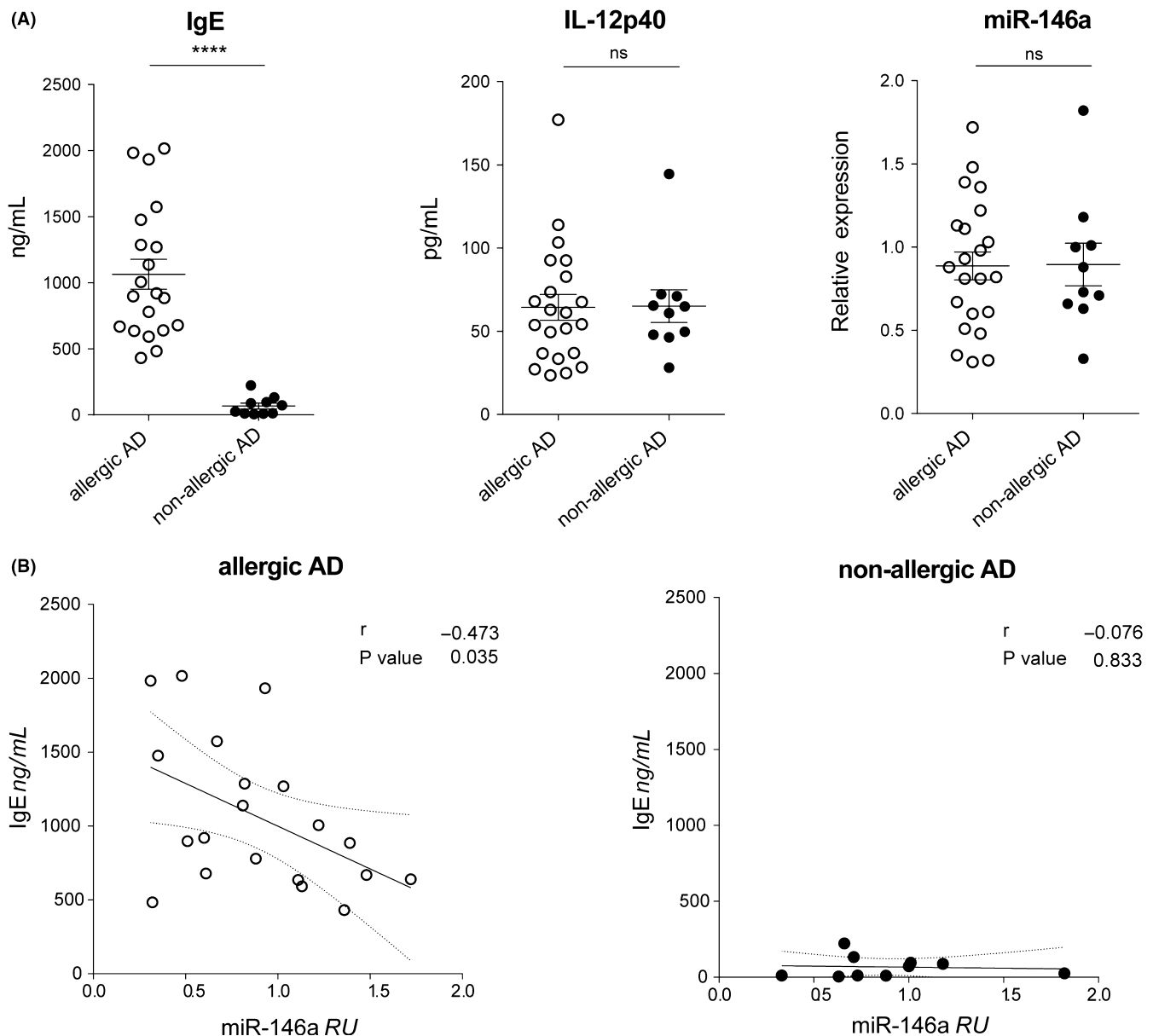


FIGURE 2 Negative correlation between miR-146a expression and serum IgE levels in allergic AD patients. (A), Serum IgE and IL-12p40 levels, and miR-146a relative levels were compared between allergic (IgE > 360 ng/mL) and nonallergic (IgE < 360 ng/mL) AD subgroups. Data are represented as mean with SEM, unpaired student *t* test, **** $P < 0.0001$. (B), Bivariate linear regression analysis graphs of miR-146a relative expression and serum IgE levels in allergic and nonallergic AD subgroups. Pearson's *r* and *P* values are shown; significance level is set at $P < 0.05$. Dotted line represents SEM

