

# **Transcriptome profiling identifies disease- and therapy-associated signatures in atopic dermatitis**

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## **Declaration**

Herewith, I confirm that apart from my supervisor's guidance and the stated sources and means, the complete submitted thesis is the result of my own work. This thesis has not been submitted to another examining body neither partially nor wholly as part of a doctoral degree. No academic degree has ever been withdrawn. Parts of this thesis have been published as research articles in scientific journals. This thesis has been carried out in strict accordance with the rules of Good Scientific Practice of the German Research Foundation.

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## Summary

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease and has a substantially detrimental impact on patients and caregivers. The disease is characterised by eczematous lesions, persistent itch, and a relapsing and remitting pattern, and a high heterogeneity with currently unpredictable disease courses and clinical outcomes. AD potentially comprises a variety of endotypes, which share common clinical characteristics, but arise from distinct molecular and cellular mechanisms. Although omics analyses have promoted the understanding of disease mechanisms and the development of innovative targeted therapies, many questions regarding the pathophysiology, disease heterogeneity, and patient-stratified therapeutic approaches remain unresolved.

So far, most of the transcriptome studies, i.e. studies investigating the gene expression, on AD relied on microarray data, were conducted in the context of clinical trials, examined one timepoint only, and rarely included healthy control subjects as reference. Further, most of the previous transcriptome studies in AD were focused exclusively on the skin, while blood was not covered, although there is robust evidence for systemic immune abnormalities in AD. Within the scope of this work, I analysed transcriptome data generated from longitudinal skin and blood samples of deeply characterised AD patients from the TREATgermany registry as well as of healthy controls using sensitive and unbiased mRNA sequencing.

In a first study, I was able to identify a stable core transcriptome signature in both lesional and nonlesional skin differentiating patients from healthy individuals and reflecting epidermal barrier dysfunction, innate immune activation and heightened itch signalling as key disease mechanism. A second dynamic signature reflects a progressive activation of immune responses with heightened inflammation in lesions. A large proportion of transcriptomic dysregulation is reverted with clinically successful systemic treatment with, however, a considerable residual genomic profile with treatment-specific differences indicating insufficient disease control on the molecular level that might give rise to flares and recurrences.

In a second study, I characterised skin transcriptomic signatures attributable to natural killer (NK) cells, which recently have been suggested to be involved in the pathophysiology of AD. The analysis showed that NK cells and related killer cells accumulate in AD lesions, and that there is a disturbed composition of resting and activated NK cells. After successful remission through systemic treatment I observed qualitative albeit not quantitative shifts of NK cell signatures.

In a third work, I analysed whole-blood transcriptome data of patients with AD and healthy controls. In a cluster analysis, I identified two potential endotypes, one of which showed a high degree of systemic transcriptomic dysregulation and an eosinophilic profile, and another showing a low degree of dysregulation and a non-eosinophilic profile. Association of eosinophil expression profiles with disease activity suggest that these endotypes might have clinical implications. Detected signatures related to NK cells indicate a reduced abundance and dysfunction of certain NK cells subsets also in the peripheral blood, underpinning the importance of this cell type for the pathophysiology of AD.

## Zusammenfassung

Bei der atopischen Dermatitis (AD) handelt es sich um die häufigste chronische entzündliche Hauterkrankung, welche sich erheblich nachteilig auf Patienten und deren Bezugspersonen auswirkt. Charakteristisch für die AD sind schubförmig oder persistierend auftretende Hautekzeme und ein intensiver Juckreiz, während der Krankheitsverlauf oft nicht vorhersagbar und extrem heterogen ist. Die AD umfasst eine potentiell große Vielfalt an Endotypen, die zwar ähnliche klinische Zeichen und Symptome zeigen, jedoch auf verschiedenen molekularen und zellulären Mechanismen basieren. Omics Analysen haben zu einem besseren Verständnis der Erkrankungsmechanismen und zur Entwicklung von neuen zielgerichteten Therapien beigetragen. Dennoch bestehen viele ungeklärte Fragen in Bezug auf die Pathophysiologie, die Heterogenität der Erkrankung, und Patienten-stratifizierte therapeutische Ansätze.

Bisher stützen sich die meisten Transkriptom-Studien, d.h. Studien zur Untersuchung der Genexpression, bei der AD auf Mikroarray Daten und wurden im Kontext von klinischen Studien mit nur einem untersuchten Zeitpunkt durchgeführt, sowie beinhalteten selten gesunden Kontrollen. Des Weiteren konzentrierten sich die meisten der bisherigen Studien auf die Untersuchung der Haut, wohingegen Transkriptom-Analysen von Blut kaum in Betracht gezogen wurden, obwohl es klare Hinweise gibt, dass die AD eine systemische Komponente aufweist. Im Rahmen der vorliegenden Arbeit habe ich longitudinale Haut- und Blut-Transkriptom-Daten von klinisch umfassend charakterisierten Patienten mit AD aus dem TREATgermany Register sowie von gesunden Kontrollen analysiert, welche mittels mRNA Sequenzierung generiert wurden.

In einer ersten Studie konnte ich eine stabile Kern-Transkriptom-Signatur sowohl in läsionaler und nicht-läsionaler Haut identifizieren, die Patienten deutlich von gesunden Kontrollen differenziert und auf eine gestörte epitheliale Barriere, eine Aktivierung des angeborenen Immunsystems und auf eine erhöhte Juckreiz-Signaltransduktion hindeutet. Eine zweite dynamische Signatur spiegelt eine progressive Aktivierung der Immunantwort mit erhöhter Entzündung in Läsionen wider. Ein Großteil der Dysregulation der Genexpression ist nach klinisch erfolgreicher systemischer Therapie normalisiert, wobei jedoch eine beträchtliche anhaltende genomisches Dysregulation mit behandlungsspezifischen Unterschieden bestehen bleibt. Dies deutet auf eine unzureichende Krankheitskontrolle auf molekularer Ebene hin und könnte die Ursache für die Krankheitsschübe und das Wiederauftreten bei der AD sein.

In einer zweiten Studie habe ich Genexpressionssignaturen in der Haut charakterisiert, welche auf natürliche Killer(NK)-zellen zurückzuführen sind. Es wurde kürzlich gezeigt, dass diese Zellen an der Pathophysiologie der AD beteiligt sein könnten. Die von mir durchgeführten Analysen zeigen, dass NK-Zellen und verwandte Zellen in den Läsionen von Patienten angereichert sind und, dass eine gestörte Zusammensetzung von ruhenden und aktivierten NK-Zellen besteht. Nach erfolgreicher Remission durch systemische Behandlung konnte ich qualitative jedoch keine quantitativen Veränderungen der NK-Zell-Signaturen beobachten.

In einer dritten Arbeit habe ich Blut-basierte Transkriptom-Daten von Patienten mit AD und gesunden Kontrollen analysiert. In einer Cluster Analyse konnte ich zwei potentielle Endotypen identifizieren, von denen einer durch ein hohes Maß an Dysregulation und ein eosinophiles Profil charakterisiert war, wohingegen der zweite ein geringes Maß an Dysregulation und ein nicht-eosinophiles Profil zeigte. Die beobachtete Assoziation von eosinophilen Expressionsprofilen mit der Krankheitsaktivität deuten darauf hin, dass diese molekularen Endotypen eine klinische Bedeutung haben könnten. Die detektierten Signaturen in Bezug auf NK-Zellen deuten auf eine reduzierte Anzahl und gestörte Funktion von bestimmten NK-Zell-Subtypen im Blut hin und unterstreichen damit die mögliche Relevanz dieses Zell-Typs für die Pathophysiologie der AD.

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# 1 Introduction

## 1.1 Atopic dermatitis: Background and epidemiology

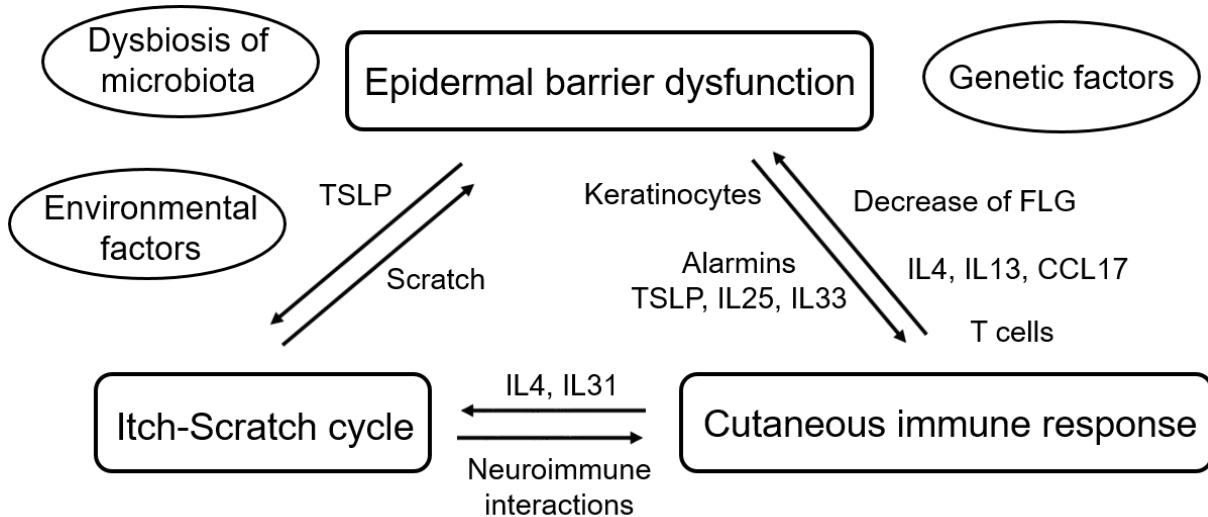
Atopic dermatitis (AD), also known as eczema or atopic eczema, is the most prevalent inflammatory skin disease affecting 230 million people worldwide (retrieved 2010), and causes the highest disease burden among non-fatal skin conditions.<sup>1</sup> AD is characterised by recurrent eczematous lesions as well as intense and persistent itching, albeit the clinical presentation is extremely heterogeneous (Figure 1). AD is a multifactorial disorder and multiple genetic as well as environmental risk factors have been identified. Several pathophysiological mechanisms contribute to the aetiology and the clinical manifestation of AD including (1) a disturbed epidermal barrier function, (2) an altered skin microbiome, (3) a T cell driven inflammatory milieu that is skewed towards an overwhelming T<sub>H</sub>2 immune response, and (4) neuroimmune interactions that promote a persistent itch-scratch cycle.<sup>2</sup> The life-time prevalence of AD is 15-20% in multiple countries, particularly in high-income countries. The typical disease onset is at an age of 3-6 months, though AD can occur at any age. AD was originally considered as a disease of early childhood, because it is affecting up to 20% of children. However, today, it is evident that AD is also a common inflammatory disorder in adults with a prevalence of 7-10%.<sup>2-4</sup>



**Figure 1: Eczematous lesions of patients with atopic dermatitis.** The typical clinical appearance of AD in infants (top left and top middle), children (top right and bottom left), and adults (bottom middle and bottom right). From Langan, Irvine & Weidinger, *Lancet*, 2020.<sup>2</sup>

## 1.2 Pathophysiology and mechanisms of disease

The pathophysiology of AD is complex and of multifactorial nature with several mechanisms contributing to its clinical manifestation (Figure 2).



**Figure 2: Contributing factors in the pathophysiology of atopic dermatitis.** The figure shows the interaction of three major mechanisms of disease and further contributing factors. A disturbed skin barrier function, persistent itch, and a chronic cutaneous inflammation are the hallmarks of AD. Genetic and environmental factors, as well as a dysbiosis of the skin microbiota substantially contribute to the AD aetiology. Figure modified from Nomura, Honda & Kabashima, *International Immunology*, 2018.<sup>5</sup>

### Genetic susceptibility

Genetic susceptibility is a major factor in the aetiology of AD demonstrated by the fact that a positive family history of an atopic disease particularly of AD itself is the strongest yet identified risk factor for developing AD.<sup>6</sup> Further, evidence from twin studies and genome-wide association studies (GWAS) underlined the importance of genetics in AD. The estimated heritability is about 75%. The sum of the genetic loci identified as being associated with AD so far account for <20% of the total heritability. Thus, there is a gap also denoted as the missing heritability, a phenomenon observed in various other multifactorial diseases as well.<sup>7-9</sup> Explanations for this gap are a generally high heterogeneity of the disease together with non-additive effects of genetic variations, gene-gene, and gene-environment interactions, as well as heritable epigenetic mechanisms and structural variations of the genome not explorable by GWAS as well as rare variants not covered by GWAS assays.<sup>9</sup>

So far, the strongest identified genetic risk factor for AD are loss-of-function (LOF) mutations in the filaggrin (FLG) gene encoding the skin barrier pre-protein profilaggrin, which undergoes proteolytic processing in the epidermis into filaggrin monomers. Filaggrin LOF mutations result in a truncated profilaggrin that cannot be cleaved into monomers.<sup>10,11</sup> Ten percent of people with European and Japanese descent carry a single LOF mutation in the FLG gene, conferring a generalised skin dryness and a three to five-fold increased risk to develop AD.<sup>12</sup> However, only ~20-40% of people with moderate-to-severe AD carry FLG LOF mutations and >50% of people with FLG LOF mutations never develop any atopic disease indicating that FLG LOF mutations are neither sufficient nor necessary to cause AD.<sup>13,14</sup> The suggested mechanism to link FLG deficiency and AD is an increased skin permeability to exogenous trigger factors

together with a dysregulation of multiple other genes downstream to FLG.<sup>15</sup> In addition, GWAS have identified several risk loci for AD, which present specific genomic regions harbouring one or more single-nucleotide polymorphisms (SNPs) that are associated with AD i.e. the frequency of those variants is significantly higher in individuals suffering from AD as compared to those who do not.<sup>2,8</sup>

### **Epidermal barrier dysfunction and the role of the microbiome**

A dysfunctional epidermal barrier is a hallmark of AD, as in both lesional (affected) and nonlesional skin (apparently healthy skin) the following epidermal changes have been observed: a reduced water retention, and an increase of pH, permeability to low-mass chemicals, and susceptibility to infections, as well as an easy irritability.<sup>2</sup> Multiple factors are involved in the disturbance of skin barrier integrity such as the itch-scratch cycle, a reduction and altered composition of lipids, a microbial dysbiosis, as well as the reduced expression of proteins crucial for barrier integrity such as structural proteins e.g. loricrin (LOR) and FLG.<sup>2,16</sup> While FLG LOF mutations lead to a primary downregulation of FLG, an elevated expression of type 2 immunity cytokines such as interleukin (IL) 4, IL13, and IL33 causes a secondary reduction of FLG and other epidermal structural proteins exacerbating the barrier defect.<sup>17</sup> In AD, the microbial diversity of the skin is markedly reduced, mainly at the extent of *Staphylococci* overgrowth particularly of *S. aureus*. Similar to other factors, it is uncertain whether the observed microbial dysbiosis in AD is causative of the disease or a consequence of epidermal barrier disruption and tissue inflammation with type 2 immune responses.<sup>18,19</sup> *S. aureus* expresses various virulence factors that exacerbate the pathogenesis of AD by acting on keratinocytes, which respond with the release of proinflammatory and pruritogenic signals (also called alarmins) such as IL1 $\beta$ , IL25, IL33 and thymic stromal lymphopoietin (TSLP). These trigger skin-resident antigen-presenting cells and drive the recruitment of type 2 inflammatory cells into the skin resulting in a chronic inflammatory milieu that further leads to tissue damage.<sup>20,21</sup> In addition, an impaired barrier enables an exaggerated percutaneous allergen (antigen) priming leading to further recruitment of immune cells into the skin and the establishment of an inflammatory milieu.<sup>22,23</sup>

### **Immunological dysregulation and cutaneous inflammation**

Both lesional and nonlesional i.e. affected and apparently healthy skin of patients with AD are characterised by abnormal accumulation of intercellular fluid (spongiosis) as well as dermal (from dermis, layer under the epidermis) immune cell infiltrates. These infiltrates mainly contain CD4+ T helper cells (T<sub>H</sub> cells) with more subtle appearance in nonlesional as compared to lesional skin.<sup>2,24</sup> Increased numbers of dendritic cells and Langerhans cells, which are resident dendritic cells of the skin, are found in lesional skin. This enables the uptake of antigens beyond the epidermal barrier and presentation to T helper cells. This process is facilitated by the dissolution of epidermal tight junctions in response to alarming signals by keratinocytes due to the disrupted skin barrier.<sup>25</sup> Cytokines produced by T<sub>H</sub>2 cells such as IL4, IL10, and IL13, promote B cell IgE class switching and the production of antigen-specific IgE antibodies.<sup>26</sup> Most patients with AD show an increased IgE-mediated reactivity towards particular antigens such as food proteins, aeroallergens, microbial antigens and/or keratinocyte-derived autoantigens, which can trigger AD flares.<sup>27-29</sup> However, T cells specific for those antigens are not accumulated in skin infiltrates of patient with AD, but instead the T cell repertoire is highly polyclonal raising open questions regarding the antigen-specificity of T cells accumulating in the skin of patients with AD.<sup>2,30</sup> Lesional skin is characterised by a dysregulated expression of many genes mainly related to keratinocyte activity and T cell infiltration. Especially genes related to T<sub>H</sub>2 (IL4, IL10, IL13) and T<sub>H</sub>22 cells (IL22) are upregulated. Both lesional and

nonlesional skin are skewed towards type-2 mediated responses, but genes associated with T<sub>H</sub>1 and T<sub>H</sub>17 cells were shown to be overexpressed as well.<sup>31–34</sup> Recently, a selective reduction of mature CD56<sup>dim</sup> CD57<sup>+</sup> natural killer (NK) cells in peripheral blood, as well as an enrichment of activated NK cells in lesional skin have been shown in independent sets of patients with moderate-to-severe AD.<sup>35</sup> Based on these findings, it was postulated that in AD, NK cells are recruited from the periphery to the inflamed skin as an endogenous counterregulatory response to the type 2 skin inflammation.<sup>36</sup> Beyond this, it was speculated that the release of type 2 cytokines such as IL4 and IL13, and a deficient interferon gamma (IFNG) release by certain NK cell subsets may contribute to immune responses with a type 2 pattern in AD. However, the role of cutaneous NK cells in the pathophysiology of AD is insufficiently understood, so far.<sup>37–39</sup>

### **Cutaneous neuroimmune interactions**

Itch is a central contributing factor in AD, as it promotes scratching, which results in skin barrier damage and the subsequent release of alarmins such as TSLP, IL33 and IL-1 $\beta$  leading to a sustained itch-scratch cycle. Several pruritogens that induce itch have been identified in the context of AD. Those pruritogens mediate itch by cutaneous pruriceptive primary sensory nerves.<sup>40,41</sup> Psychological stress is known to exacerbate hypersensitivity and hyperinnervation to pruritus, and accordingly, stress can cause flares in patients with AD.<sup>2,42</sup> Histamine, which is released from mast cells and basophils, is the best studied pruritogen. Though histamine is an important pruritogen in AD, acting via stimulation of histamine receptors H1 and H4 on a subset of sensory neurons, the effectiveness of antihistamines to ameliorate AD signs and symptoms including itch has not been proven. Histamine-independent itch-inducing signalling includes TSLP released by keratinocytes and activating sensory neurons, type 2 cytokines IL4 and IL13, which bind to the IL4 receptor alpha (IL4RA) on afferent neurons, as well as IL31, a cytokine released by several type 2 immune cells, which stimulates neurons expressing IL31 receptor alpha (IL31RA).<sup>40,43–45</sup> Animal models of AD skin indicated an increased density, sprouting and thickness of epidermal neurons. However, it is insufficiently understood if itch is a causal factor for AD or a consequence of the exaggerated release of pruritogens by type 2 immune cells, basophils and mast cells.<sup>22</sup>