MC903 treatment (Figure S1). Decreased serum IgE level in miR-146a^{-/-} mice demonstrates that miR-146a may have a role in the production of IgE, which is in concordance with a previous study showing increased serum IgE due to class switch in B cells in miR-146a-overexpressing transgenic mice.⁷ The observation that IL-12p40, a component of both IL-12 and IL-23 cytokines,⁹ was increased in MC903-treated miR-146a^{-/-} mice indicates that mice lacking miR-146a enhance either IL-12 and/or IL-23 signalling related to type-1/17-cell-mediated immunity. However, as we did not observe any correlation between the levels of IgE and IL-12p40 in miR-146a^{-/-} mice with MC903-induced AD-like symptoms (Figure S2), these characteristics appear to be at least partially independent. A recent study has demonstrated reduction of marginal zone B cells in miR-146a deficient mice indicating that miR-146a is also needed for proper development of B cells.¹⁰ Taking into account our and previous findings, we suggest that miR-146a may affect the production of IgE both through its direct effect on B cell development and influence on type-1/17-cell-mediated immune responses.

As previous⁷ and current miR-146a-deficient mice results suggested a link between serum IgE level and miR-146a expression, we next analyzed a set of AD patients for serum miR-146a and IgE levels. First, serum samples from 32 AD patients were split into allergic and nonallergic subgroups according to the IgE levels ($P < 0.0001$, Figure 2A). No significant differences in miR-146a and IL-12p40 levels (Figure 2A) and other characteristics between these groups were found aside from higher female representation in nonallergic AD ($P < 0.05$, Table S1). However, relative serum miR-146a levels were negatively associated with serum IgE levels in allergic AD patients ($P < 0.05$, Figure 2B). In this subgroup, miR-146a expression remained independently associated with IgE levels ($\beta = -0.483$, $P < 0.05$) after adjusting for confounding variables as gender, age of onset, and severity by multiple regression analysis (Table S2). No other correlations with miR-146a expression were found (Table S3). Although we observed limited production of IgE in mice lacking miRNA-146a, we did not observe differences in serum miR-146a levels between allergic and nonallergic patients; and also not between moderate and severe patients (data not shown). Both allergic and nonallergic subgroups presented similar characteristics for age of onset, duration of disease and severity in our study, hence, observed similar miR-146a levels are plausible. Thus, we suggest that in allergic patients with already high IgE levels, miRNA-146a could be acting as a feedback suppressor limiting type-2-cell-mediated responses, which would be in concordance with its already known feedback suppressor activity in inflammation. Alternatively, strong type-2-cell-mediated immune responses characterized by high IgE levels may limit the expression of miR-146a. Further studies will be needed as we cannot indicate causality with the present cross-sectional study. Finally, the lack of correlation between serum miR-146a and IL-12p40 as well as IgE and IL-12p40 levels in AD patients (Figure S3) points out that AD has a more complex regulatory network in humans as compared to mice.

In conclusion, low serum IgE and high serum IL-12p40 levels in miR-146a^{-/-} mice indicate that miR-146a is needed for the production of IgE and associated with the regulation of type-1/17-cell-mediated immune responses in mice. Negative association of miR-

146a with serum IgE in allergic AD patients suggests that miR-146a might have capacity to limit type-2-cell-mediated immune responses in a subgroup of AD patients with increased IgE. In future studies, related cytokines and cell types involved in the relationship between miR-146a expression and serum IgE levels should be examined in a larger cohort to ascertain the hypotheses made in the current report and to delineate the function of miR-146a in the regulation of immune responses in AD.

ACKNOWLEDGMENTS

This work was supported by European Regional Development Fund (Project No. 2014-2020.4.01.15-0012) from European Union, institutional research grant IUT2-2 and personal research grants PUT1669 and PUT1465 from Estonian Research Council, Leo grant (LF17040) from Leo Foundation; and the Christine Kühne-Center for Allergy Research and Education (CK-CARE) in Davos, Switzerland.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ORCID

Gemma Carreras-Badosa  <http://orcid.org/0000-0003-2100-0221>
Ana Rebane  <http://orcid.org/0000-0001-6051-1361>

Gemma Carreras-Badosa¹ 
Toomas Runnel¹
Mario Plaas¹
Jaanika Kärner^{1,2}
Beate Rückert³
Freddy Lättekivi¹
Sulev Kõks¹
Cezmi A. Akdis³
Küllü Kingo⁴
Ana Rebane¹ 

¹Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

²Department of Medicine, Karolinska Institutet, Solna, Sweden

³Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Davos, Switzerland

⁴Department of Dermatology, University of Tartu and Dermatology Clinic, Tartu University Hospital, Tartu, Estonia

Correspondence: Ana Rebane, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 14B, Tartu 50411, Estonia (ana.rebane@ut.ee).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Staphylococcus aureus small colony variants: Prevalence in chronic rhinosinusitis and induction by antibiotics

To the Editor,

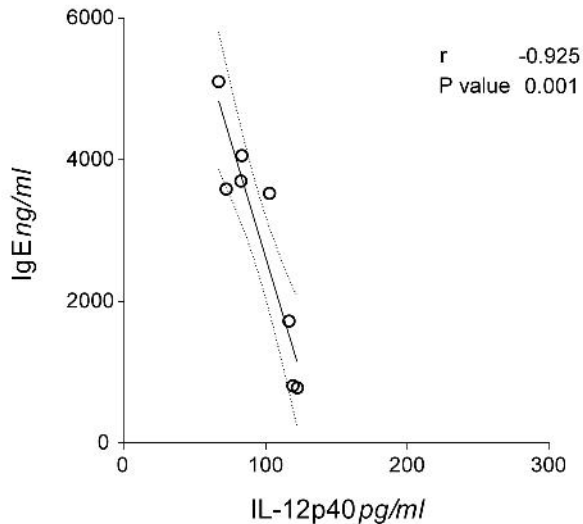
Staphylococcus aureus (*S. aureus*) small colony variants (SCVs) are an altered bacterial phenotype characterized by slow growth rate and small colony size. Due to reduced antimicrobial sensitivity and a capacity to invade host cells, *S. aureus* SCVs can escape immune defense mechanisms and contribute to therapy-refractory Staphylococcal infections (reviewed in Ref. 1). *Staphylococcus aureus* SCVs have been isolated from sites of several persistent and relapsing chronic infections such as osteomyelitis² and from within the nasal polyps of patients with chronic rhinosinusitis (CRS)³; however, the triggers and mechanisms of SCV formation are largely unknown. Previous work has demonstrated *S. aureus* can be induced into the SCV phenotype by exposure to different stressors in vitro^{2,4,5} and SCVs are also isolated from patients with ongoing antibiotic treatment.^{1,6-8} However, the incidence and characteristics of SCVs amongst *S. aureus* clinical isolates (CIs) from CRS patients and whether these isolates are induced into a SCV phenotype by clinically relevant antibiotics are not known. As antibiotics form the mainstay of treatment for CRS patients, this information is important to advise treatment options.

The purpose of this study was to determine the prevalence of *S. aureus* SCVs in CRS CIs, analyze the capacity of clinically relevant antibiotics to induce SCVs, and compare the characteristics of induced SCVs with their normal colony phenotype (NCP) parents. To achieve this, the SCV status of 66 CRS *S. aureus* CIs was determined and 11 types of antibiotics tested for their capacity to induce SCVs in representative CIs and the reference strain ATCC51650.