## **1.3 Clinical presentation and disease management**

### **Clinical presentation**

No clinical tests are currently available for the diagnosis of AD. Thus, the diagnosis is made based on clinical parameters assessed by a physician.<sup>46</sup> According to the Hanifin and Rajka criteria, which are widely used for diagnosis, essential features of AD are 1) eczematous lesions, either acute, subacute or chronic, 2) intense pruritus, and 3) a chronic or relapsing disease course. Features not being prerequisite for diagnosis of AD but frequently seen in patients are 1) early disease onset (typically < age of two years), 2) generalised skin dryness, and 3) a personal or family history of atopic conditions (e.g., specific IgE reactivity, asthma, allergic rhinitis, atopic dermatitis).<sup>2,47</sup> Lesions can occur on any body part, but besides diffuse eczema, predilection sites are the face, cheeks, and trunk in infants, and the hands, flexor surfaces, eyelids, head, and neck in adolescents and adults. AD can have heterogeneous trajectories such as an early transient disease course, a relapsing-remitting course, long periods of remission followed by recurrence or a chronic persistent course.<sup>2</sup> AD severity can



be measured by validated scoring systems such as the Scoring of Atopic Dermatitis (SCORAD) and the Eczema Area and Severity Index (EASI), while both scores integrate the following clinical signs into a formula: infiltration (swelling), erythema (red flush), lichenification (thickening) and excoriations (scratch marks) as well as the extent of the area affected. While the SCORAD attaches more weight on the intensity of the lesions, the EASI does so on the skin area affected by lesions.<sup>48–50</sup> Beside these physician-based scoring systems, patient-reported scorings such as the Patient-Oriented Eczema Measure (POEM) exist. These are often used in clinical trials to evaluate symptoms of the disease, e.g. extent of itch and disturbance of sleep due to eczema.<sup>22,51</sup>

### **Comorbidities and complications**

AD is strongly associated with other atopic conditions such as asthma, allergic rhinitis as well as food allergy, and the disease is often accompanied by increased IgE serum levels with reactivity against specific antigens such as pollen, mould, dust mite, and food antigens. The occurrence of those comorbid conditions can be explained mainly by shared risk factors such as genetic risk variants and environmental risk factors, as indicated by twin and sibship studies.<sup>4,52–56</sup> The theory of the so-called atopic march, which suggests the progressive occurrence of AD, asthma and hay fever, is a matter of debate, because only few patients show this atopic disease progression.<sup>22,57</sup> Patients with AD often suffer from bacterial, fungal, and viral cutaneous infections, which further complicates the clinical picture of AD. In the majority of patients, a colonisation with *Staphylococcus aureus* together with a reduced overall diversity of microbial communities can be observed.<sup>18,19</sup> Viral infections commonly seen in AD include herpes simplex virus infections, which can develop to life-threatening forms of eczema herpeticum, as well as herpes zoster infections and cutaneous warts.<sup>58</sup> Among the non-atopic AD comorbidities, mental health disorders are the most frequent illnesses caused by the impact of AD on daily life. Psychological stress caused by visible disease, stigma, itch, and sleep deprivation contributes to adverse psychological outcomes such as depression and anxiety.<sup>22,59,60</sup>

### **Prevention**

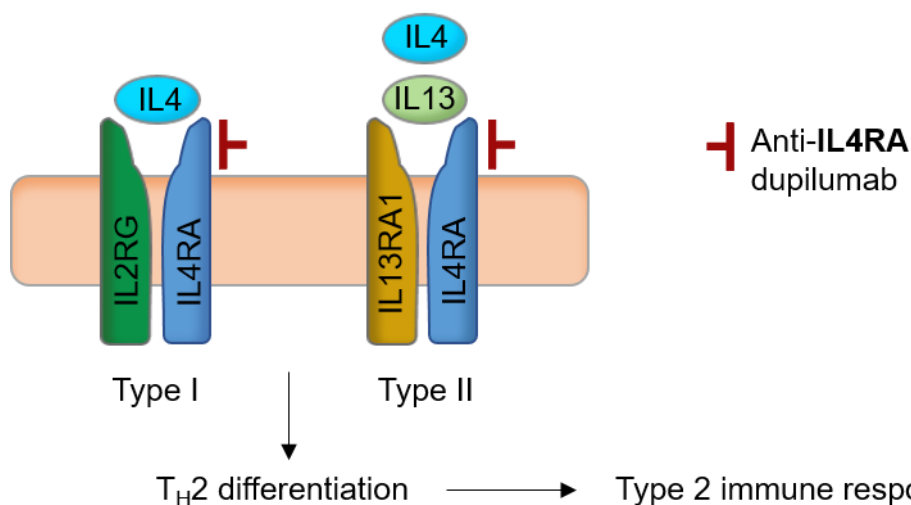
Since AD is common, has detrimental effects on the quality of life of patients and their families, and causes high health-care costs, there is a need to better understand the causes and outcomes of AD and comorbid diseases in order to prevent or adequately treat the disease.<sup>1,61</sup> Prevention strategies of AD naturally have limitations as the disease aetiology has a genetic component of polygenic nature that cannot be influenced. There are no official guidelines for the prevention of AD. However, pilot studies demonstrated the benefit of daily use of skin emollients in infants with a positive family history of AD or in infants carrying a FLG LOF mutation, which is the strongest identified genetic risk factor for AD so far.<sup>22,62,63</sup> The focus of secondary prevention strategies lies on the avoidance of factors that trigger AD flares, such as air pollutants (e.g. traffic exhaust), indoor pollutants (e.g. tobacco smoke and organic compounds, such as propylene glycol and glycol ether), food allergens, as well as environmental allergens such as house dust mite, pollen or animal fur.<sup>22,64</sup>

### **Therapy**

Therapy strategies aim to reduce the symptoms of AD and establish a persistent disease control in order to limit the debilitating effects of AD and comorbidities on daily life and achieve long-term health. Mild AD can often be controlled by topical treatment with skin moisturising creams and anti-inflammatory treatment on demand such as with topical corticosteroids or

calcineurin inhibitors (ointment or cream).<sup>2,65,66</sup> Moderate-to-severe AD often requires a multistep approach combining the restoration of skin barrier by moistures, phototherapy and systemic immunomodulatory therapy, while the latter is usually avoided as long as topical and phototherapy are sufficiently effective.<sup>67</sup> Systemic therapy can be classified into conventional immunosuppressants such as cyclosporine, azathioprine, and methotrexate, and immunomodulating biologics such as monoclonal antibodies. Cyclosporine, which decreases the activity of lymphocytes, particularly of T cells, was the most effective treatment for severe AD before the approval of targeted monoclonal antibodies. However, due to potential toxic side effects of broad immunosuppressants, guidelines recommend only short-term use of maximum 1-2 years.<sup>22,64,68,69</sup>

In 2017, the monoclonal antibody dupilumab was the first biologic approved as a treatment for adult patients with moderate-to-severe AD. Meanwhile, dupilumab is approved in the United States of America for patients with an age of  $\geq 6$  years and for patients aged  $\geq 12$  years in the EU. The antibody is directed against the IL4 receptor subunit alpha (IL4RA) and therefore blocks IL4 and IL13 signalling, which are the major effector cytokines of type 2 immune responses, which are exaggerated in the skin of AD (Figure 3). In clinical trials, and also in large real-world patient cohorts, dupilumab led to a significant improvement of disease severity in adult patients, and revealed a good safety profile with manageable adverse effects such as conjunctivitis being the most frequent and adverse event.<sup>2,70-72</sup>



**Figure 3: The mode of action of dupilumab.** The monoclonal antibody dupilumab binds to the IL4 receptor alpha (IL4RA) and prevents the binding of IL4 to the IL2RG/IL4RA receptor complex (Type I) as well as the binding of both IL4 and IL13 to the IL13RA1/IL4RA receptor complex. IL4 and IL13 signalling promotes T helper cell differentiation into T<sub>H</sub>2 cells. An exaggerated type 2 immune response is a hallmark of the cutaneous inflammation in AD. Modified from Litman, Journal of Pathology, Microbiology and Immunology, 2019.<sup>73</sup>

Other biologics targeting cytokine pathways relevant in AD are currently tested in clinical trials such as antibodies targeting IL13, IL31RA, and IL22. Besides therapeutic antibodies, multiple small molecule antagonists such as Janus kinases (JAK) inhibitors are currently in clinical trials and show promising effects. JAKs are essential for intracellular signal transduction, such as for downstream signalling of cytokine receptors relevant in AD.<sup>74-77</sup>

## **The TREATgermany atopic dermatitis registry**

AD has a high socio-economic impact demanding the implementation of official national guidelines in Germany and more effective care.<sup>1</sup> In order to evaluate effectiveness of new therapy approaches under real-life conditions, systematic and longitudinal data from routine care are necessary. To this end, the German Atopic Dermatitis Registry TREATgermany was initiated in 2011 on the initiative of the German Society for Dermatology and conceptualised by university professors.<sup>78</sup> In 2016, the registry was relaunched under new inclusion criteria and with an additional biosampling module. By May 2021, 1,180 patients have already been included in the registry. TREATgermany is a prospective non-interventional multi-centre study in form of a nation-wide clinical registry. The patients are recruited at university and non-university hospitals as well as at dermatological practices with new recruiting sites being continuously initiated.<sup>79</sup> Patients with an age of more than 18 years who are diagnosed with moderate-to-severe AD (objective SCORAD >20) are included from dermatological routine care and undergo longitudinal monitoring during physician visits. Various biosamples including skin biopsies, blood samples, skin swabs, and stool samples are collected with an additional declaration of consent of the patient. The main objectives of TREATgermany are to record medical care and therapies of moderate-to-severe AD in Germany, and to evaluate the sequence of treatments, changes in treatment as well as the perspective of the patient. In addition, this research network aims to examine and compare the effectiveness, tolerability and safety of systemic therapies approved for moderate-to-severe AD<sup>78,79</sup>. Further, TREATgermany represents a resource for molecular research of AD, given the systematic and standardised collection of multiple biosamples. To date, 50 study centres have been initiated with 20 centres collecting biosamples (<http://www.treatgermany.org/>). In this work, transcriptomic data generated from skin biopsies as well as from blood samples collected at five different TREATgermany study centres (Kiel, Buxtehude, Dresden, Hannover, Berlin) have been used to examine molecular signatures of moderate-to-severe AD as well as changes under systemic treatment.

### **1.4 Omics analyses in atopic dermatitis**

Over the last decades there has been a tremendous advance in high-throughput omics techniques for different molecular layers, and genomic, epigenomic, transcriptomic and proteomic applications are indispensable scientific tools in today's medical research on complex and heterogeneous disease conditions like AD. The analysis of large amounts of data produced by these omics tools have essentially promoted the understanding of the pathophysiology of AD. The aim of using omics technologies is the identification of genes, proteins or regulatory pathways involved in disease pathophysiology, that might serve as predictive, diagnostic or prognostic biomarkers e.g. for the prediction of the response to a certain therapy, provide starting points for the development of new targeted treatments, as well as the identification of disease and drug endotypes, which eventually pave the way for the development of innovative therapeutic options and precision medicine.<sup>22,73,80,81</sup>

Multiple studies of genomics and transcriptomics, and a smaller number of studies of epigenomics and proteomics have been performed on patients with AD and yielded novel insights into genetic and epigenetic variation, as well as dysregulation on the transcript and protein level that are associated with the disease. A comparatively small number of studies

addressed metabolomics or the microbiome of the skin, but also here, characteristic alterations could be linked to AD.<sup>82-84</sup> In the following, the main omics findings in AD are described in more detail.

## Genomics

In the last decade, genomics have been extensively studied in AD, and a total of 62 genes has been described as associated with AD by candidate gene studies, GWAS, and next-generation sequencing (NGS)-based studies such as whole-exome sequencing (WES) studies.<sup>85</sup> So far, GWAS have identified 31 genomic loci associated with AD susceptibility in multiple ethnicities.<sup>86</sup> In those genomic regions, genes involved in the regulation of immune system functions such as T cell activation (IL2, CARD11), T<sub>H</sub>2 cell differentiation (IL4, IL13), and innate host defence (IL6R, CD207), as well as genes involved in extracellular matrix organisation (CCDC80, ADAMTS10) and transcription regulation (ZNF652, DMRTA1) are overrepresented. This indicates that genetic variants contribute to immune and skin structural abnormalities in AD. However, it has to be mentioned that the majority of the identified risk variants are proxies and do probably not represent the causal risk variant itself. Here, an exception is the FLG gene located in the “epidermal differentiation complex” (EDC) on chromosome 1q21.3, for which several LOF mutations have been identified. Functional studies showed that those mutations lead to a lower or completely abolished expression of the protein, finally resulting in dry skin and an increased skin barrier permeability leaving carriers more susceptible to develop AD.<sup>22,85,86</sup> The reported risk variants identified by large-scale GWAS are common (minor allele frequency (MAF)  $\geq 5\%$ ) and explain only 20% of the estimated disease heritability of about 75%.<sup>7,8</sup> Thus, it was argued that rare protein-coding variants could further explain the estimated heritability, and a recent study suggested that rare protein-coding variants might contribute about 12%.<sup>87</sup>

## Epigenomics

Epigenetic mechanisms present a further explanation for the missing disease heritability, as various epigenetic changes have been linked to AD.<sup>88,89</sup> The field of epigenomics comprises the investigation of histone modifications, non-coding RNA-mediated gene silencing e.g. by microRNAs, as well as DNA methylation. Especially DNA methylation has been addressed by several studies of AD, though in contrast to genomic studies, systematic and epigenome-wide epigenetic studies of AD are rare.<sup>80,85</sup> Although methylation is generally stable and might even be heritable, it can be altered by environmental and lifestyle factors such as diet, tobacco smoking, pollutants, and physical activity.<sup>88,90</sup> In an epigenome-wide methylation analysis of skin from patients with AD and healthy controls, Rodriguez *et al.* observed differentially methylated sites in genes related to epidermal integrity (IVL), genes encoding inflammatory markers (S100A5, S100A9), genes encoding keratins (KRT6C, KRT77), as well as genes related to immune responses (IL23A, CD28, TNFSF4) to be significantly associated with the disease.<sup>91</sup> In our recent review on the current state of epigenetic AD research based on twelve studies identified by careful and intense literature research, we could show that differentially methylated genes are enriched in biological processes of B-cell-mediated immunity and T<sub>H</sub>-cell differentiation.<sup>89</sup> In many cases, a negative correlation of methylation and gene transcription could be shown, indicating that DNA methylation indeed represents a regulatory mechanism of transcription and might as well be involved in the dysregulation of gene expression in the skin of AD.<sup>89</sup> As addressed by a candidate gene study, TSLP, a cytokine produced by epithelia as “alarmin” that triggers T<sub>H</sub>2 inflammatory responses, showed hypomethylation of the promoter and concomitantly elevated gene expression in AD.<sup>92,93</sup> The gene was shown to be upregulated in the skin of AD by multiple studies.<sup>94-96</sup> Besides the

association with AD, TSLP promoter hypomethylation was associated with prenatal tobacco smoke exposure in a birth cohort, providing a possible link between environmental factors and the risk to develop AD.<sup>97</sup>

## Transcriptomics

Besides genomics, transcriptomics account for the most studied omics level in AD. Skin transcriptome studies in AD have mainly been conducted using whole-skin biopsies, while other sampling strategies such as laser capture microdissection and tape stripping were applied less frequently.<sup>94,98,99</sup> Skin biopsies are usually collected by punching out a cylindrical piece of the skin with its layers epidermis and dermis. Thus, transcriptome analysis denoted as from whole-skin will mostly reflect gene expression from epidermal and dermal cells. While the epidermis consists mainly of tightly-packed keratinocytes, the thicker dermis harbours various cell types such as fibroblast, endothelial cells, adipocytes, Schwann cells as well as immune cells with varying frequencies.<sup>100,101</sup> Beside the degree of inflammation, the body-site was shown to have an influence on the immunological setup of the skin.<sup>102</sup>

So far, 945 genes have been associated with AD by RNA expression according to Open Targets, a comprehensive data integration platform.<sup>103</sup> According to an analysis of a compilation of several transcriptome data sets, the top upregulated genes in lesional skin of AD encode epidermal anti-microbial proteins such as DEFB4A, PI3, and S100A9, while the top downregulated genes such as FABP7, ELOVL3, and CLDN8 are related to lipid metabolism and epidermal differentiation. Further, it was shown that the suppression of lipid-related genes is linked to elevated T<sub>H</sub>2 activation, one of the hallmarks of AD pathophysiology.<sup>104,105</sup> Gene expression signatures detected in the skin of AD reflect a specific T helper cell activation which is skewed towards T<sub>H</sub>2 cells and driven by elevated expression of type 2 cytokines such as IL4, IL13, and IL9 as well as chemokines such as CCL17 (TARC), CCL18, CCL22, and CCL26.<sup>32,94,95,104</sup> Besides type 2 immune responses, T<sub>H</sub>17-/T<sub>H</sub>22- and T<sub>H</sub>1-associated gene profiles showed an upregulation in AD as characterised by elevated expression of genes such as CCL20, IL22, IL23, IL36, and IL36RN, as well as IFNG and CXCL10. Several studies indicated that differences in those T helper cell expression profiles are related to ethnicity, age, as well as chronicity of the skin lesions.<sup>31–34,98,106</sup> Further, general inflammation markers such as IL34 and MMP12, genes encoding proteins from the S100 and late-cornified envelop (LCEs) family, which are involved in barrier defence and repair mechanisms, as well as alarmins such as TSLP and IL25 known to be expressed by stressed keratinocytes are upregulated in the skin of AD as demonstrated by several transcriptome studies. Epidermal barrier dysfunction is displayed on the gene expression level by a downregulation of genes encoding structural proteins such as FLG, FLG2, and LOR with a concomitantly upregulated expression of markers of epidermal proliferation such as KRT16 and MKi67 together with a high extent of dysregulation of genes involved in proteolytic processes such as SERPINB3, SERPINA1, and KLK5.<sup>32,80,94,95,104</sup> Recently, Tsoi *et al.* have reported that both nonlesional and lesional skin show clear enrichment of tumour necrosis factor (TNF), T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 response signatures, with a lower degree of dysregulation in the nonlesional skin. Further, their study demonstrated that there is a continuum in dysregulation from nonlesional to acute to chronic lesional skin, demonstrating quantitative rather than qualitative differences between the different stages of skin inflammation.<sup>95,96</sup>

Before the approval of biologics, cyclosporine was the most effective systemic drug for the treatment of moderate-to-severe AD, and it was shown that the clinical improvement under cyclosporine is associated with a normalisation of gene expression related to T<sub>H</sub>2-, T<sub>H</sub>22-, and T<sub>H</sub>17 responses as well as epidermal hyperplasia.<sup>107</sup> Studies of dupilumab, the first biologic

approved for AD, showed that the AD skin transcriptomic signature improved in a dose-dependent manner. Of note, genes upregulated in AD lesions at baseline normalised by 65% as compared to nonlesional skin under the highest administered and finally approved dose of dupilumab. The clinical improvement of the disease was associated with a reduction in genes related to type 2 inflammation such as IL13 and CCL17 as well as epidermal hyperplasia. Overall, it was shown that the lesional transcriptome shifted towards a nonlesional signature under systemic treatment.<sup>108,109</sup>