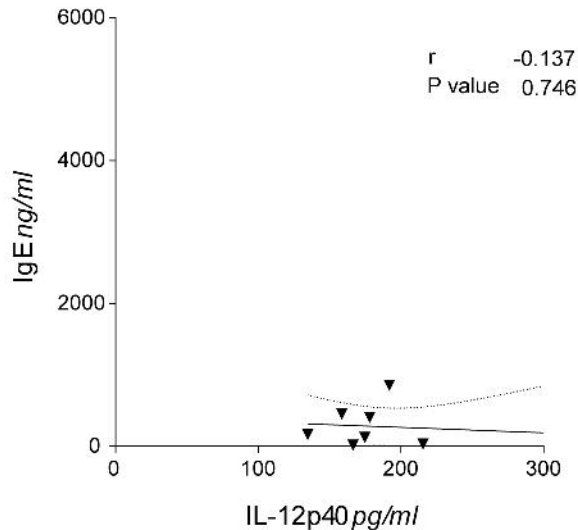
SCVs were defined by decreased colony size and reduced pigmentation compared to parent NCP. Biochemical activity as well as infection ability, antibiotic sensitivity, and stability was also tested. Details of materials and methods are found in this article's online repository. Of the 66 *S. aureus* CIs from CRS patients assessed for SCV phenotype, half (33/66) grew large-sized white or yellow colonies, typical of *Staphylococci* and 26 of 66 *S. aureus* CIs grew small (≤ 1 mm) colonies with some of these (5/26) appearing white in color and lacking hemolytic activity (SCVs). Mixed populations of small and large colonies were present in the remaining 7 of 66 CIs with three of these containing colonies with SCV phenotype (Table S1). Representative images of *S. aureus* CIs are shown in Figure S1.

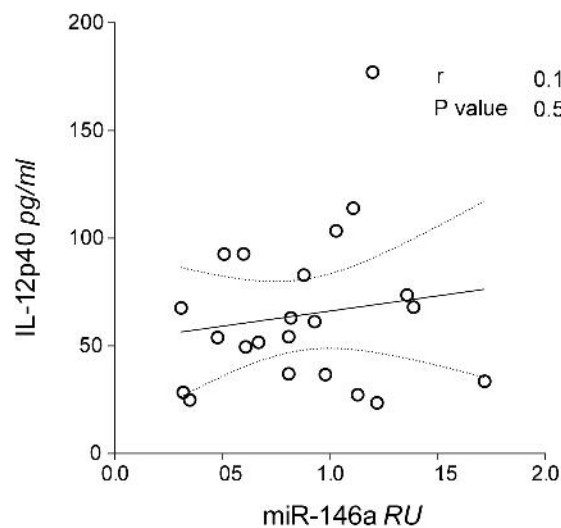
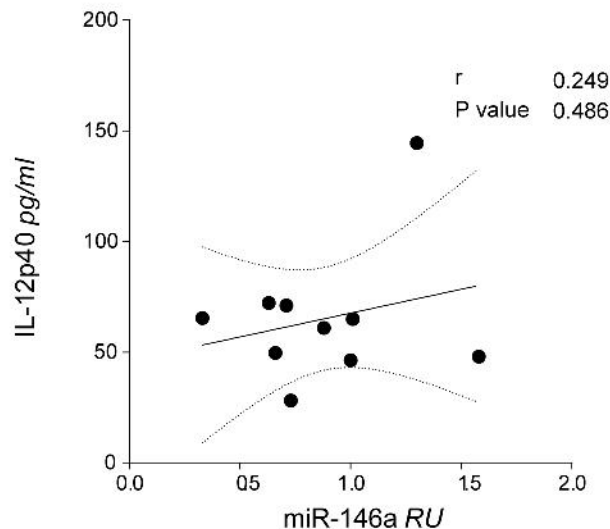
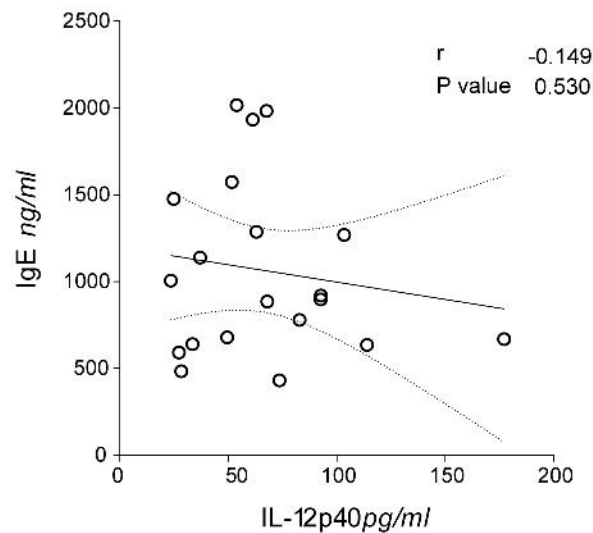
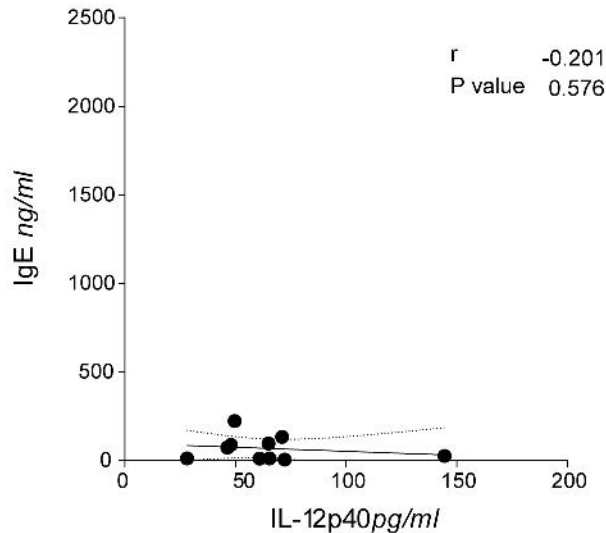
After SCV status was determined, we then tested 11 antibiotics for their capacity to induce SCVs of ATCC51650 and three representative CIs. The MIC of these antibiotics was determined and is shown in Table S2. Incubation with gentamicin was particularly effective at inducing SCVs. This was consistent with previous reports that have shown gentamicin exposure induces reversible rapid switching of gentamicin-sensitive strains into gentamicin-resistant SCVs.⁵ Isolates CI1 and ATCC51650 showed switching to SCV phenotype after as little as 1 hour at $\frac{1}{2}$ MIC whilst all isolates showed a gradual increase in SCVs over the 7-day period in continuous culture with two MIC gentamicin (Figure 1A and Figures S2 and S3). The other antibiotics tested did not induce SCVs at short exposure times (<24 hours). However, SCVs were induced by 24-hour incubation with clindamycin from three MIC to 10 MIC and by rifampicin from eight MIC to 10 MIC (Figure 1B-D). Using different protocols, Tuchscher et al² similarly showed gentamicin- and clindamycin-induced *S. aureus* SCV formation and reported a high