In AD, it is obvious to investigate the transcriptome of the skin, as it is the primary organ of disease manifestation and easily accessible. Nonetheless, investigating the blood transcriptome offers the clear advantage of being less invasive than skin biopsies. Moreover, previous analysis of inflammatory markers in the blood indicated that AD has a systemic component, which however is still insufficiently explored.<sup>110</sup> There exist only few studies of the blood transcriptome of AD so far. Brunner *et al.* investigated blood transcriptome signatures of children aged under six years who had an AD onset within the previous six months. They observed elevated levels of eosinophil and T<sub>H</sub>2 markers such as IL5RA, IL1RL1, histamine receptor H4 (HRH4), CCR3, SIGLEC8, PRSS33, and CLC whereas T<sub>H</sub>1 markers such as IFNG and TNF alpha (TNFA) were decreased, indicating that the immune response is skewed towards a type-2 dominated pattern as observed in skin as well.<sup>111</sup> Interestingly, the genes identified by Brunner *et al.* partially overlap with the genes associated with a multimorbid phenotype of allergic diseases including AD in a study of Lemonnier *et al.* indicating that the blood transcriptomic signatures of AD are linked to a generalised atopic phenotype.<sup>112</sup>

## Proteomics

Several dysregulations observed on the transcript level in the skin of patients with AD could be shown on the protein level as well. Mass spectrometry of proteins extracted from tape strips, a sampling technique where the upper layers of the skin are peeled off, identified lower levels of skin barrier proteins such as FLG and FLG2, several keratins (KRT1, KRT10), corneodesmosin, desmoglein-1, desmocollin-1, and transglutaminase-3, as well as natural moisturizing factor (ARG1, CASP14, GGCT) in patients with AD as compared to healthy skin, whereas proteins from the serine protease inhibitor family (SERPINB3, SERPINB4), and antimicrobial proteins (S100A8, S100A9) were upregulated in AD.<sup>113–116</sup> However, those observations were gathered from stratum corneum (the horny layer of the skin) proteome datasets, since only the outermost layers of the epidermis are peeled off by tape strips. Those datasets are not readily comparable with datasets generated from full-thickness punch biopsy skin samples as used for most of the transcriptomic studies.<sup>80,81</sup> In the blood of patients with AD, increased levels of proteins related to immune responses and inflammatory processes such as C5, LBP, CRP, IgE, CCL17, and CCL22 have been identified, while the latter three showed a positive correlation with disease activity as measure by the SCORAD.<sup>117</sup> In a recent study comparing sera of patients with AD and healthy controls, protein analysis using the Olink technology identified increased levels of inflammatory and cardiovascular risk markers in AD such as CX3CL1, CCL8, M-CSF, HGF and VEGF-A underscoring the systemic nature of AD. Further, AD sera revealed elevated protein levels of specific T cell responses including T<sub>H</sub>2, T<sub>H</sub>1, and T<sub>H</sub>17 markers such as IL-13, CCL17, eotaxin-1/CCL11, CCL13, CCL4, and IL-10, CXCL10, CXCL11, IFN- $\gamma$ , CXCL9, and TNF- $\beta$ , as well as IL-12/IL-23p40 and CCL20.<sup>110</sup>

## Scientific rational of this thesis

Transcriptomics account for one of the most studied omics in AD. However, as transcriptional profiles underly dynamic processes, they fluctuate with time in response to a variety of intrinsic and extrinsic factors. Thus, every transcriptomic analysis in AD and other disorders presents only a snapshot of the current molecular state at a particular body-site or organ.<sup>118</sup> Various findings by transcriptome studies in AD have not been validated by other studies, given that a slight change of conditions, whether biological (disease heterogeneity and activity, body-area, treatment, demographic variables) or technical (sampling, experimental procedures), can influence the outcome substantially. Nonetheless, every study of transcriptomics in AD can add valuable information that will lead to a better understanding of disease pathophysiology, disease heterogeneity, as well as how and why individual patients respond to certain treatments. Those are the preconditions of precision medicine.<sup>73,81</sup>

With NGS becoming affordable due to technical advances and the associated drop of costs, NGS-based methods ultimately replaced microarray-based methods to study different omics. Today, transcriptomes are analysed mainly by RNA sequencing (RNA-Seq), because it generates sensitive genome-wide high-quality data and enables a less biased view on the transcriptome than microarray-based methods.<sup>119</sup>

In AD, most of the previous transcriptome studies were based on microarrays, investigated only one timepoint, were conducted in the context of clinical trials with very specific settings, or lacked the inclusion of healthy controls. Further, studies addressing the blood transcriptome of patients with AD are rare. To this end, I performed a comprehensive analysis of RNA-Seq transcriptomic datasets from whole-skin and whole-blood samples from patients with AD before the start of systemic treatment as well as three months after, and from healthy controls. Patient biosamples were available from the TREATgermany registry, offering to study the cutaneous and peripheral-blood AD transcriptome and its changes under systemic therapy in a routine-care patient cohort. Healthy control samples were available from another high-quality case-control cohort. Comprehensive investigations of the molecular signatures of AD are indispensable in order to identify new therapeutic targets and risk predictors, to disentangle the clinical heterogeneity of AD, and to determine patient endotypes, which eventually can help to identify drug endotypes and promote precision medicine approaches.

### 1.5 Objectives of this thesis

The primary objective of this thesis was to identify molecular signatures of AD, AD subtypes, and relevant outcomes based on transcriptomic profiling. To this end, I performed analyses of skin and blood transcriptome data generated by RNA-Seq of patients from the TREATgermany atopic dermatitis registry, as well as of healthy controls. Skin and blood transcriptome data from two timepoints were available: (1) before the start of a systemic treatment with either anti-IL4RA dupilumab or immunosuppressant cyclosporine, and (2) three months after the start of treatment. The focus of this work was to differentiate stable from more dynamic cutaneous expression signatures in AD, as well as to elaborate on signatures attributable to NK cells, which just recently have been hypothesised to play a role in the pathophysiology of AD. Further, I aimed at conducting the first sequencing-based whole-transcriptomic profiling of blood from patients with AD. The secondary objective was to analyse the interdependence of

gene expression and clinical variables such as disease activity and responsiveness to treatment.

## 1.6 Summary of article I

L. Möbus, E. Rodriguez, I. Harder *et al.* „Atopic dermatitis displays stable and dynamic skin transcriptome signatures“. In: *Journal of Allergy and Clinical Immunology* (2021)

With this article, we provide an in-depth characterisation of gene expression in the skin of patients with moderate-to-severe AD as well as the impact of systemic treatment with anti-IL4RA dupilumab and cyclosporine. Lesional and nonlesional skin biopsies have been sampled from 59 AD patients participating in the TREATgermany registry before the start of systemic treatment as well as three months after. Skin samples have been subjected to mRNA sequencing. The resulting data has been complemented with transcriptomic data sets from 31 healthy volunteers who were included in a previous skin transcriptome study. We show that the AD cutaneous transcriptome comprises stable as well as dynamic signatures. Transcriptomic shifts under treatment were observed, including the downregulation of inflammatory markers and the upregulation of epidermal barrier markers. Despite clinical improvement after three months of therapy, we detected a substantial residual transcriptomic dysregulation in AD patients which could be causal for the relapse of AD.

## 1.7 Summary of article II

L. Möbus, E. Rodriguez, I. Harder *et al.* „Elevated NK-cell transcriptional signature and dysbalance of resting and activated NK cells in atopic dermatitis“. In: *Journal of Allergy and Clinical Immunology* (2021)

In this work, we re-analysed the skin transcriptome data of the afore-mentioned study of patients from the TREATgermany registry in order to characterise gene expression signatures attributable to NK cells and related cytotoxic cells in the skin of patients diagnosed with AD. NK cells belong to the family of innate lymphoid cells and mediate cellular cytotoxicity through releasing apoptosis-inducing molecules such as granzymes. Alterations in these cell populations have been previously observed in moderate-to-severe AD patients, including a reduction of total peripheral blood NK cells and elevation of activated NK cell transcriptomic signatures in lesional skin. In our study, we report an accumulation of NK cells in lesional skin of AD as demonstrated by immunofluorescence staining and upregulated NK-cell related gene expression signatures that included an upregulation of NK-cell inhibitory receptors. The NK-cell signature remained upregulated after three months of systemic treatment, despite clinical improvement of AD. Overall, we observed qualitative rather than quantitative changes in gene expression under therapy suggesting a compositional shift of NK cell subsets. The observations strongly support an involvement of NK cells in the skin pathophysiology in AD.



## 1.8 Summary of article III

L. Möbus, E. Rodriguez, T. Werfel, J. Schmitt, S. Weidinger *et al.* and the TREATgermany study group „Blood endotypes of atopic dermatitis with potential therapeutic implications“. (in preparation)

In this work, we analysed blood transcriptomic data of 60 patients with AD from the TREATgermany registry. Whole-blood PAXgene samples have been collected before and three months after the initiation of a systemic treatment as well as from healthy controls. Using a hierarchical clustering approach, we identified and characterised two clusters of patients that indicated an eosinophilic and a non-eosinophilic blood endotype. We observed a correlation of endotype-related signatures with clinical variables, such as disease activity and intensity of itch. The eosinophilic endotype showed strong transcriptional dysregulation, while the non-eosinophilic endotype showed weak transcriptional dysregulation as compared to healthy controls. Additionally, we observed downregulated expression of NK cell related genes. This is in line with recent reports about the reduction of peripheral NK cells in AD. Furthermore, we show that clinical improvement within three months of therapy with anti-IL4RA dupilumab is accompanied by shifts in the blood transcriptome such as the decrease of inflammatory profiles.

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## 2 Publications

The following article was published in the Journal of Allergy and Clinical Immunology, Vol 147(1), Lena Möbus, Elke Rodriguez, Inken Harder, Dora Stölzl, Nicole Boraczynski, Sascha Gerdes, Andreas Kleinheinz, Susanne Abraham, Annice Heratizadeh, Christiane Handrick, Eva Haufe, Thomas Werfel, Jochen Schmitt, Stephan Weidinger, and the TREATgermany study group, Atopic dermatitis displays stable and dynamic skin transcriptome signatures, 213-223, Copyright Elsevier (2021). [doi.org/10.1016/j.jaci.2020.06.012](https://doi.org/10.1016/j.jaci.2020.06.012)

# Atopic dermatitis displays stable and dynamic skin transcriptome signatures



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**Background:** Skin transcriptome studies in atopic dermatitis (AD) showed broad dysregulation as well as “improvement” under therapy. These observations were mainly made in trials and based on microarray data.

**Objectives:** Our aim was to explore the skin transcriptome and the impact of systemic treatment in patients of the TREATgermany registry.

**Methods:** Biopsy specimens from 59 patients with moderate-to-severe AD before and 30 patients 12 weeks after start of systemic treatment (dupilumab [n = 22] or cyclosporine [n = 8]) and from 31 healthy controls were subjected to mRNA sequencing.

**Differential expression, pathway enrichment, correlation, and coexpression network analysis were conducted.**

**Results:** Both lesional and nonlesional skin showed a stable “core” signature characterized by disturbed epidermal differentiation and activation of IL-31/IL-1 signaling. A second dynamic signature showed progressive enrichment for type 2 inflammation, T<sub>H</sub>17 signaling, and natural killer cell function. Markers correlated with disease activity have functions in epidermal barrier properties and immune modulation. *IL4RA* was among the top 3 central dysregulated genes. Cyclosporine led to a more pronounced global transcriptome reversion and normalized T<sub>H</sub>17 cell/IL23 signaling, whereas dupilumab led to a stronger increase in level of epidermal

differentiation markers. Both treatments strongly decreased levels of type 2 markers, but overall the residual profile was still profoundly different from that of healthy skin. Lower levels of *IL4RA* and *IL13* and high *IL36A* expression were related to a stronger clinical response to dupilumab.

**Conclusion:** The AD core signature is characterized by dysregulation of genes related to keratinocyte differentiation and itch signaling. A dynamic signature reflects progressive immune responses dominated by type 2 cytokines with an additional role of T<sub>H</sub>17 and natural killer cell signaling. (*J Allergy Clin Immunol* 2021;147:213-23.)

**Key words:** Atopic dermatitis, transcriptome, RNA-sequencing, gene expression, dupilumab, cyclosporine, type 2 inflammation, residual signatures

Atopic dermatitis (AD) is the most common chronic inflammatory skin disorder, affecting up to 20% of children and 10% of adults.<sup>1</sup> Its pathophysiology results from a complex interaction of genetic and environmental factors leading to epidermal dysfunction and cutaneous inflammation, which is driven by excessive T-cell activation and an increased production of inflammatory cytokines.<sup>2</sup>

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Aventis, Schering-Plough, Sienna Biopharmaceuticals, Takeda, Teva, UCB Pharma, VBL Therapeutics, and Wyeth Pharma. T. Werfel is coprincipal investigator of the German Atopic Eczema Registry TREATgermany; has received institutional research grants from LEO Pharma and Novartis; performed consultancies for AbbVie, Janssen, Galderma, LEO, Sanofi-Genzyme, and Novartis; has also lectured at educational events sponsored by AbbVie, Janssen, Celgene, Galderma, LEO Pharma, Sanofi, and Novartis; and is involved in performing clinical trials for various pharmaceutical enterprises that manufacture drugs used for the treatment of and atopic dermatitis. S. Weidinger is coprincipal investigator of the German Atopic Eczema Registry TREATgermany; has received institutional research grants from Sanofi Deutschland GmbH, Leo Pharma, and La Roche Posay; has performed consultancies for Sanofi-Genzyme, Regeneron, LEO Pharma, AbbVie, Pfizer, Eli Lilly, Kymab, and Novartis; has also lectured at educational events sponsored by Sanofi-Genzyme, Regeneron, LEO Pharma, AbbVie, Novartis, and Galderma; and is involved in performing clinical trials with many pharmaceutical enterprises that manufacture drugs used for the treatment of psoriasis and atopic eczema. The rest of the authors declare that they have no relevant conflicts of interest.

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**Abbreviations used**

AL:	Lesional AD skin
AN:	Nonlesional AD skin
NN:	Healthy skin
DEG:	Differentially expressed gene
EASI:	Eczema Area and Severity Index
FDR:	False discovery rate
LFC:	Log <sub>2</sub> fold change
NK:	Natural killer
oSCORAD:	Objective Scoring of AD
POEM:	Patient-Oriented Eczema Measure
RNA-Seq:	RNA sequencing

Microarray and quantitative RT-PCR studies on skin specimens have revealed a large number of dysregulated genes and pathways, in particular in lesions such as type 2 cytokines and chemokines (such as IL-13, IL-31, CCL17, CCL18, CCL22, and CCL26), epidermal differentiation and proliferation markers (such as KRT16, FLG, LOR), and T<sub>H</sub>17/T<sub>H</sub>22 cell activity-related genes (such as IL-17A, IL-22, IL-23A/p19, IL-12B/p40, CCL20, DEFB4A, and STAT3).<sup>3-5</sup> A recent meta-analysis also identified lipid abnormalities (FA2H, FAR2, and ELOVL3) and their correlation with T<sub>H</sub>2 cell immune activation in lesional skin (AL) versus nonlesional skin (AN).<sup>6</sup> Several clinical trials have indicated that systemic treatment with biologics and immunosuppressants modifies cutaneous gene expression and that clinical improvements are associated with a shift in expression levels of many of the genes dysregulated in marker lesions toward the levels observed in AN skin of patients with AD.<sup>7-11</sup> Most of these analyses were done in the context of clinical trials and their specific settings, were based on microarray profiling, and so far lack independent replication. Here, we have set out to study the cutaneous AD transcriptome and its changes under systemic therapy in a routine care patient population from the German TREATgermany registry by using RNA sequencing (RNA-Seq) as a powerful alternative to microarrays, thus providing higher precision and accuracy.<sup>12-17</sup> To date, there are only few AD transcriptome studies based on RNA-Seq data.<sup>5,18,19</sup> We have also included healthy controls to distinguish between inflammation-related signatures and basal AD signatures.

TREATgermany is a real-world academia-led disease registry that longitudinally collects clinical and lifestyle data from adult patients with moderate-to-severe AD at more than 30 study centers in Germany.<sup>20</sup> Biosamples were collected at baseline and after 24 months, as well as before and 3 months after initiation of a systemic treatment.

**METHODS****Patient characteristics and skin samples**

This study included adult patients with moderate-to-severe AD from the German TREATgermany registry<sup>20</sup>; they were recruited between July 2017 and February 2019 at 6 of the 17 study centres that agreed to participate in the additional and optional bioanalytics module. Intrapersonal AL and AN biopsy specimens (4 mm) were collected from 59 patients before the initiation of systemic therapy under a protocol approved by the Medical Faculty of the Christian-Albrechts-University, Kiel, Germany (B 261/16), and the responsible local ethics committees at the other participating sites. Of those patients, 34 and 11 received anti-IL-4R $\alpha$  dupilumab and cyclosporine, respectively; follow-up biopsy specimens at month 3 were available from 22 and 8 of those patients at the time when molecular analysis was conducted. AN samples were

taken at least 5 cm from the active lesion. Biopsy specimens were preferably collected from the upper extremities, otherwise from the trunk. In addition, RNA from biopsy specimens of 31 healthy volunteers with no history of atopic or chronic inflammatory disease who were included in a previous skin transcriptome study<sup>5</sup> under an institutional review board-approved protocol (A 110/12) was available for this study. Biopsy specimens from healthy controls had been taken from the upper extremities. Disease activity was evaluated by using the following assessment instruments: Eczema Area and Severity Index (EASI), Investigator's Global Assessment (IGA), objective Scoring of AD (oSCORAD), and Patient-Oriented Eczema Measure (POEM). Response to therapy was defined as achieving at least a 50% improvement in EASI, and high response was defined as achieving at least a 75% improvement of EASI plus an EASI of 7 or lower.

**RNA-Seq**

RNA later-fixed skin tissue samples were disrupted by using innuSPEED Lysis Tubes W (1.4- to 1.6-mm steel beads and 3.5-mm ceramic beads) (Analytik Jena, Jena, Germany) in a SpeedMill Plus (3 times at 1-minute intervals) (Analytik Jena) in Lysis/Binding Buffer (Life Technologies, Carlsbad, Calif). Total RNA was isolated by using the mirVanaTM miRNA Isolation Kit (Life Technologies) following the manufacturers specifications with an additional on-column DNase treatment. Quality control of concentration and integrity of the isolated RNA was performed with the Qubit 2.0 Fluorometer (Qubit RNA HS Assay [Life Technologies]) and the 2200 Tape Station (R6K ScreenTape Assay [Agilent, Santa Clara, Calif]) following the manufacturer's instructions. RNA libraries were prepared by using the Illumina Truseq Stranded mRNA protocol including poly-A enrichment. Libraries were sequenced on the HiSeq4000 with 2  $\times$  75 bp, producing paired-end reads according to the manufacturer's protocol (Illumina, San Diego, Calif). Raw mRNA sequencing data were processed by using Cutadapt (version 1.15),<sup>21</sup> Tophat2 (version 2.1.1)<sup>22</sup> together with Bowtie 2 (version 2.3.2),<sup>23</sup> Samtools (version 1.5),<sup>24</sup> and HTSeq (version 0.10.0),<sup>25</sup> and annotated according to the Gencode version 27 annotation gtf file.