MC903-treated wild-type

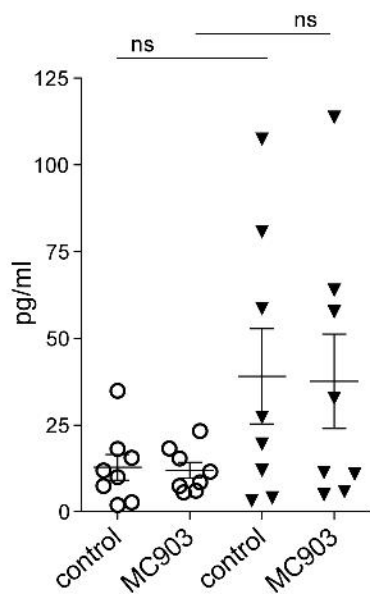


MC903-treated 146a^{-/-}

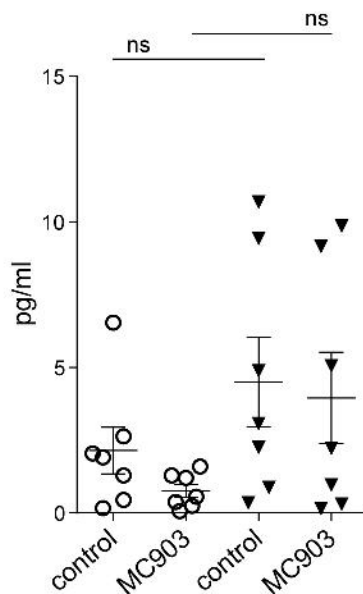


A**allergic AD****non-allergic AD****B****allergic AD****non-allergic AD**

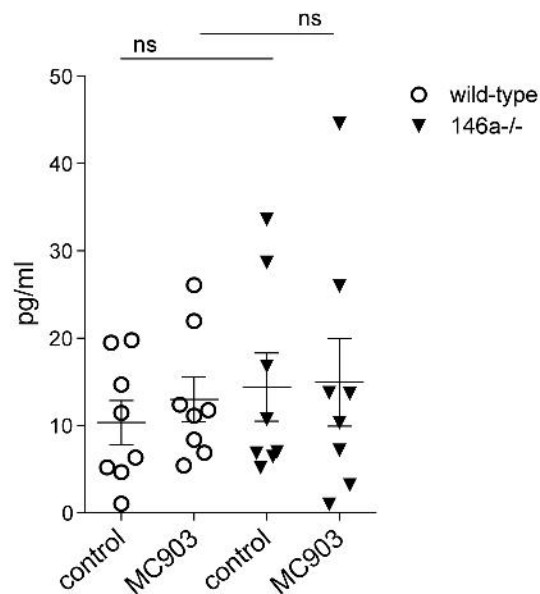
IL-5



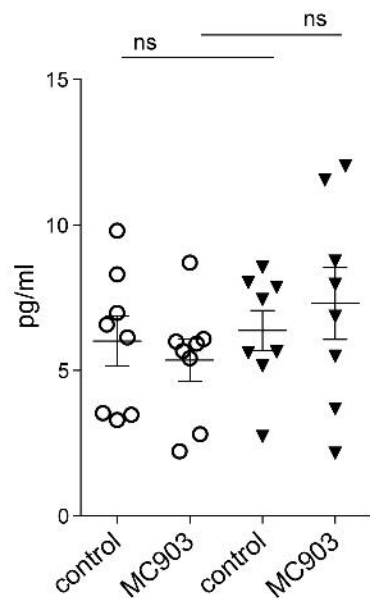
IL-6



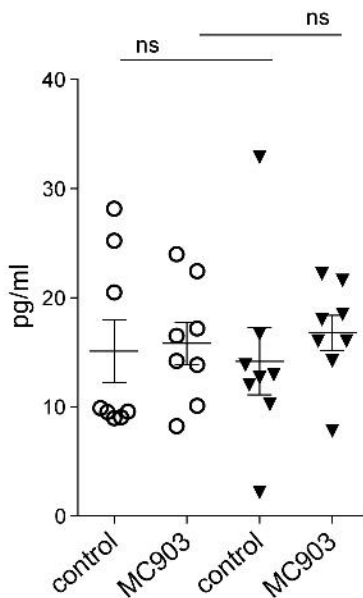
IL-17



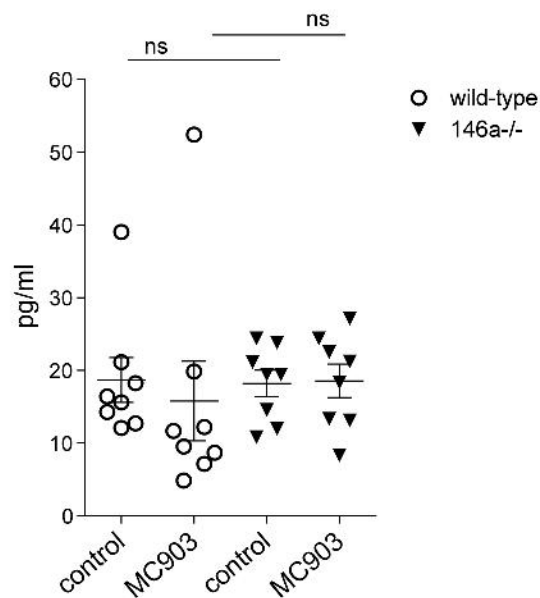
CCL5



CXCL1



G-CSF



SUPPLEMENTARY DATA

EXPERIMENTAL PROCEDURES

Serum samples from MC903-induced mouse model of AD

Serum samples were collected from the MC903-induced mouse model experiment described in regards of skin inflammation in our previous report (1). Briefly, nine-to-ten week-old female miR-146a^{-/-} knockout mice in C57BL/6J background and wild-type C57BL/6J mice (Jackson Laboratory, Bar Harbor, Me, USA) were used. To induce AD, the vitamin D3 analogue MC903 (calcipotriol hydrate, C4369, Sigma-Aldrich, St Louis, Mo, USA) dissolved in EtOH or the same amount of EtOH was topically applied on both ears every other day 10 times starting at day 0. Serum samples were collected on day 19. The sample size of each experimental group was 8 mice. The mice were maintained and bred in the animal facility at the Institute of Molecular and Cell Biology, University of Tartu, in accordance with the institute's regulations. Animal experiments were approved by the Animal Ethics Committee of the University of Tartu.