**Statistical analysis**

Technical batch effects and outliers were assessed by using principal component analysis. Differential expression analysis between different conditions was conducted by using the DESeq2 Bioconductor package (version 1.24.0).<sup>26</sup> Analysis was performed by using the parametric Wald test and independent filtering of the results. A false discovery rate (FDR) less than 5% was used to declare significance, and genes with an absolute log<sub>2</sub> fold change (LFC) of 1 or more were defined as differentially expressed genes (DEGs). LFC estimates were corrected by using the DESeq2 inbuilt Log<sub>2</sub> Fold Change shrinkage function with the *apeglm*<sup>27</sup> method. For descriptive statistics and visualization, R software (version 3.6.1) with various R and Bioconductor packages and normalized or transformed gene counts were used. Pathway analysis was performed by using FunMappOne software<sup>28</sup> (for more details, see the Methods section in the [Online Repository at www.jacionline.org](http://www.jacionline.org)). Coexpression network analysis was performed using InFORM.<sup>29</sup>

**RESULTS**

After quality control, full data sets were available for 57 AL and 54 AN pretreatment transcriptomes, as well as for 30 AL and 27 AN posttreatment transcriptomes. In all, 31 healthy skin (NN) (ie, control skin) transcriptomes were available for comparison. Demographics and clinical characteristics of the participants from the TREATgermany and the control cohort are shown in [Table E1](#) (in this article's [Online Repository at www.jacionline.org](http://www.jacionline.org)). An average of 25,139, 25,230, and 25,090 genes were expressed in AL, AN, and NN (control) samples, respectively, whereas there were 985, 5787, and 3739 DEGs (FDR-adjusted  $P < .05$  by

pairwise Wald test and absolute LFC  $\geq 1$ ) between AL and AN, AL and NN, and AN and NN, respectively (see [Tables E2–E4](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). On a transcriptome-wide level, AL, AN, and NN samples clustered distinctly ([Fig 1, A](#)), whereby the distribution of pairwise distances (each among the NN, AN, and AL samples) showed that the AL specimens in this study were more heterogeneous than were AN from the same patients ( $P = 1.487e-10$ ), and both were significantly more heterogeneous than the NN ( $P < 2.2e-16$ ;  $P < 2.2e-16$ ) (see [Table E5](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The top 10 expressed genes across all samples (highest expression in AL, AN, and NN) are known skin/epithelial specific keratins, as well as mitochondrial and ribosomal “housekeeping” genes such as the *keratin* genes *1*, *2*, *5*, *10*, and *14*; the mitochondrial genes *MT-CO1*, *MT-CO3*, *MT-ND4*, and *MT-RNR2*; and the *EEF1A1* gene encoding a protein involved in protein synthesis. Two genes, *DEFB4B* and *CLEC3A*, were detected in AN but not in NN. Both were detected in AL as well, whereby *DEFB4B* showed a distinctly higher expression in AL than in AN. We detected a notable expression of *IL19* and *SLC18A3* in AL, but not in AN and NN (for *IL19*, FDR-adjusted  $P = .02$  in AL vs in AN by the paired Wald test;  $P = 1.8e-124$  in AL vs NN by the unpaired Wald test).

### The AD core transcriptome signature is characterized by disturbed epidermal differentiation and activation of itch pathways

Several genes were dysregulated to a comparable extent in both AL and AN as compared with in NN (“core signature” AL = AN  $\neq$  NN, based on FDR-adjusted  $P < .05$  by the pairwise Wald test). This core signature comprised a total of 7466 genes (see [Table E6](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), of which 2162 revealed an absolute LFC of 1 or more in AL versus in NN. The core transcriptomic signature included genes involved in skin homeostasis, such as *KRT7* (upregulated), occurring in basal and suprabasal layers as well as in sebaceous glands; *KRT10*, a marker for early keratinocyte differentiation in suprabasal layers of the epidermis; genes involved in lipid metabolism such as *ELOVL1* (downregulated) and *FABP3*, *FABP4*, and *FABP9* (all upregulated); and the kallikrein genes *KLK5*, *KLK9*, *KLK11*, and *KLK14* (all downregulated), encoding proteases. Together, these observations indicate that a disturbance of epidermal homeostasis is a general feature of AD ([Fig 1, B](#)). Further, several interleukin receptor signaling complexes showed a dysregulation in both AL and AN (eg, upregulation of IL-1R1 along with a slight increase in IL-1B level and a decrease in level of the IL-1 antagonist IL-1RN). Likewise, the level of IL-31RA was upregulated in both AL and AN (along with a gradual [AL > AN > NN] increase of the receptor partner oncostatin M receptor [OSMR] and a slight increase of IL-31 level in AL, but not in AN). Further, both AL and AN showed an increased expression of JAK1 and STAT5B, which encode downstream molecules of the IL31RA/OSMR receptor complex. We identified *IL12A/p35* among the core gene set, indicating elevated T<sub>H</sub>1 cell differentiation signaling in AD. In contrast, *IL12B/p40* and the receptor subunit genes *IL12RB1* and *IL12RB2* showed a stronger upregulation in AL skin than in AN skin, indicating progressive upregulation of the IL-23 receptor signaling complex, where *IL12B/p40* (besides *IL23A/p19*) encodes a necessary ligand for T<sub>H</sub>17 cell signal transduction. Further genes that showed dysregulation to

a comparable extent in both AL and AN include *IL6ST/CD130*, *TSLP*, and *IL1RL2* (upregulated), and *NFKBIL1* (downregulated). Together, these observations indicate that the transcriptome in AD shows a generally disturbed expression of genes mainly related to keratinocyte differentiation and skin barrier function, as well as to itch signaling.

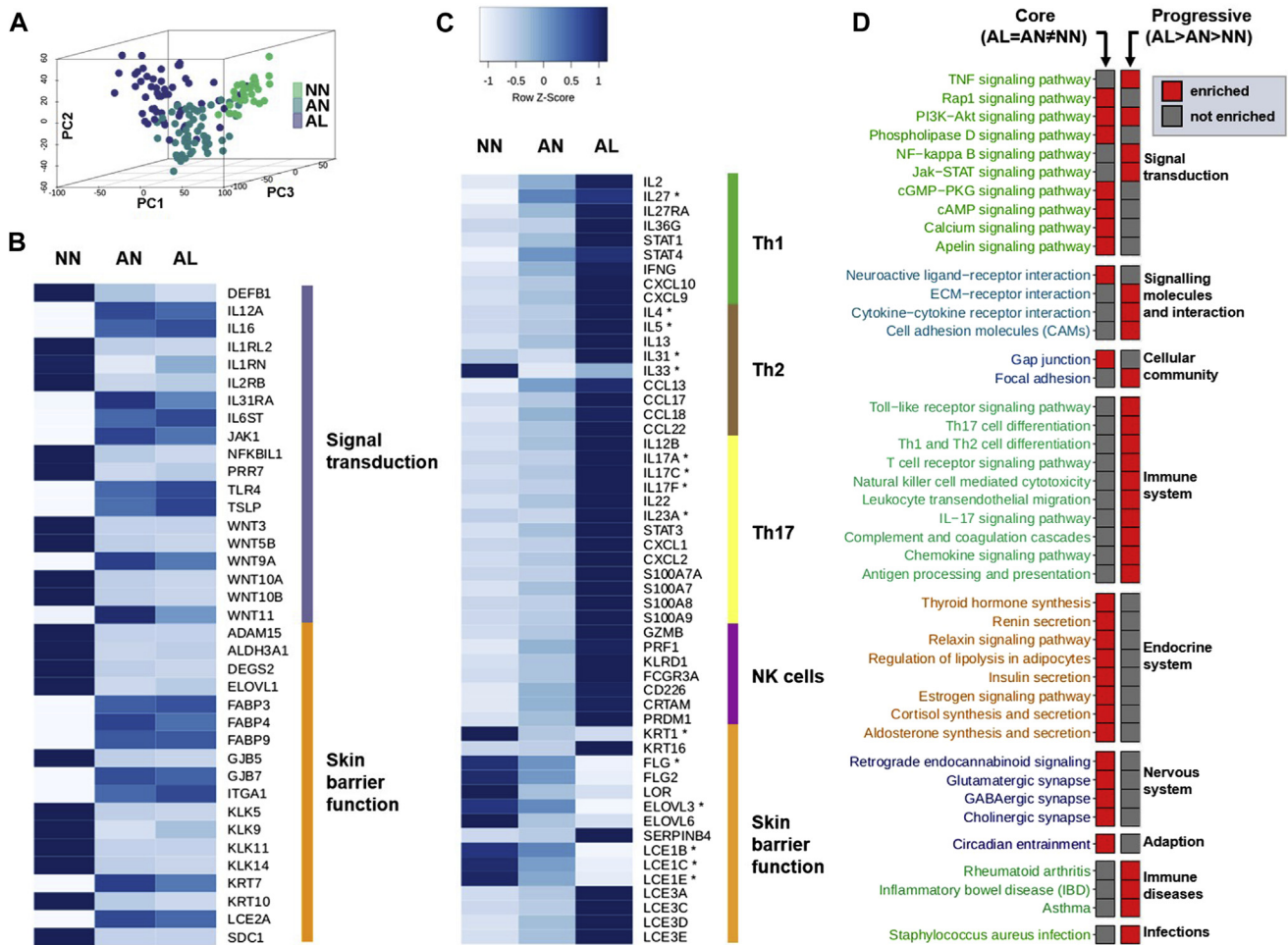
### The AD dynamic transcriptome signature is characterized by progressive activation of type 2 and T<sub>H</sub>17 cell signaling

A more progressive dysregulation was observed for 4713 genes (“dynamic signature”; AL > AN > NN or AL < AN < NN, according to FDR-adjusted  $P < .05$  by pairwise Wald test) (see [Table E7](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), of which 2603 genes revealed an absolute LFC of 1 or more in AL versus NN.

A progressive upregulation (AL > AN > NN) was seen for classical type 2 genes such as *IL13*, *IL22*, *CCL13*, *CCL17*, *CCL18*, *CCL22*, and *CCL26*, but also T<sub>H</sub>1 and T<sub>H</sub>17 cell genes, and genes involved in natural killer (NK) cell function, such as granzyme B (*GZMB*), perforin (*PRF1*), *KLRD1/CD94*, *FCGR3A/CD16A*, *CD226/DNAM-1*, *CRTAM*, and *PRDM1* ([Fig 1, C](#)). Along with this, there was a gradual downregulation of several structural genes related to epidermal barrier function, such as *LOR* (LOR being the main protein component of the cornified envelope), *FLG* and *KRT1* (genes of the LCE group 1), and *ELOVL3* and *ELOVL6* (enzymes important for skin lipid homeostasis),<sup>30</sup> whereas the expression of *LCE3* genes (which have antimicrobial activity<sup>31</sup>) showed an upregulation ([Fig 1, C](#)).

On the pathway level, this was reflected in a gradual upregulation of immune system pathways such as activation of cytokine and chemokine signaling, cell adhesion, T<sub>H</sub>1 and T<sub>H</sub>2 cell differentiation pathways; TNF, NF- $\kappa$ B, and PI3K-Akt signal transduction pathways; and signatures associated with other immune-mediated diseases such as asthma and rheumatoid arthritis, as well as infectious diseases ([Fig 1, D](#)). In contrast, the AD core transcriptome signature was not enriched for any pathway directly related to immune system but was instead characterized by an upregulation of several signal transduction pathways related to cell proliferation, remodeling, trafficking, and junction formation, pathways related to the nervous system such as glutamatergic,  $\gamma$ -aminobutyric acid-ergic, and cholinergic synapse, as well as neuroactive ligand-receptor interaction pathways ([Fig 1, D](#)).

Of note, the most discriminating feature (with regard to gene expression fold change) between AL and AN was the upregulation of IL17 signaling. Although some genes related to NK cell function were part of the core signature (*CD56/NCAM1* and *Nkp80/KLRF1*), a much more pronounced and progressive upregulation of markers related to NK cell function was seen in lesional skin, and a gene set enrichment analysis by using differences (LFC) in all genes covered by sequencing between AL and AN (see [Table E8](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) revealed the natural killer NK cell-mediated cytotoxicity pathway as 1 of the top enriched upregulated pathways, along with the IL-17 signaling pathway and T<sub>H</sub>17 cell differentiation ([Fig 2, A](#)), indicating that genes involved in those pathways separate AL from AN. Unsupervised hierarchical clustering based on genes related to NK cell function indeed



**FIG 1.** AD skin transcriptome signatures. **A**, First 3 principal components based on all measured genes (excluding genes on the X chromosome and Y chromosome). **B**, Heatmap of NN, AN, and AL mean expression values of selected genes of the AD core signature. Color code represents row z scores. **C**, Heatmap of NN, AN, and AL mean expression values of selected genes of the AD dynamic signature. Color code represents row z scores. \*Genes show progressive dysregulation but do not fulfil predefined criteria of dynamic signature. **D**, Pathway enrichment analysis of genes of the AD core transcriptomic signature ( $AL = AN \neq NN$ , based on FDR-adjusted  $P < .05$  by the pairwise Wald test plus absolute LFC  $\geq 1$  in AL vs in NN) and progressively dysregulated genes ( $AL > AN > NN$  or  $AL < AN < NN$ , based on FDR-adjusted  $P < .05$  by pairwise Wald test plus absolute LFC  $\geq 1$  in AL vs in NN). All significantly enriched pathways were upregulated.

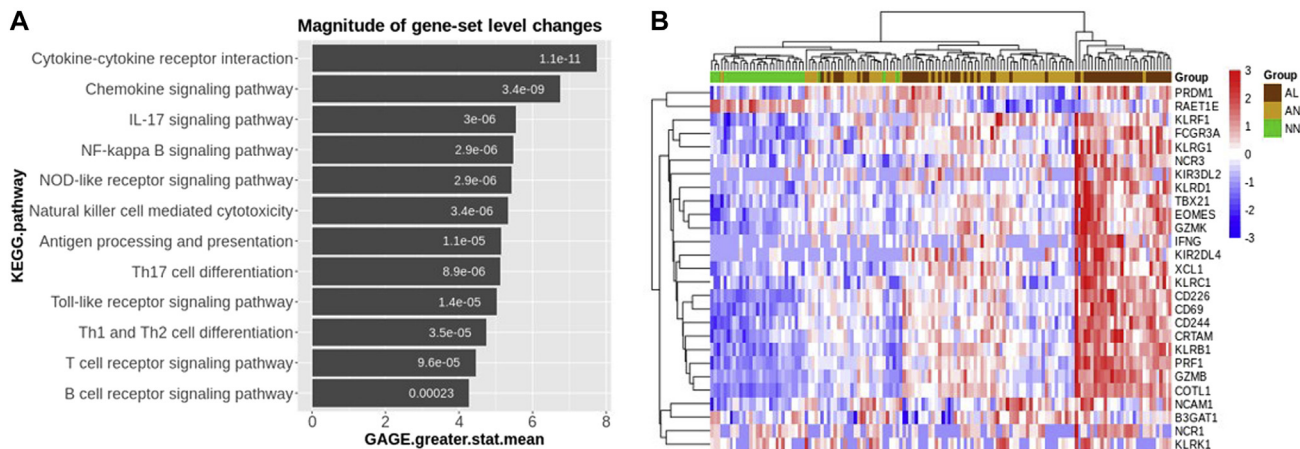
separated NN skin from AN and AL almost perfectly, and AN from AL to a reasonable extent (Fig 2, B).

Because AD is known to be dominated by type 2 mechanisms, we specifically examined the expression pattern of key type 2 markers such as IL-4, IL-5, IL-9, IL-13, TSLP, and CCL17/TARC and their correlation with each other and with markers of epidermal barrier function (see Table E9 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). IL-13, TSLP, and CCL17/TARC were expressed at median levels of 10, 35, and 203 detected gene transcripts in AL and 3, 30, and 38 transcripts in AN, respectively, whereas IL-4, IL-5, and IL-9 were virtually absent in both AL and AN. Lesional expression levels of IL-13 correlated strongly and positively with other type 2 markers such as IL-22 (Spearman rho [ $\rho$ ] = 0.79) CCL17/TARC ( $\rho$  = 0.77), and CCL22 ( $\rho$  = 0.77), and negatively with genes related to epidermal differentiation such as *FLG* ( $\rho$  = -0.53), *LOR* ( $\rho$  = -0.43), and *KLK5* ( $\rho$  = -0.37), *KRT1* ( $\rho$  = -0.29), and *KRT10* ( $\rho$  = -0.35) (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Additional genes showing strong positive

correlation with *IL13* were *MMP12* ( $\rho$  = 0.85), an enzyme involved in tissue remodeling and skin fibrosis,<sup>32</sup> *ALOX15* ( $\rho$  = 0.80), which contributes to corneocyte lipid envelope formation and affects the differentiation and function of dendritic cells,<sup>33</sup> and *IL4I1* ( $\rho$  = 0.81), which has antibacterial and immunomodulatory properties.<sup>34</sup> There was also a positive correlation of *IL13/IL4* receptor genes with type 2 markers, albeit of moderate strength, and a negative correlation with epidermal differentiation markers. Of note, *IL4RA* in particular showed a strong positive correlation with established AD marker genes expressed by keratinocytes such as the S100A genes *KRT16*, *KRT6A*, and *SERPINB4* (mean  $\rho$  = 0.76) and a strong negative correlation with *LOR* ( $\rho$  = -0.72), whereby type 2 markers IL-13, CCL17, and CCL22 showed a distinctly weaker correlation, indicating a strong link of *IL4RA* with markers of disturbed epidermal conditions.

To relate transcript levels to disease activity (as determined by EASI, oSCORAD, and POEM), we correlated AL and AN gene expression with AD severity measures. Generally, AL expression





**FIG 2.** AD skin transcriptomic NK cell signature. **A**, Magnitude of gene set–level changes in AL vs in AN. The x-axis shows the greater statistical mean value, and white numbers show the *q* values from the gene set enrichment analysis (performed with Generally Applicable Gene Set Enrichment [GAGE]) for top enriched pathways. **B**, Unsupervised hierarchic clustering of AL, AN, and NN samples based on genes related to NK cell function. Color code represents row *z* score, which was restricted to values from -3 to 3.

levels were more strongly correlated with severity than AN expression levels were; thus, only correlation in AL is reported (see Table E10 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)). We observed moderate correlations of EASI with genes related to cell junction and adhesion; water and urea transport across cell membranes; innate immune system; and signaling pathways involved in itch, such as aryl hydrogen receptor (AHR), CST6, aquaporin 3 (AQP3), IL36A, and OSMR ( $\rho = 0.52, -0.5, 0.47, 0.46,$  and  $0.46$ , respectively) (Fig 3, A). The POEM score yielded multiple additional correlations (eg, with tight junction protein 2 [TJP2] [ $\rho = 0.58$ ], protocadherin 7 [PCDH7] [ $\rho = 0.57$ ], NPEPPS [ $\rho = 0.56$ ], apolipoprotein E [APOE] [ $\rho = -0.53$ ], and MALT1 [ $\rho = 0.52$ ]) that were significant after FDR correction (Fig 3, B). The genes most strongly correlated with disease activity (EASI) did not correlate strongly with type 2 marker genes (eg, although associated with AD *per se*, type 2 markers such as IL-13, IL-22, and CCL17/TARC overall showed only weak correlations with severity scores). In contrast, moderate-to-strong correlations of the genes most strongly correlated with EASI were seen with T<sub>H</sub>17 cell/IL-23 markers. The strongest correlation was observed between PI3 and IL-36A ( $\rho = 0.71$ ), and between IL-23A and IL-36A ( $\rho = 0.64$ ). A correlation analysis of transcript levels in AL with itch intensity as determined by numerical rating scale (NRS) did not reveal any striking correlations (see Table E10). Of note, neither of the genes most strongly correlated with the EASI or POEM showed marked correlations with itch.

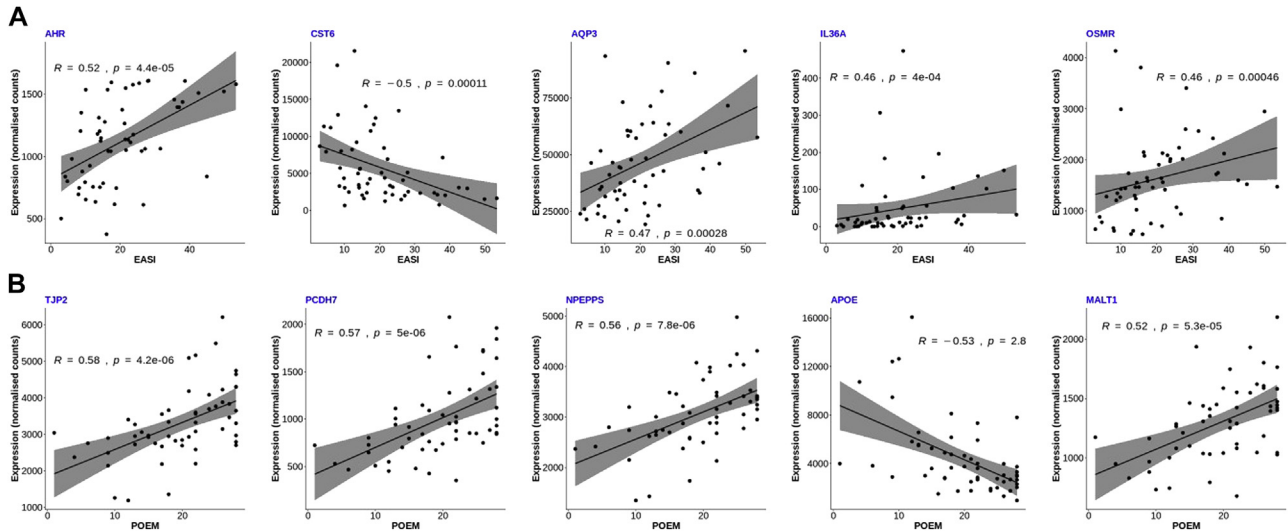
### The *IL4RA* gene is a central gene in the lesional AD transcriptome network

Coexpression network analysis was performed to relate DEGs in AL versus in AN ( $n = 985$  DEGs [see Table E2]) with each other. After the network had been computed, genes were ranked by the combined influence of network centrality properties and differential expression scores (see Table E11 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)). According to our analysis, *SH2D2A* encoding a T-cell–specific adapter protein

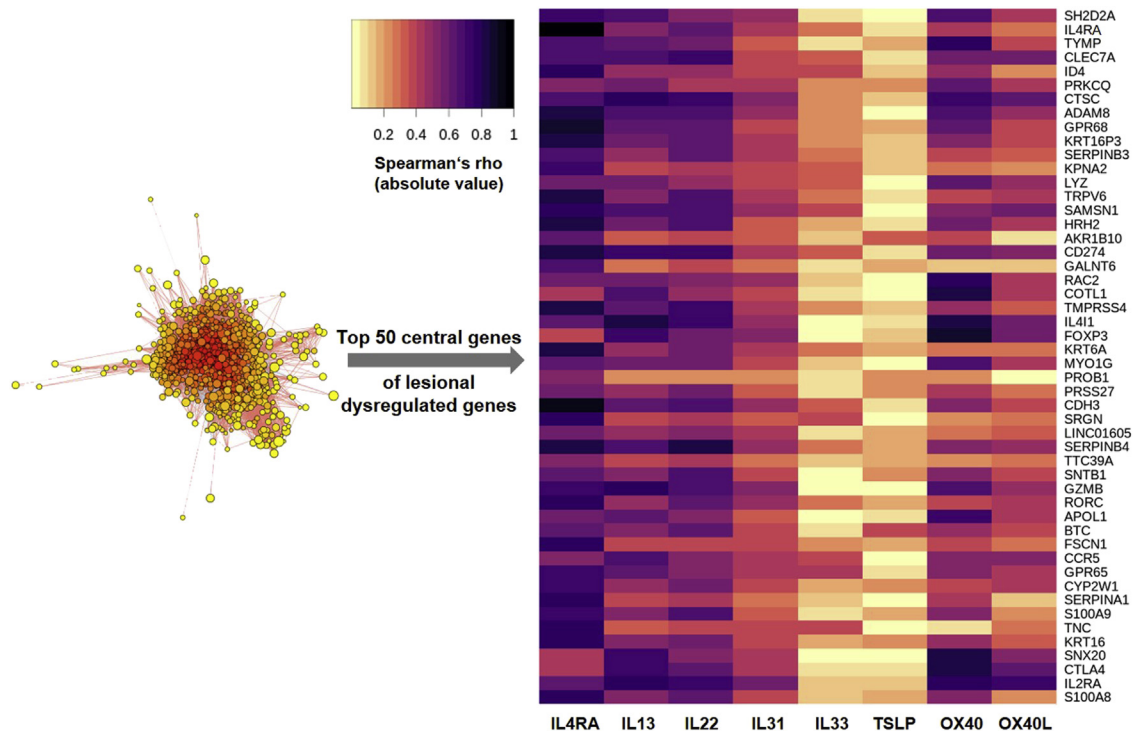
involved in T-cell activation,<sup>35</sup> *IL4RA* encoding the IL4R  $\alpha$ -chain, and *TYMP* encoding an angiogenic factor attracting and promoting the growth of endothelial cells<sup>36</sup> were the top 3 central genes of transcriptome dysregulation in AL. Notably, the consensus network allocated a central role to *IL4RA*, but not to those genes encoding its receptor partners (*IL13RA1* and *IL2RG*) or to its ligand IL13. Other highly ranked genes included cathepsin C (*CTSC*), histamine receptor H2 (*HRH2*), *FOXP3*, *KRT6A*, cadherin 3 (*CDH3*), and granzyme B (*GZMB*). We correlated the top 50 central genes with 8 specific target proteins (IL-4RA, IL-13, IL-22, IL-31, IL-33, TSLP, OX40, and OX40L) that are targets of mAbs under development as AD therapies (Fig 4). The transcription levels of *IL4RA* on average showed the strongest correlation with central genes (mean  $\rho = 0.51$ ), which is explained by the fact that *IL4RA* itself was a top central gene, indicating that blocking *IL4RA* affects the expression of top central dysregulated genes in AD lesions (Fig 4). We observed weaker correlations for *IL13* (mean  $\rho = 0.45$ ), *IL22* (mean  $\rho = 0.44$ ), *OX40/TNFRSF4* (mean  $\rho = 0.43$ ), *IL31* (mean  $\rho = 0.32$ ), and *OX40L/TNFRSF4* (mean  $\rho = 0.31$ ), and very weak correlations for *IL33* (mean  $\rho = 0.15$ ) and *TSLP* (mean  $\rho = 0.09$ ).

### Dupilumab treatment leads to a stronger normalization of skin barrier–related genes, whereas cyclosporine treatment has more pronounced effects on T<sub>H</sub>17 cell/IL-23 signaling in lesional skin

The mean reduction in EASI was 75% in patients treated with dupilumab and 48% in those treated with cyclosporine ( $P = .02$  [see Tables E12 and E13 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)]). In all, 86.4% and 62.5% of the patients treated with dupilumab and cyclosporine, respectively, reached at least a 50% reduction in EASI at month 3. There were no marked differences in the pretreatment transcriptome profiles between groups (see Fig E2 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)). Despite the lower overall clinical efficacy,



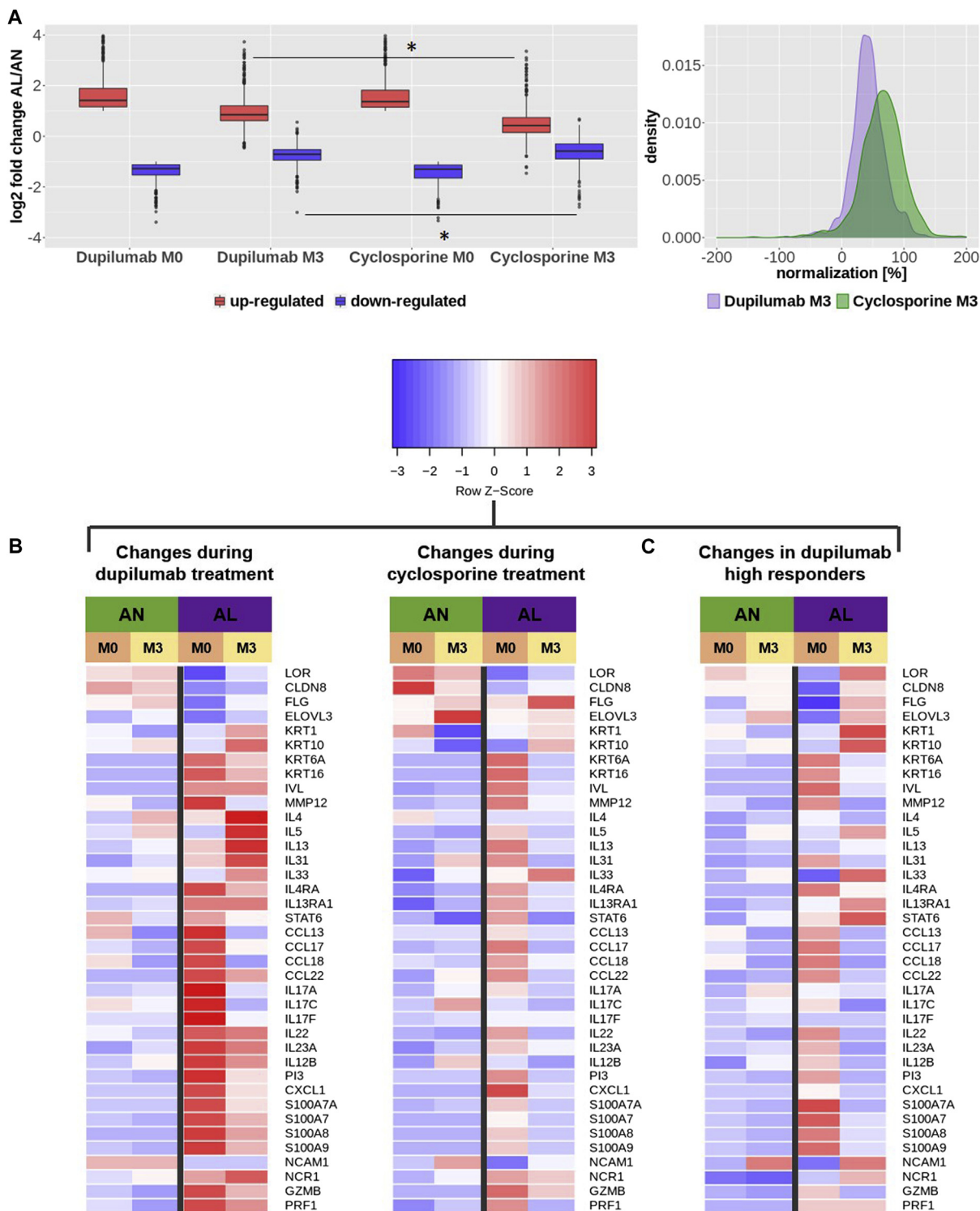
**FIG 3.** Gene expression associated with AD disease severity scores. **A**, Top Spearman correlations of pretreatment AL gene expression with the EASI. **B**, Top Spearman correlations of pretreatment AL gene expression with POEM.



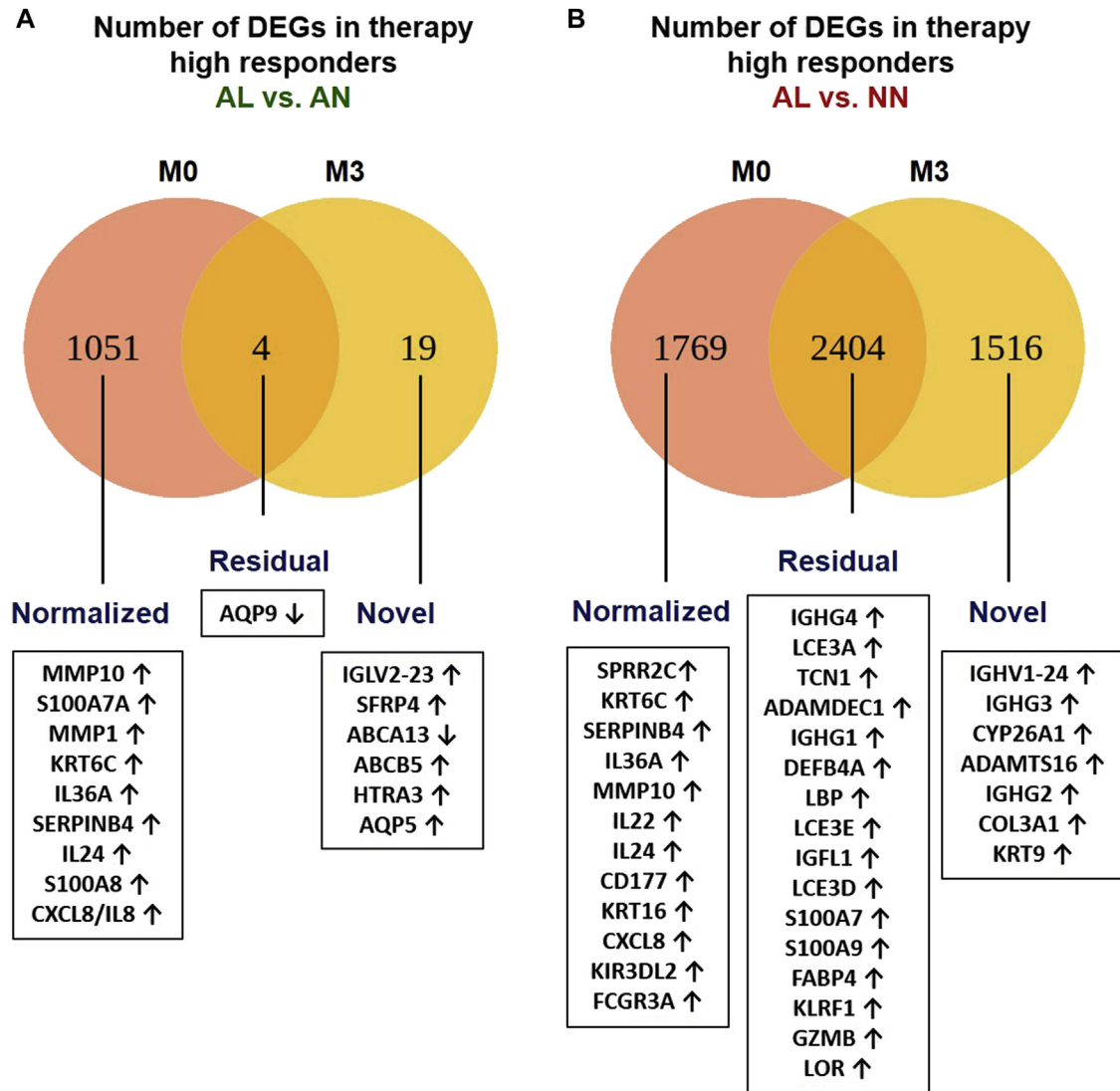
**FIG 4.** Coexpression network and its relation to (potential) therapeutic targets. *Left*, INFORM coexpression network with DEGs in AL and AN. Nodes are colored by rank from high rank (red) to low rank (yellow). The genes are ranked by the combined influence of network centrality properties and differential expression scores. *Right*, Top 50 central genes from the network analysis and their correlation with genes that can be blocked by targeted treatments and were tested in clinical trials of AD. Color code of the heatmap represents Spearman  $\rho$  (absolute value).

on a global level the shift of the AL transcriptome toward an AN-like transcriptome was significantly more pronounced under cyclosporine treatment (a 65% mean reduction in expression fold change between AL and AN) than under dupilumab treatment (a 43% mean reduction) (for upregulated genes,  $P < 2.2e-$

16; for downregulated genes,  $P = 7.2e-05$ ) (Fig 5, A). In the coexpression network analysis, the levels of the top 50 central genes changed by 50% in patients treated with cyclosporine and by 34% in patients treated with dupilumab (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).



**FIG 5.** Transcriptomic changes under therapy with dupilumab and cyclosporine. **A, Left,** Shift of LFCs of DEGs in AL and AN (with absolute LFC  $\geq 1$ ) from baseline (month 0 [M0]) to month 3 (M3) in patients treated with dupilumab and cyclosporine, respectively. The upregulated and downregulated genes in AL compared with in AN are depicted in red and blue, respectively.  $*P < .05$  by Wilcoxon test. **Right,** The distribution of normalization of AL gene expression toward AN expression levels. A 100% normalization means that the expression fold change between AL and AN after 3 months of therapy was 0. **B,** Heatmaps showing mean expression values (mean normalized counts) of selected AD-related genes in patients treated with dupilumab or cyclosporine, grouped according to time point and treatment. Rows are ordered by annotations of genes to functions/immune axes. **C,** Heatmap showing the same as in **(B)** for high responders to dupilumab only ( $\geq 75\%$  improvement in EASI and EASI  $\leq 7$ ;  $n = 7$  patients). Color code represents row z score above all 3 heatmaps together.



**FIG 6.** Reverted and residual genomic profile in responders to dupilumab. **A**, The number of DEGs in AL and AN before (month 0 [M0] [orange]) and after 3 months of treatment (M3) (yellow), and the overlap of both. All genes with an FDR-adjusted *P* value less than .05 were considered DEGs. **B**, The number of DEGs in AL and NN before (M0 [orange]) and after 3 months of treatment (M3 [yellow]), and the overlap of both. All genes with an FDR-adjusted *P* value less than .05 and an absolute LFC of 1 or more were considered DEGs.

Treatment with dupilumab in particular decreased the expression of type 2-related chemokines such as CCL13, CCL17, CCL18, and CCL22 and had a strong effect on normalization of skin barrier function-related markers such as LOR, CLDN8, FLG, KRT1, KRT10, and in particular ELOVL3 (533%), whereas the expression levels of type 2 cytokines such as IL-4, IL-5, IL-13, and IL-31 showed an increase (Fig 5, B and see Table E14 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). In general, expression changes correlated with the degree of clinical response and were most pronounced in "high responders" (Fig 5, C and see Table E14). Interestingly, patients with a particularly strong response to dupilumab (EASI75 plus EASI  $\leq 7$ ) had lower baseline IL-4RA and IL-13 expression, a more pronounced decrease in IL-4RA expression, and less pronounced increases in IL-13

expression (Fig 5, B and C and see Table E14). Further, these patients showed a distinct increase (41%) in level of general NK cell markers such as CD56/NCAM1 and Nkp46, whereas levels of NK cell activity markers (granzymes, perforin/PRF1, TRAIL/TNFSF10, FAS, FASLG, and granulysin/GNLY) showed no marked changes (see Table E14).