Patients

The study population consisted of 32 Caucasian patients diagnosed with AD. Subjects were recruited among those seen within a setting of a Dermatology clinic in Tartu University Hospital (Estonia). The protocol was approved by the Ethics Review Committee on Human Research of the University of Tartu. Informed written consent was obtained from all patients. Clinical examination was performed according to the protocol followed by venous blood sampling. Subjects were split into allergic (N=22) and non-allergic (N=10) AD subgroups according to IgE threshold of 150 IU/ml (equivalent to 360 ng/ml) (2). Age, gender, age of onset, disease duration, severity and information on comorbidities (respiratory diseases and others) is provided in Supplementary Table 1. All serum samples were collected at exacerbation stage prior to hospitalization of the patients. None of the patients used neither topical nor systemic medications for at least two weeks prior to the time of serum sample collection. Investigator's Global Assessment (IGA) overall assessment of the skin lesions ranging from clear (=0) to severe (=4) and focusing on signs of erythema, induration/papulation, lichenification and oozing/crusting was used to define the level of severity (3).

Analytical methods

Mouse blood samples were obtained by standard cardiac puncture and human serum samples were obtained by standard venepuncture. Separation of serum was performed by centrifugation at 1500g for 15 minutes at 4°C after 60 minutes clotting process at room temperature. All serum samples were kept frozen at -80°C until assay. Mouse serum IgE levels were analysed by ELISA using Mouse IgE ELISA MAX™ Deluxe kit (Biolegend, San Diego, CA, USA). Mouse serum cytokines were analysed using Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Biorad, Hercules, CA, USA). Human serum IgE and IL-12p40 levels were analysed by ELISA using Human IgE Platinum ELISA-BMS2097 (ThermoFisher, Waltham, MA, USA) and IL-12/IL-23 ELISA MAX™ Deluxe Human (BioLegend, San Diego, CA, USA). The sensitivity of IgE was 0.5 ng/ml and the sensitivity of IL-12p40 was 30 pg/ml. Both ELISA kits' intra-assay CVs were less than 8%.

Circulating miRNAs

Total RNA (including miRNAs) was isolated from 150 µl of serum samples using Qiazol (Qiagen, Hilden, Germany) reagent and Total RNA Zol-out kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions with minor modifications. 0.8 µg of MS2 RNA carrier to improve the recovery and 2.5 nM Cel-miR-39 spike-in mimic for additional control were included per one sample. To maintain small RNA fraction, a 5:4 ratio of isopropanol was added to the samples before loading onto kit columns. 2 µl out of total 100 µl of recovered RNA were reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit and the following RT TaqMan primers: cel-miR-39 (ID-000200), hsa-miR-146a (ID-000468) and hsa-let-7a (ID-000377) (Applied Biosystems, Foster City, USA). MicroRNA gene expression was assessed by real-time PCR carried out on ViiA™ 7 Real-Time PCR system (Life Technologies). Real time PCR was performed in duplicates using 5× HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) and the following Taqman microRNA assays: cel-miR-39 (ID-000200), hsa-miR-146a (ID-000468) and hsa-let-7a (ID-000377) (Applied Biosystems, Foster City, USA). Hsa-miR-let7a was used as the reference. Relative miRNA levels were calculated according to the $2^{-\Delta Ct}$ method, a randomly chosen nonallergic patient was set to one.

Statistical analysis

Mouse IgE and cytokine serum levels are shown as mean with standard error of the mean (SEM). Patient characteristics are expressed as percentage (calculated from absolute numbers) and IgE, IL-12p40 and miRNA serum levels as mean ± standard deviation (SD) in Supplementary Tables and as mean ± SEM in Figure 2. Variables were compared between groups by Chi-square (qualitative) or unpaired Student's *t* test (quantitative) at confidence interval (CI) of 95%. The

relation between variables was analysed by Pearson bivariate correlations followed by multivariate linear regression analyses. The enter method was used for computing the independent variables. Significance level was set at $P<0.05$. Data analyses were performed with the SPSS (v.21) statistical software and GraphPad (v.6).