Treatment with cyclosporine was also associated with a strong decrease in levels of key type 2 markers, including IL-13, IL-4RA, IL-13RA1, and IL-31, and a stronger reduction in levels of  $T_H17$  cell/IL23 signaling markers such as IL-22, IL-23A/p19, IL-12B/p40, and PI3/elafin, as well as in levels of S100A8, and S100A9 (Fig 5, B [see Table E14]). The level of expression of general NK cell markers did not show marked changes, but there was a decrease in level of NK cell activity markers by 24%. To



work out discriminating features in terms of expression changes induced by treatment, we examined genes that showed a difference of at least 30% between dupilumab and cyclosporine in terms of improvement as compared to healthy controls (see Table E15 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Overall, a total of 313 genes showed a stronger improvement in response to cyclosporine. These genes have annotations mainly related to immune responses, T-cell response, and cytokine and chemokine signaling (eg, *IL2RA*, *IL9R*, *IL12B*, *IL26*, *CXCL3*, *CXCL5*, *CXCL8*; *CXCR1* and *CD80*; and *CTLA4* and *OX40-R/OX40*), as well as to keratinocyte differentiation (eg, *KRT16*, *KRT6A*, *PI3*, and *LCE3A*). A total of 58 genes showed a considerably stronger improvement with dupilumab; these genes were mainly related to leukocyte recruitment (eg, *CCL3* and *CCL4*), linoleic acid metabolism (eg, *ALOX15* and *CYP1A2*), and regulation of T-cell responses (eg, *TNFSF18* and *TNFRSF13B*). To identify baseline signatures associated with the degree of clinical response, we compared AL of patients achieving the greatest reduction of EASI (upper quartile) with patients showing the least reduction of EASI (lower quartile). The top gene (according to fold change and *P* value) discriminating high from low responders was *IL36A*, which revealed a 5 times higher level of expression in high responders and decreased to levels seen in NN skin after 3 months of treatment (see Fig E4 and Table E16 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). To determine the residual disease genomic profile ("molecular scar") in target lesions, we compared dysregulated genes before and 3 months after treatment, whereby we discriminated the residual profile as compared with those of AN and NN, respectively. In patients treated with cyclosporine, less than 1% and 53% of dysregulated genes remained significantly dysregulated whereas 38% and 73% of dysregulated genes remained dysregulated after 3 months of dupilumab treatment. When the analysis was restricted to high responders to dupilumab, of the 1055 genes differentially regulated between AL and AN at baseline, only 1 (skin-relevant) gene, namely *AQP9* (aquaporin 9), was still characterized by significant (albeit improved) downregulation after 3 months of treatment (Fig 6, A). Of the 4173 genes differentially regulated at baseline between AL and NN, 2404 (58%) were still dysregulated after treatment (Fig 6, B). Thus, in patients achieving a high response to dupilumab, the transcriptome in lesions resembled the transcriptome in AN, but still showed large differences compared to NN. Notably, 41% of the AD core signature and 54% of the dynamic signature remained largely unchanged after 3 months of therapy. Interestingly, AN and AL may respond differently to treatment. Although  $T_H1$ -,  $T_H2$ -, and  $T_H17$  cell-related genes mostly showed a decrease in AL during both dupilumab and cyclosporine treatment, some markers, such as *CCL22*, *IL17A*, *IL23A*, and *IL12B*, were slightly upregulated in AN (Fig 5, B and see Table E14). In contrast, in both AL and AN, *IL33* expression increased strongly under both treatments.

## DISCUSSION

We provide evidence for a "core" AD gene expression profile found in both AL and AN, which is characterized by an activation of pathways related to epidermal barrier dysfunction and itch signaling. Of note, the AD core signature is not enriched for pathways directly related to immune response and inflammation. We further observed a progressive upregulation of immune responses, in particular, type 2,  $T_H17$ , and NK cell signaling

with inflammation ("dynamic" signature), along with a complex discoordinated expression of genes important for epidermal differentiation and skin lipid metabolism. In line with the findings of our previous RNA-Seq study on AD,<sup>5</sup> the type 2 phenotype in AD skin is largely attributable to IL-13, whereas IL-4 and IL-5 are virtually absent. Interestingly, activation of IL-17 and NK cell signaling as part of the dynamic signature constitute a strong discriminating feature between AL and AN, which is in line with the findings of previous studies suggesting that IL-17 signaling is not a unique feature of psoriatic skin but is also dysregulated in AD,<sup>4,6,37,38</sup> and with recent reports on an elevated NK cell transcriptional signature in AD skin lesions.<sup>39</sup> The NK cell signature observed appears to be derived from both NK1 and NK2 subsets previously defined on the basis of single-cell RNA-seq.<sup>40</sup> Key type 2 markers, in particular IL-13, were negatively correlated with structural genes related to barrier function and fatty acid metabolism, and positively correlated with each other, supporting previous observations of a close interaction between barrier abnormalities and cutaneous inflammation as well as progressively heightened and type 2-dominated immune signaling.<sup>4,5,18</sup> Despite their strong correlation with disease status, type 2 markers such as IL-13, IL-22, and CCL17/TARC overall showed only a weak correlation with disease activity. In contrast, markers related to epidermal barrier properties and immune modulation, such as *AQP3*,<sup>41</sup> *TJP2*,<sup>42</sup> *CST6*,<sup>43</sup> *AHR*,<sup>44</sup> and *IL-36A*,<sup>45</sup> as well as *OSMR* encoding a subunit of the *IL31RA/OSMR* receptor complex involved in itch signaling,<sup>46</sup> showed a stronger correlation. The overall only moderate strength of correlation ( $\rho = 0.4$ - $0.6$ ) is in accordance with that in other studies involving correlation analysis of molecular markers with EASI and oSCORAD.<sup>10,37</sup> Among the top 3 central genes in the lesional AD transcriptome network was *IL4RA*. Its transcription levels on average showed the strongest correlation with other central genes, implying that blocking *IL4RA* might positively influence the expression of top central dysregulated genes, as previous studies demonstrated that the activation level of a biologic signature can be characterized by its overall state of correlation, and that genes showing similar expression patterns may share biologic functions under common regulatory control.<sup>47-50</sup> Indeed, in the coexpression network analysis, levels of the top 50 central AD network genes changed by 34% in all patients treated with dupilumab and by 63% in patients showing a high response (defined as  $\geq 75\%$  improvement in EASI and absolute EASI  $\leq 7$  at month 3) to dupilumab. Interestingly, despite lower clinical efficacy, cyclosporine overall normalized the top 50 central genes to an even greater extent (50%). On the basis of our TNFRSF analyses, blockade of IL-13, IL-22, and OX40/TNFRSF4 should also positively affect central dysregulated genes, whereas blockade of IL-31 and OX40L, and in particular IL-33 and TSLP, may have only little effect on the lesional AD transcriptome network.

Both treatment with the *IL4RA*-blocking antibody dupilumab and the immunosuppressant cyclosporine resulted in a shift of the global AL transcriptome toward a profile seen in AN, an effect that was slightly more pronounced under cyclosporine treatment. Both treatments caused a strong decrease in cutaneous type 2 chemokine expression, as well as an increase in skin barrier-related genes, the latter of which was more pronounced for dupilumab. In addition, cyclosporine caused a decrease in the type 2 signature cytokines IL-4, IL-5, and IL-13, whereas they increased under treatment with dupilumab. This is to be expected,



given that cyclosporine blocks  $T_H2$  cell activation (and therefore cytokine expression), whereas dupilumab blocks IL-4 and IL-13 action. For cyclosporine, stronger effects were observed on DEGs related to the  $T_H17$  cell/IL23 axis, particularly IL-22, IL-12B/p40, and PI3/elafin. Dupilumab showed more specific effects and, for example, caused stronger improvement of ALOX15, which is involved in the cellular response to IL-13 and related to linoleic acid metabolism.<sup>51,52</sup> In line with that, we observed a high correlation of IL-13 and ALOX15 expression levels in lesions at baseline. Lower baseline IL-4RA and IL-13 levels and high IL-36A expression levels were related to a more pronounced clinical response to dupilumab. Interestingly, in patients with a high response to dupilumab, we observed an increase in NK cell marker expression, whereas the expression of NK cell activity markers remained largely stable, implying effects on a potential counterregulatory mechanism to aberrant type 2 inflammation, which warrants further investigation. Likewise, the increase in IL-33 expression levels under both treatments and in both lesional and nonlesional AD skin deserves further study. Overall, dupilumab and cyclosporine showed largely comparable effects in reverting the dysregulation of the transcriptome in lesions toward the profile seen in nonlesional AD skin. Although not all individual molecular changes were correlated with clinical improvements, the overall degree of changes was correlated with the degree of clinical improvement, indicating that these changes relate to healing of lesions rather than to a specific treatment. However, compared with the residual transcriptomic profile in healthy control skin, the residual transcriptomic profile in target lesions of high responders to dupilumab still showed profound dysregulation of 41% of the DEGs of the AD core signature and 54% of the DEGs of the dynamic signature, indicating that a fraction of the AD-associated transcriptome remains unchanged despite clinical improvement. It should also be noted that the interpretation of molecular changes is not straightforward, and that molecular changes do not necessarily have immediate clinical implications.

Our study has several strengths, but also some limitations. We utilized samples collected in a highly standardized fashion with accompanying deep clinical information from the academia-led prospective TREATgermany registry as well as carefully matched controls, and we applied highly sensitive sequencing and novel analytic approaches with higher sensitivity and versatility compared with those of array-based approaches. However, the sample size in the current analysis is still rather low, which precluded robust responder analyses, and whole biopsy specimens composed of a mixture of cellular components were used rather than single-cell transcriptomics.

In summary, our study provides novel insights into core and dynamic transcriptome signatures in AD, as well as insights into the specific changes associated with targeted treatment with dupilumab compared with the broader immunosuppression with cyclosporine. With growing numbers of patients recruited to TREATgermany and novel drugs becoming available and used, future analyses should allow verification and extension of the preliminary findings and assumptions reported here.

We would like to thank all participating patients and all study physicians and study staff in the participating clinics and offices for their support of the German Atopic Dermatitis Registry TREATgermany. As of 2020, the TREATgermany registry has been financially supported by Sanofi-Aventis Deutschland GmbH.

### Key messages

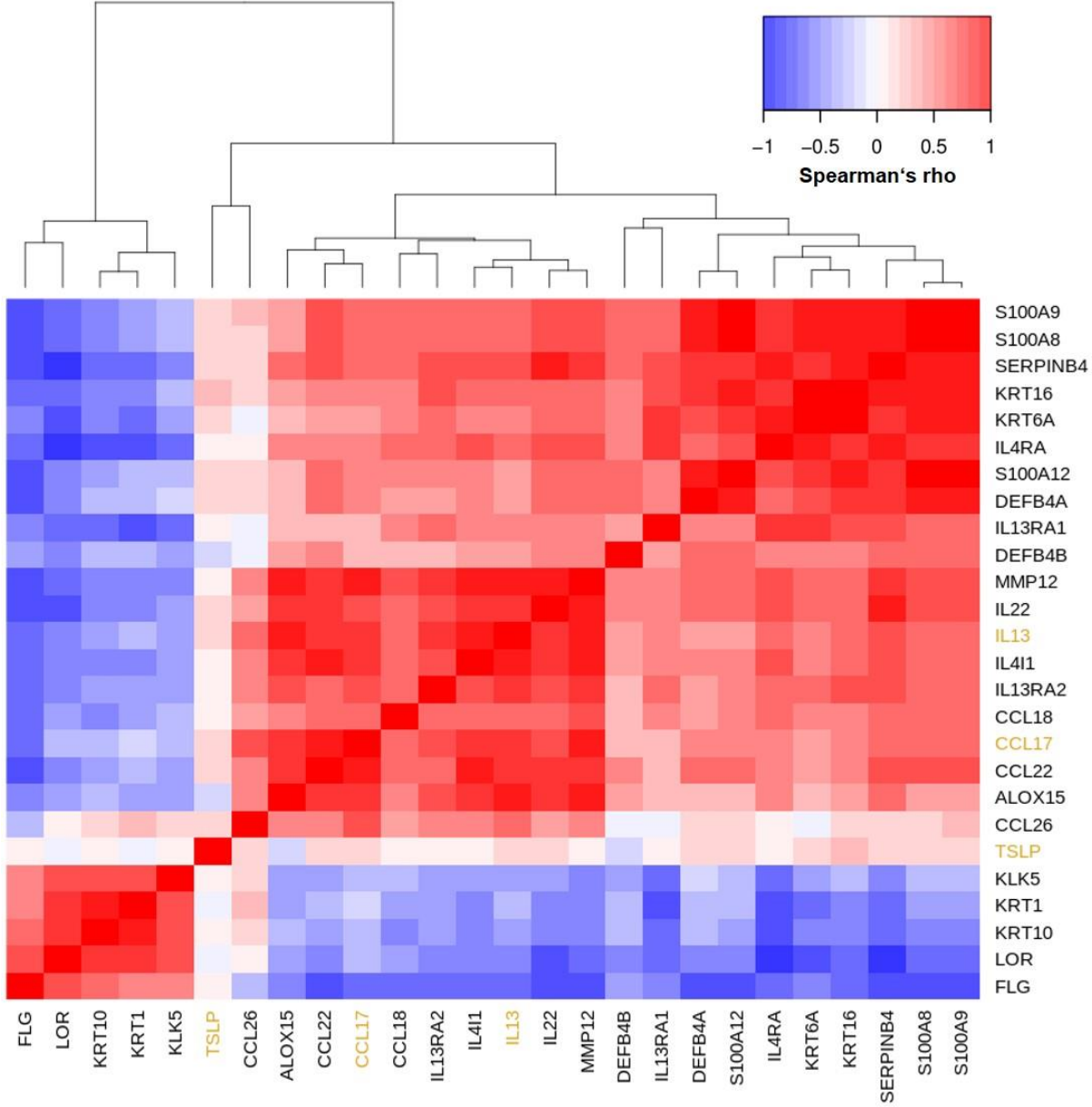
- The skin in AD displays a core transcriptomic signature characterized by a dysregulation of genes driving skin barrier dysfunction and itch signaling, whereas a second, more dynamic signature reflects progressive inflammatory mechanisms such as activation of type 2,  $T_H17$ , and NK cell function.
- Both dupilumab and cyclosporine led to a strong downregulation of type 2 markers and genes involved in  $T_H17$  cell/IL23 signaling.
- After 3 months of therapy, the transcriptome in target lesions resembled the nonlesional transcriptome; however, despite clinical improvement, a profound fraction of the AD-associated transcriptome remained dysregulated as compared with that of healthy skin.

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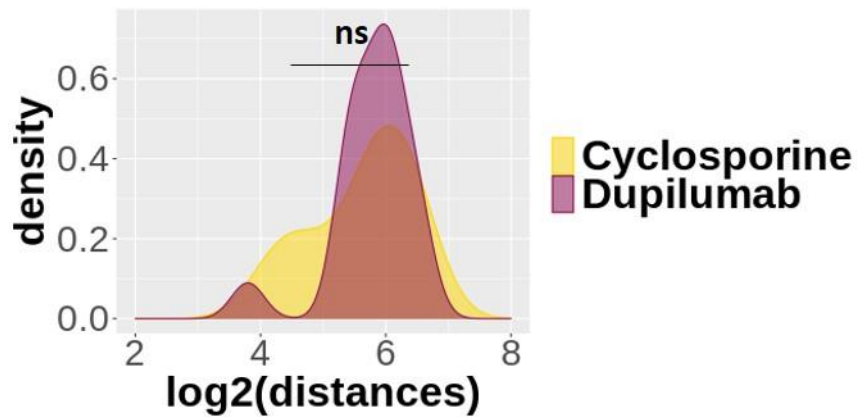
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Supplementary figures

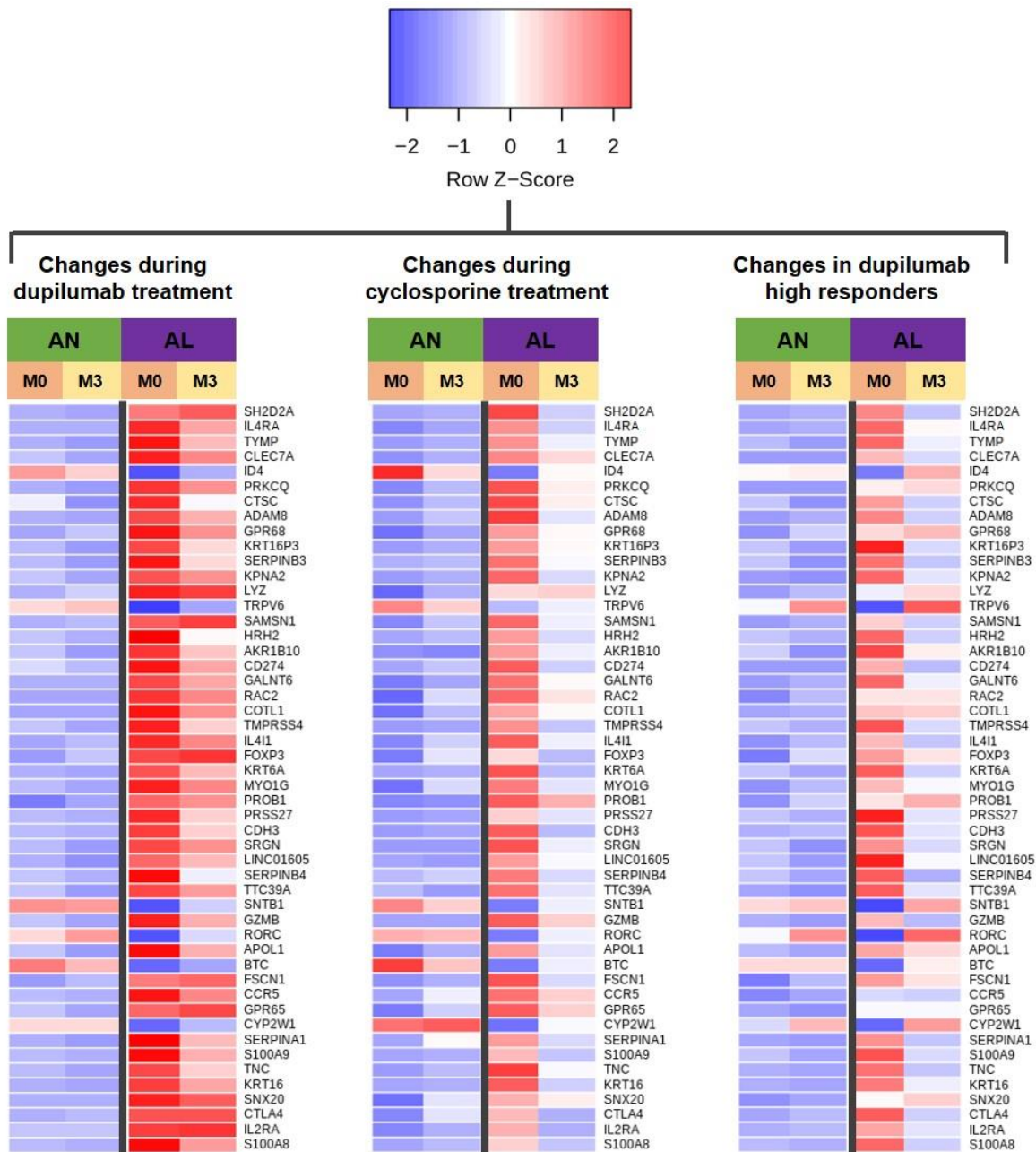


**FIG E1. Correlation of type 2 cytokines with other AD-related genes.** Heatmap of Spearman correlation of type 2 cytokines IL13, TSLP and CCL22/TARC with other AD-related genes. Colour code denotes Spearman's rho. Rows and columns were ordered by unbiased hierarchical clustering. Dendrogram is shown for columns.