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Supplementary Figure 1. Serum levels of multiple detected cytokines and chemokines in miR-146a^{-/-} and wild-type mice did not differ between the groups. Serum IL-5, IL-6, IL-17, CCL5, CXCL1 and G-CSF levels in MC903- and control treated wild-type and miR146a^{-/-} mice. Results from cytokines and chemokines detected in at least 7 out of 8 samples per group are shown. Data are represented as mean with SEM, unpaired student t-test was used.

Supplementary Figure 2. Negative correlation between serum IgE and IL-12p40 levels in MC903-treated wild-type mice. Correlation graphs between serum IgE and IL-12p40 levels in MC903-treated wild-type mice and MC903-treated miR-146a^{-/-} mice. Pearson r and P values are shown; significance level was set at $P < 0.05$. Dotted line represents SEM.

Supplementary Figure 3. Lack of correlation between miR-146a expression and IL-12p40 levels as well as IgE and IL-12p40 levels in serum samples of AD patients. Linear regression analysis graphs of miR-146a relative expression and serum IL-12p40 levels **(A)** and of serum IgE and IL-12p40 levels **(B)** in allergic and non-allergic AD subgroups. Pearson's r and P values are shown; significance level is set at $P < 0.05$. Dotted line represents SEM.

Supplementary Table 1. Demographic, clinical and laboratory characteristics of AD patients and subgroups.

	AD	Allergic AD	Non-allergic AD
	N=32	N=22	N=10
Clinical variables^A			
Gender <i>female</i> % (<i>n</i>)	62.5 (20)	50.0 (11)	90.1 (9)*
Age <i>years</i>	31.1 ± 15.3	31.1 ± 14.7	31.2 ± 17.4
Age of onset			
Early-onset (< 2 years) % (<i>n</i>)	53.1 (17)	54.4 (12)	50.0 (5)
Late-onset (> 2 years) % (<i>n</i>)	46.9 (15)	45.5 (10)	50.0 (5)
Disease duration <i>years</i>	21.4 ± 11.7	21.1 ± 12.5	22.2 ± 10.3
Disease severity			
Moderate (IGA=3) % (<i>n</i>)	40.6 (13)	31.8 (7)	60.0 (6)
Severe (IGA=4) % (<i>n</i>)	59.4 (19)	68.2 (15)	40.0 (4)
Rhinitis % (<i>n</i>)	15.6 (5)	13.6 (3)	20.0 (2)
Asthma % (<i>n</i>)	15.6 (5)	18.2 (4)	10.0 (1)
Impetigo % (<i>n</i>)	6.3 (2)	4.5 (1)	10.0 (1)
Hypertension % (<i>n</i>)	3.1 (1)	0 (0)	10.0 (1)
Anaemia % (<i>n</i>)	6.3 (2)	9.1 (2)	0 (0)
Laboratory variables^B			
IgE <i>ng/ml</i>	732.3 ± 629.6	1064.6 ± 504.1	67.7 ± 66.5 ***
IL-12p40 <i>pg/ml</i>	64.6 ± 34.2	64.36 ± 36.3	65.12 ± 31.0
miR-146a relative expression <i>RU</i>	0.89 ± 0.39	0.88 ± 0.39	0.89 ± 0.40

^A Data are shown as percentage and absolute numbers in brackets. Chi Square test, **P* < 0.05.

^B Data are shown as mean ± SD. Student's *t* test, ****P* < 0.001.

IGA: Investigator's Global Assessment score for overall severity of atopic dermatitis signs.

Supplementary Table 2. Multiple regression analysis for IgE as dependent variable in allergic AD subgroup.

Allergic AD ^A			
IgE ng/ml	β	p	R ²
Gender <i>female</i>	0.103	0.654	
Age of onset	-0.330	0.137	
Disease severity	0.051	0.823	
miR-146a relative expression <i>RU</i>	-0.483	0.039	
			0.173

^A Enter method was used and model adjusted R² value is shown.

Supplementary Table 3. Characteristics of correlation analyses between relative miR-146a serum levels and related variables in allergic and non-allergic AD subgroups.

	Allergic AD		Non-allergic AD	
miR-146a relative expression ^A	r	p	r	p
Age <i>years</i>	-0.075	0.740	-0.326	0.358
IgE <i>ng/ml</i>	-0.473	0.035	-0.076	0.833
IL-12p40 <i>pg/ml</i>	0.146	0.516	0.249	0.486

^A Pearson's r and P values are shown. Significance level is set at $P<0.05$.