## AL vs. AN global distances at baseline

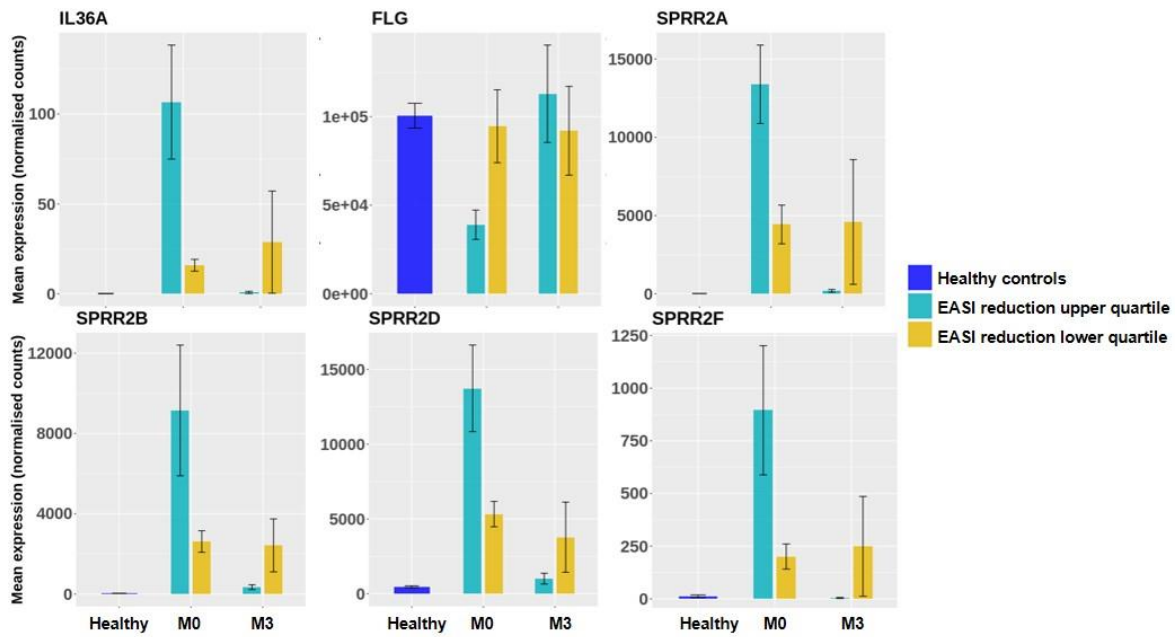


**FIG E2. AL-AN global distances for dupilumab and cyclosporine treatment group.** Distribution of pre-treatment lesional (AL) - non-lesional (AN) Euclidean distances (in log2 scale) for patients treated with dupilumab and cyclosporine, respectively (only patients for who post-treatment biopsies were available). The distance was computed based on the top three principal components and all measured genes (excluding genes on chrX and chrY). ns p-value >0.05 by Wilcoxon test.



**FIG E3. Transcriptomic changes of top central genes under therapy with dupilumab and cyclosporine.** Heatmaps showing mean expression values (mean normalised counts) of the top 50 central genes dysregulated in lesional skin grouped according to time point and treatment (dupilumab, cyclosporine, and dupilumab high responders). Rows are ordered by rank of the genes in the co-expression network. Colour code represents row Z-Scores.





**FIG E4. Baseline dysregulation related to dupilumab response.** Differentially expressed genes in baseline (M0) lesional (AL) skin of dupilumab high responders (patients achieving a reduction of EASI in the upper quartile) and dupilumab low responders (patients achieving a reduction of EASI in the lower quartile). Expression levels of healthy skin, baseline (M0) and dupilumab month 3 (M3) lesional skin are depicted. Error bars show standard error of the mean.

## Supplementary tables

**TABLE E1. Patients and healthy subject basis characteristics**

Characteristic	Patients (n=57)	Controls (n=31)
Female sex, no. (%)	16 (28.1)	17 (54.8)
Age, mean $\pm$ SD	43.6 $\pm$ 15.3	32 $\pm$ 10.6
BMI, mean $\pm$ SD	26.1 $\pm$ 4.3	23.9 $\pm$ 3.8
EASI, mean $\pm$ SD	20.2 $\pm$ 11.7	-
IGA, mean $\pm$ SD	3.7 $\pm$ 0.8	-
POEM, mean $\pm$ SD	19.4 $\pm$ 6.9	-
Age of onset, mean $\pm$ SD (min, max)	8.1 $\pm$ 15.2 (0,77)	-
Duration of AD, mean $\pm$ SD (min, max)	35.2 $\pm$ 16.5 (1,78)	-

NA, Not available. SD, Standard deviation.

**TABLE E2. Baseline differentially expressed genes in AL vs. AN**

see separate excel file

**TABLE E3. Baseline differentially expressed genes in AL vs. NN**

see separate excel file

**TABLE E4. Baseline differentially expressed genes in AN vs. NN**

see separate excel file

**Table E5. Euclidean distance between all pairs of samples within AL, AN, and NN skin**

see separate excel file

**Table E6. Genes of the AD core transcriptomic signature**

see separate excel file

**Table E7. Genes of the AD dynamic transcriptomic signature**

see separate excel file

**Table E8. Complete results of the baseline differential expression analysis in AL vs. AN**

see separate excel file

**TABLE E9. Spearman correlation matrix of type 2 cytokines and AD-related genes**

see separate excel file

**TABLE E10. Spearman correlation of disease activity scores and baseline AL gene expression**

see separate excel file

**TABLE E11. Rank table of DEGs from the co-expression network analysis**

see separate excel file

**TABLE E12. Characteristics of patients with dupilumab treatment (n=22)**

Characteristic	Baseline	Month 3
EASI, mean $\pm$ SD	21 $\pm$ 11.5	4.9 $\pm$ 4.3
oSCORAD, mean $\pm$ SD	43.8 $\pm$ 13	20.3 $\pm$ 8.2
IGA, mean $\pm$ SD	3.6 $\pm$ 0.7	1.9 $\pm$ 0.7
% EASI50	-	86.4
% EASI75	-	63.6
% EASI $\leq$ 7	0	81.8

**TABLE E13. Characteristics of patients with cyclosporine treatment (n=8)**

Characteristic	Baseline	Month 3
EASI, mean $\pm$ SD	21.3 $\pm$ 9.6	9.5 $\pm$ 5.1
oSCORAD, mean $\pm$ SD	47 $\pm$ 7.3	34.1 $\pm$ 7.1
IGA, mean $\pm$ SD	3.9 $\pm$ 0.6	2.5 $\pm$ 0.5
% EASI50	-	62.5
% EASI75	-	25
% EASI $\leq$ 7	0	37.5

**TABLE E14. Mean expression values of all genes separated for time point and treatment**

see separate excel file

**TABLE E15. Gene expression normalisation in AL vs. NN skin after treatment with dupilumab and cyclosporine**

see separate excel file

**TABLE E16. Baseline differentially expressed genes in AL skin of dupilumab upper vs. lower quartile of EASI reduction**

see separate excel file

**TABLE E17. Baseline AL vs. AN DEGs in patients treated with dupilumab**

see separate excel file

**TABLE E18. M3 AL vs. AN DEGs in patients treated with dupilumab**

see separate excel file

**TABLE E19. Baseline AL vs. NN DEGs in patients treated with dupilumab**

see separate excel file

**TABLE E20. M3 AL vs. NN DEGs in patients treated with dupilumab**

see separate excel file

**TABLE E21. Baseline AL vs. AN DEGs in patients treated with cyclosporine**



see separate excel file

**TABLE E22. M3 AL vs. AN DEGs in patients treated with cyclosporine**

see separate excel file

**TABLE E23. Baseline AL vs. NN DEGs in patients treated with cyclosporine**

see separate excel file

**TABLE E24. M3 AL vs. NN DEGs in patients treated with cyclosporine**

see separate excel file

**TABLE E25. Baseline AL vs. AN DEGs in dupilumab high responders**

see separate excel file

**TABLE E26. M3 AL vs. AN DEGs in dupilumab high responders**

see separate excel file

**TABLE E27. Baseline AL vs. NN DEGs in dupilumab high responders**

see separate excel file

**TABLE E28. M3 AL vs. NN DEGs in dupilumab high responders**

see separate excel file

## Supplementary methods

### RNA sequencing

Isolation of mRNA was performed in batches of 10-12 samples after block randomisation based on the following variables in order to avoid batch effects: lesional skin, non-lesional skin, time point of sampling, individual patient. RNAlater fixed skin tissue samples were disrupted using innuSPEED Lysis Tubes W (1.4 – 1.6 mm steel beads & 3.5 mm ceramic beads) (Analytik Jena, Jena, Germany) in a SpeedMill Plus (3x 1min intervals) (Analytik Jena) in Lysis/Binding Buffer (Lifetechnologies). Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Lifetechnologies, Carlsbad, CA) following the manufacturers specifications with an additional on-column DNase treatment. Quality control on concentration and integrity of the isolated RNA was performed with the Qubit 2.0 Fluorometer (Qubit® RNA HS Assay) (LifeTechnologies, Carlsbad, CA) and the 2200 Tape Station (R6K ScreenTape Assay) (Agilent, Santa Clara, CA) following the manufacturer's instructions. RNA libraries were prepared using the Illumina Truseq® Stranded mRNA protocol including poly-A enrichment. Libraries were sequenced on the HiSeq4000 with 2x75bp, producing paired-end reads according to the manufacturer's protocol (Illumina, San Diego, CA). RNA samples were sequenced in pools of 12 samples per lane, with 8 lanes per flow cell and 1-2 flow cell per sequencing run on the HiSeq4000 machine.

### RNA-seq data processing

Low quality reads were filtered from the data using the Illumina CASAVA FASTQ filter. Data quality was assessed using Picard (<http://broadinstitute.github.io/picard>), FastQC (version 0.11.5) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC<sup>1</sup>. Illumina standard adapters were trimmed using Cutadapt (version 1.15)<sup>2</sup>. Paired reads were mapped to the human reference genome (GRCh38, Ensembl release 91) using Tophat2 (version 2.1.1)<sup>3</sup> and Bowtie 2 (version 2.3.2)<sup>4</sup>, only uniquely mapped read pairs were retained. Mapped reads were cleaned and sorted using Samtools (version 1.5)<sup>5</sup>. Number of reads for each gene was counted using HTSeq (version 0.10.0)<sup>6</sup> and annotated according to the Gencode version 27 annotation gtf file. The RNA processing was conducted according to the following parameter settings:

```
#!/bin/bash
```

```
cutadapt \
```

```
-m 35 -q 20 -O 3 \
```

```
-a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC \
```

```
-A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT \
```

```
-o AD1_R1.cutadapt.fastq -p AD1_R2.cutadapt.fastq \  
AD1_R1.filtered.fastq AD1_R2.filtered.fastq
```

tophat2 \

```
--transcriptome-index=tophat_transcriptome_index_Ensembl_release_91 \  
--library-type fr-firststrand \  
--b2-very-sensitive \  
Ensembl_release_91/Homo_sapiens.GRCh38.dna.primary_assembly \  
AD1_R1.cutadapt.fastq AD1_R2.cutadapt.fastq
```

```
samtools view -h -F 0x0008 accepted_hits.bam | samtools view -Shb -F 0x0004 - > AD1.cleaned.bam
```

```
samtools sort -n -o AD1.cleaned_sorted.bam AD1.cleaned.bam
```

htseq-count\_0.10.0 \

```
-f bam -s reverse -m union -a 20 \  
AD1.cleaned_sorted.bam \  
gencode.v27.annotation.gtf \  
> AD1.htseq \  

```

## Statistical analysis

Principle component analysis (PCA) was performed based on all measured genes excluding genes from the sex chromosomes to check for technical batch effects due to sequencing run (n=2), technician who isolated the RNA (n=3), and season in which the RNA was isolated (n=4). In order to identify samples that exceptionally vary from the other samples the following approach was used. PCA was performed for all subsets of samples (lesional (AL) and non-lesional (AN), each pre- and post-treatment, and healthy (NN)) based on all genes excluding genes from the sex chromosomes. Samples were defined as outliers if they deviated from the mean by more than three times the standard deviation for each principle component (PC) of those PCs that summed up to 60% of total variance (the first 5-9 PCs). Outliers were excluded from all analyses.

The pairwise Euclidean distances each among NN, AN, and AL samples were computed using the top three principal components. Reported p-values were computed by Wilcoxon test. Differential expression analysis between different conditions was conducted using DESeq2<sup>7</sup> Bioconductor package (version 1.24.0). Analysis was performed using the

parametric Wald test and independent filtering of the results. A false discovery rate (FDR) <5% was used to declare significance. If not stated otherwise, significant genes with an absolute log<sub>2</sub> fold change (LFC) ≥1 were defined as differentially expressed genes (DEGs). Log fold change estimates were corrected using DESeq2 inbuilt lfcshrinkage() function with the *apegglm*<sup>8</sup> method in order to improve on the previous effect size estimator. The following R code described the general DESeq2 steps performed (biopsy as exemplary conditional variable):

In case of unpaired tests:

```
> ddsHTSeq = DESeqDataSetFromHTSeqCount(..., design = ~ biopsy)
```

In case of paired tests:

```
> ddsHTSeq = DESeqDataSetFromHTSeqCount(..., design = ~ patient + biopsy)
```

```
> dds = DESeq(ddsHTSeq, betaPrior = FALSE)
```

```
> res = results(dds, independentFiltering = TRUE, alpha = 0.05)
```

```
> resLFC <- lfcShrink(dds, coef="biopsy_AL_vs_AN", type="apeglm", res = res)
```

For descriptive statistics and visualisation, R (version 3.6.1) with various R and Bioconductor packages and normalised (DESeq2::estimateSizeFactors()) or transformed (DESeq2::vst(), also includes normalisation) gene counts were used (see details below).

Differential gene expression was performed for lesional (AL) vs. non-lesional (AN), AL vs. healthy skin (NN), and AN vs. NN samples, whereby for AL vs. AN comparison, a paired test according to patient was performed (DESeq2 design formula: design = ~ patient + biopsy). The core signature was defined as genes differentially expressed in the same direction (FDR adjusted p-value <0.05) for AL vs. NN and AN vs. NN without being different (FDR adjusted p-value >0.05) between AL and AN in the same direction. The progressive signature was defined as genes being differentially expressed in the same direction for AL vs. NN, AN vs. NN, and AL vs. AN (FDR adjusted p-value >0.05). For gene enrichment analysis, we considered genes from the core and dynamic signature with an absolute LFC ≥1 in AL and NN. Gene enrichment analysis was performed using FunMappOne<sup>9</sup> software with default settings. Enriched KEGG pathways were displayed on the third level of BRITE functional hierarchy. The input for FunMappOne was provided as Excel file containing two lists of DEGs (the core and the progressive DEG set) with gene modifications (shrunken LFCs according to DESeq2 lfcshrinkage() see above). Gene set enrichment analysis was performed based on the LFCs between AL and AN samples of all genes using GAGE (version 2.34.0)<sup>10</sup> Bioconductor package with default settings and the set of KEGG signalling and metabolic pathways (kg.hsa\$sigmet.idx). Heatmaps of core and progressive genes were created

plotting row Z-scores of mean NN, AN, and AL expression values. Disease activity scores were correlated with gene counts using the following R function and parameters:

```
> cor.test(x, y, method = "spearman", alternative = "two.sided")
```

whereby x was provided as numeric vectors of the activity scores (oSCORAD, EASI, and POEM) and itch score (NRS) and y was provided as vectors of normalised counts of the genes with the highest variance (upper quartile). Correlation analysis was performed separately for pre-treatment AL and AN samples.

Co-expression network was computed using the INfORM<sup>11</sup> software which combines multiple network inferences from an ensemble of methods to derive a gene co-expression network. Further, INfORM determines gene modules and ranks genes by the combined influence of network centrality properties and differential expression scores. The input for INfORM was provided as a table of DEGs (FDR adjusted p-values <0.05 and abs(LFC) >=1) (we included IL13RA1 despite an abs(LFC) <1) between pre-treatment AL and AN samples, as well as a table of normalised counts of the respective samples. INfORM was run with default settings, but without the use of the MRNETb inference algorithm to reduce computation time.

In order to identify baseline signatures that are associated with the strength of response to dupilumab, we compared AL skin of patients achieving the highest reduction of EASI (upper quartile, n=6 patients) with patients showing the lowest reduction of EASI (lower quartile, n=6 patients). Genes with a p-value <0.05 and an absolute LFC >=0.5 were examined.

In order to determine the residual disease genomic profile of patients who achieved a high response to dupilumab, we compared dysregulated genes before and 3 months after treatment, whereby we discriminated the residual profile as compared to AN and NN, respectively. For the AL vs. AN comparison, we accepted all differentially expressed genes with an FDR adjusted p-value <0.05 as DEGs. Due to the plenty of significant DEGs between AL and NN, we additionally filtered genes with an absolute LFC >= 1.

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# Elevated NK-cell transcriptional signature and dysbalance of resting and activated NK cells in atopic dermatitis



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**Background:** Altered quantities, activity, and composition of natural killer (NK) cells in blood as well as expression changes of genes involved in NK-cell function in skin lesions of patients with atopic dermatitis (AD) were recently reported.

**Objectives:** We sought to comprehensively analyze cutaneous NK-cell transcriptomic signatures in AD, and to examine changes under treatment.

**Methods:** We analyzed NK-cell signatures in skin transcriptome data from 57 patients with moderate to severe AD and 31 healthy controls. In addition, changes after 12 weeks of systemic treatment (dupilumab  $n = 21$ , cyclosporine  $n = 8$ ) were analyzed. Deconvolution of leucocyte fractions was conducted. Immunofluorescence staining of NK cells was performed on paraffin-embedded skin sections.

**Results:** Immunofluorescence staining revealed a relatively high abundance of both NK cells and CD3<sup>+</sup>CD56<sup>+</sup> cells in lesional as

compared with nonlesional and healthy skin. Lesional and to a lesser extent nonlesional skin showed a strong upregulation of NK-cell markers together with a dysbalanced expression of inhibitory and activating receptors, which was not reverted under treatment. Digital cytometry showed a decrease in activated and an increase in resting NK cells in both lesional and nonlesional skin, which was reverted by both treatment with dupilumab and cyclosporine. The NK-cell transcriptomic signature remained upregulated after treatment, but there was a shift on the qualitative level, indicating a compositional change in NK-cell subsets toward CD56<sup>bright</sup> NK cells.

**Conclusions:** Lesional AD skin shows a NK-cell dysregulation, which despite clinical improvement under systemic therapy was only partially reverted, and which may represent a yet underappreciated disease mechanism. (*J Allergy Clin Immunol* 2021;147:1959-65.)

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**Key words:** Atopic dermatitis, natural killer cells, skin transcriptome, RNA-Sequencing, gene expression

## INTRODUCTION

Natural killer (NK) cells belong to the family of innate lymphoid cells, and constitute 5% to 15% of peripheral blood lymphocytes. Tissue-resident NK cells are known to populate various organs such as lymphoid tissues, lung, liver, uterus, and gut, whereas only a few studies demonstrated that they are homing healthy skin (HS) in small numbers.<sup>1-3</sup> NK cells can kill pathogen-infected or malignant cells by mediating cellular cytotoxicity through releasing apoptosis-inducing molecules such as granzymes and perforin stored in their lytic granules, which is the same mechanism cytotoxic T lymphocytes (CD8<sup>+</sup> T cells) exert to kill target cells.<sup>1,4</sup> In contrast to the activation of cytotoxic T lymphocytes via the T-cell receptor, the NK-cell activation status is controlled by a combination of signaling of activating and inhibitory receptors.<sup>5,6</sup>

So far, relatively little is known about the role of NK cells in atopic dermatitis (AD). A few studies reported quantitative and qualitative alterations in NK cells in peripheral blood, in particular reduced relative proportions of peripheral blood NK cells and a reduced cytotoxicity in AD.<sup>7-11</sup> Furthermore, NK cells were detected in dermal infiltrates of AD lesional skin (LS), whereas in nonlesional skin (NLS) and HS only few or no NK cells were detected.<sup>2</sup> More recently, a selective reduction of mature CD56<sup>dim</sup> CD57<sup>+</sup> NK cells in peripheral blood, which was largely reverted by treatment with the anti-IL-4Ra antibody dupilumab, as well as an enrichment of activated NK-cell transcriptomic signatures in LS was reported in a small cohort of adults with moderate to severe AD.<sup>7,12</sup> On the basis of these findings, it was postulated that in AD, NK cells are recruited from the periphery to the inflamed skin as an endogenous counterregulatory response to type 2 skin inflammation.

To gain deeper insights into the potential role of NK cells in the regulation of type 2 inflammation, we conducted sensitive immunofluorescence staining of NK cells and an in-depth characterization of NK-cell–related expression signatures including *in silico* cytometry in transcriptome data on lesional and nonlesional skin biopsies from patients of the TREATgermany AD registry<sup>13</sup> before and 3 months after systemic treatment as well as in healthy controls.<sup>14</sup> For detailed [Methods](#), please see the [Methods](#) section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## RESULTS AND DISCUSSION

### Lesional AD skin shows an enrichment with NK cells

It has been proposed that the elevated NK-cell transcriptional signature in LS of patients with AD is due to an increase in CD3<sup>-</sup>CD56<sup>+</sup> NK cells.<sup>7</sup> Using indirect immunofluorescence, we analyzed CD3<sup>+</sup>CD56<sup>-</sup> (T cells), CD3<sup>-</sup>CD56<sup>+</sup> (NK cells), and CD3<sup>+</sup>CD56<sup>+</sup> cells (including NK T cells) ([Fig 1](#); see [Fig E1](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) in lesional, nonlesional, and healthy skin. CD3<sup>-</sup>CD56<sup>+</sup> and CD3<sup>+</sup>CD56<sup>+</sup> cells were mainly located in dermal infiltrates near the dermoepidermal junction. We calculated a ratio of 2:3 for CD3<sup>+</sup>CD56<sup>+</sup> (40%) and CD3<sup>-</sup>CD56<sup>+</sup> cells (60%) in LS. CD3<sup>+</sup>CD56<sup>+</sup> cells were not detected in NLS, whereas in HS,

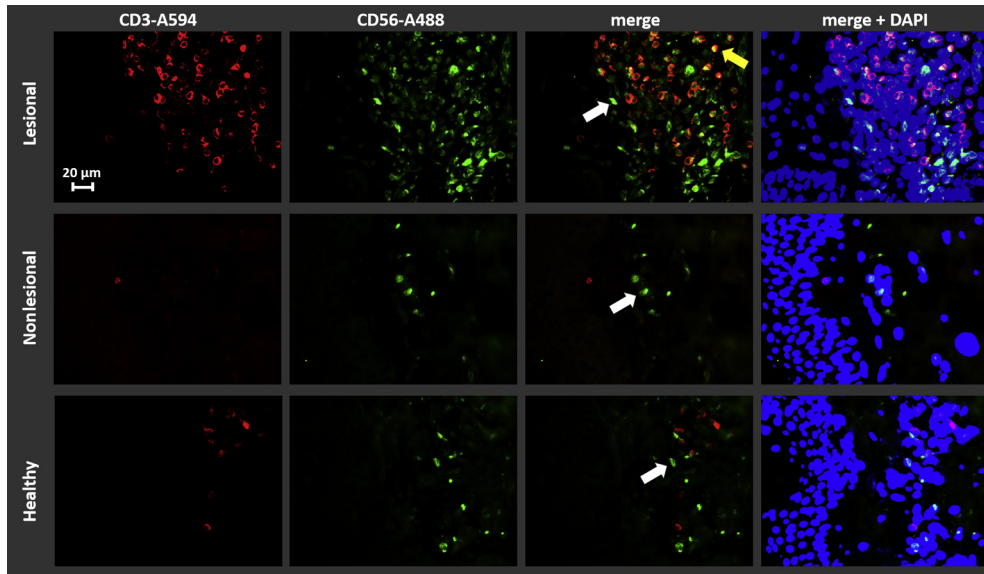
### Abbreviations used

AD:	Atopic dermatitis
EASI:	Eczema Area and Severity Index
HS:	Healthy skin
LS:	Lesional skin
NK:	Natural killer
NLS:	Nonlesional skin
oSCORAD:	Objective scoring of AD

20% of the analyzed CD56<sup>+</sup> cells were identified as CD3<sup>+</sup>CD56<sup>+</sup> cells. In LS, CD3<sup>-</sup>CD56<sup>+</sup> cells were 8- and 4-fold more abundant as compared with NLS and HS, respectively. Because CD56 is expressed with varying frequency on different NK-cell subtypes with different stage of maturity, we suspect that the actual number of NK cells deviates slightly from CD56<sup>+</sup> cells detected by immunostaining.<sup>1,15,16</sup>

### AD skin shows an elevated NK-cell transcriptomic signature with dysbalanced NK-cell inhibitory and activating receptor expression

To analyze NK-cell transcriptome signatures, we curated a set of 33 genes that are (1) classifying naive or infection-stimulated NK cells on the basis of single-cell RNA-Sequencing reference data<sup>17</sup> and (2) not detected in the skin cell lines HaCaT, SK-MEL-30, WM-115, and A-431.<sup>18</sup> Expression of all these 33 genes was detected in our data set ([Table I](#)). In a principle-component analysis, HS clustered apart from LS and NLS, whereas NLS and LS showed some overlap ([Fig 2, A](#)). The large majority of these genes was strongly upregulated (log2 fold change, 1.31-3.5) in LS versus HS (median log2 fold change, 2.57; median-adjusted *P* value,  $1.54 \times 10^{-33}$ ) at baseline, confirming our immunostaining results and previous reports of enriched NK-cell expression in AD LS ([Table I](#)).<sup>7,19</sup> Interestingly, the NK-cell inhibitory receptors NKG2A, KLRB1, and KLRG1 showed a strong upregulation in LS as compared with HS, whereas activating receptors such as NKG2D, NKG2C, and Nkp46 showed no difference ([Fig 2, B](#)).<sup>5,6</sup> Although on a global level, NK-cell gene expression was not associated with disease activity as measured by the Eczema Area and Severity Index, the expression of inhibitory NK-cell receptor genes such as NKG2A and KLRB1, and NK-cell effector molecules such as granzyme and perforin correlated positively with established type 2 markers such as IL-13, IL-4, CCL17, and CCL22 (Spearman  $\rho = 0.19-0.69$ ; median *P* value =  $9.6 \times 10^{-06}$ ) at baseline ([Fig 2, C](#)). Although single NK-cell genes such as granulysin and granzymes showed a slight downregulation under treatment, on the global level, treatment with neither dupilumab nor cyclosporine resulted in a marked normalization of the increased NK-cell–related gene expression (curated 33-gene set median log2 fold change LS vs HS, 2.13; median-adjusted *P* value,  $1.50 \times 10^{-20}$ ). In particular, the NK-cell inhibitory receptors KLRB1, KLRG1, and NKG2A remained upregulated. A previous study demonstrated increased protein levels of the key inhibitory receptor NKG2A in purified circulating NK cells that had been *in vitro* stimulated with IL-4 in individuals with AD as compared with healthy individuals, suggesting that the increased expression of NKG2A in AD skin arises from continued exposure to IL-4, the expression levels of which even increase under short-term dupilumab treatment.<sup>19,20</sup> Correlation of IL-4 and NKG2A was weak by our data; however, IL-4



**FIG 1.** CD56<sup>+</sup> NK cells in dermal infiltrates of AD. Immunofluorescent staining with anti-human CD3 (red) and anti-human CD56 (green) antibody, and DAPI (nuclear staining) of paired lesional and nonlesional skin biopsies from patients with AD and healthy controls in 40×. Yellow arrows show double-positive cells (CD3<sup>+</sup> CD56<sup>+</sup> cells such as NK T cells). White arrows show CD3<sup>-</sup> CD56<sup>+</sup> NK cells. Epidermis is oriented to the left of the picture, and in case of the lesional section, to the bottom left corner. A quantification of CD3<sup>+</sup> CD56<sup>+</sup> and CD3<sup>-</sup> CD56<sup>+</sup> cells was performed by counting double-positive and single CD56-positive cells. DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride.

**TABLE I.** Baseline differential expression of NK-cell genes

Gene	Log2 fold change LS vs HS	P value (BH-adjusted)
SRGN	3.5	1.69 × 10 <sup>-103</sup>
FCER1G	2.92	1.43 × 10 <sup>-93</sup>
PLEK	3.18	6.77 × 10 <sup>-92</sup>
ALOX5AP	2.72	1.93 × 10 <sup>-68</sup>
GIMAP7	1.98	7.00 × 10 <sup>-58</sup>
CD48	2.85	2.44 × 10 <sup>-52</sup>
HCST	2.72	7.52 × 10 <sup>-49</sup>
KLRB1	2.76	7.32 × 10 <sup>-46</sup>
TYROBP	2.06	2.22 × 10 <sup>-45</sup>
IL2RG	2.57	2.45 × 10 <sup>-43</sup>
MYO1F	1.71	3.24 × 10 <sup>-42</sup>
CST7	2.72	4.22 × 10 <sup>-38</sup>
GZMA	2.9	5.74 × 10 <sup>-38</sup>
HOPX	-1.68	4.83 × 10 <sup>-35</sup>
TRAF3IP3	2.33	3.35 × 10 <sup>-34</sup>
CD69	2.91	1.54 × 10 <sup>-33</sup>
CD247	2.29	3.65 × 10 <sup>-33</sup>
PYHIN1	3.19	3.95 × 10 <sup>-33</sup>
RGS1	3.29	5.53 × 10 <sup>-32</sup>
PRF1	2.02	1.16 × 10 <sup>-30</sup>
NKG7	2.41	1.28 × 10 <sup>-28</sup>
CCL4	3.05	2.13 × 10 <sup>-27</sup>
CORO1A	1.64	1.09 × 10 <sup>-26</sup>
CD7	2.21	2.25 × 10 <sup>-26</sup>
IRF7	1.31	4.88 × 10 <sup>-26</sup>
GNLY	2.54	5.31 × 10 <sup>-26</sup>
CTSW	1.98	5.65 × 10 <sup>-21</sup>
CCL3	2.99	1.21 × 10 <sup>-19</sup>
TRDC	1.69	4.95 × 10 <sup>-19</sup>
LSP1	-0.89	9.31 × 10 <sup>-14</sup>
KLRC1	2.24	7.61 × 10 <sup>-13</sup>
RORA	-0.65	3.91 × 10 <sup>-07</sup>
IL2RB	-0.75	1.82 × 10 <sup>-06</sup>

BH, Benjamini-Hochberg correction.

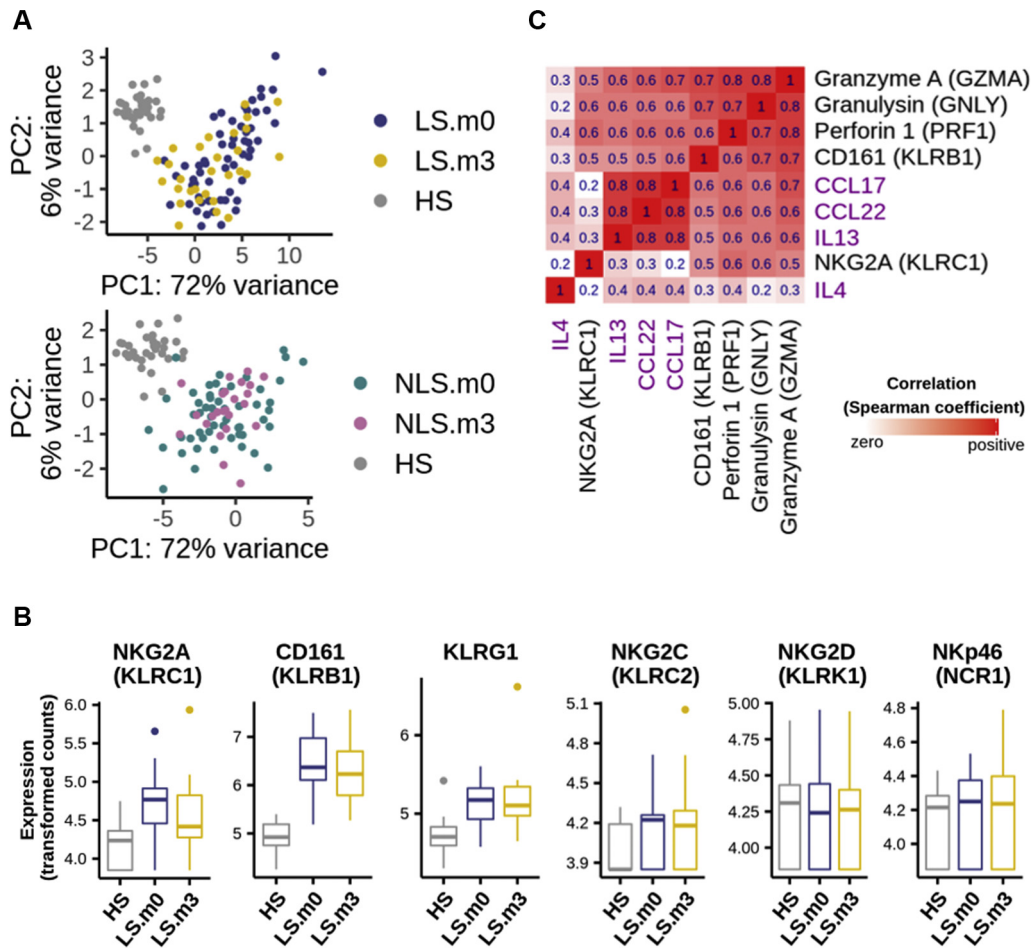
Ordered according to P value.

Log2 fold change: calculated by parametric Wald test.

detection by RNA-Sequencing in skin was poor, and thus measures might be unreliable.

### Digital cytometry shows a dysbalance of resting and activated NK cells in AD skin, which is reverted by systemic treatment

We performed an *in silico* quantification of resting and activated NK cells in the skin using CIBERSORTx. The total estimated fraction of cells consistent with an NK-cell transcriptomic signature among 22 estimated immune cell subtypes was larger in HS (5.3%) as compared with LS (3.4%) and NLS (2.8%) (Fig 3, A), indicating that NK cells represent a smaller fraction of the cutaneous immune cell population in AD as compared with HS, although absolute NK-cell numbers appear to be increased in AD skin as shown by our histological and transcriptomic analysis. The reference used for the *in silico* quantification comprises the expression signature of CD56<sup>+</sup> NK cells isolated from normal human donors' blood (resting NK cells) and CD56<sup>+</sup> CD69<sup>+</sup> NK cells treated with IL-2 or IL-15 after isolation (activated NK cells).<sup>21</sup> The estimated fraction of activated NK cells based on transcriptomic signatures was significantly lower in LS (0.8%) and NLS (1.1%) as compared with HS (3.9%) (LS < NLS < HS), whereas the estimated fraction of resting NK cells was significantly larger in LS as compared with NLS and HS (LS, 2.6%; NLS, 1.7%; HS, 1.5%) (Fig 3, A). Furthermore, there was a tendency toward a positive correlation between the fraction of resting NK cells and disease activity, whereas the fraction of activated NK cells correlated inversely with disease activity (Fig 3, B). These findings are contrary to recent findings by Mack et al,<sup>7</sup> who used the same methodology. However, we here analyzed a considerably larger number of AD samples (n = 57 vs n = 6), and also included healthy control skin for comparison.<sup>7</sup> Treatment with neither dupilumab nor cyclosporine altered the estimated proportion of NK cells on the basis of transcriptomic signatures among the other



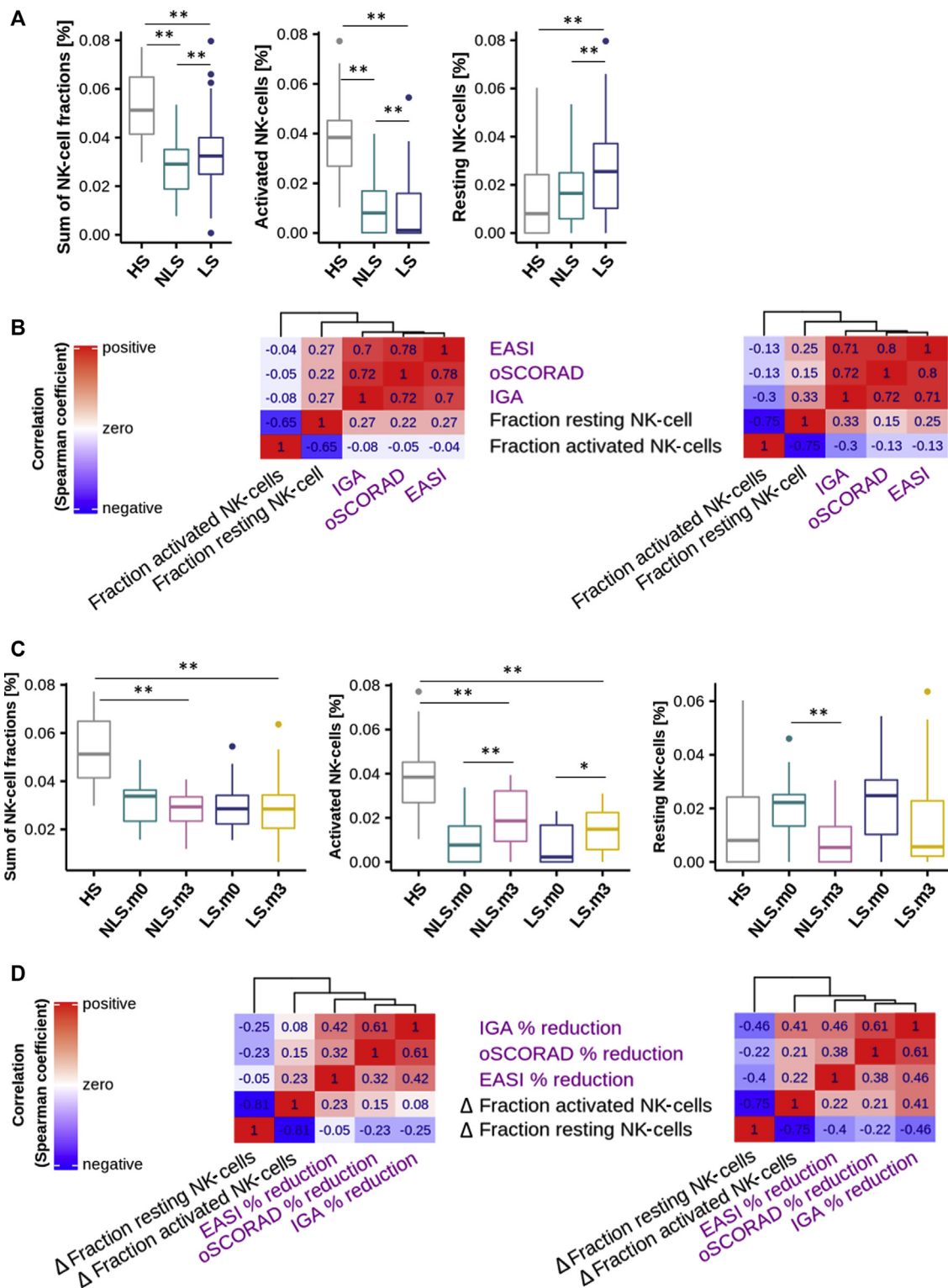
**FIG 2.** NK-cell gene expression in AD skin. **A**, Principle-component analysis based on expression of a curated set of NK-cell-related genes in baseline lesional (LS.m0) and nonlesional (NLS.m0) skin, and lesional and nonlesional skin 3 months after the start of systemic treatment (LS.3m and NLS.3m) (dupilumab  $n = 21$ , cyclosporine  $n = 8$ ), as well as HS. **B**, Expression of inhibitory (1-3) and activating (4-6) NK-cell receptors in HS, baseline LS (LS.m0), as well as LS 3 months after the start of treatment with dupilumab (LS.m3). Gene expression is stated in variance stabilizing transformed sequencing counts. **C**, Spearman correlation coefficients for selected type 2 inflammation markers and genes encoding NK-cell receptors and NK-cell effector molecules in baseline LS. *PC*, Principle-component.

immune cell subtypes (Fig 3, C). However, the fraction of activated NK cells increased considerably, whereas the fraction of resting NK cells decreased in both LS and NLS and reached proportions seen in HS. The magnitude of these changes correlated with the reduction in disease activity under treatment as measured by the Eczema Area and Severity Index (Fig 3, D). These findings indicate that successful treatment reverts the dysbalance between resting and activated NK-cell proportions rather than impacting the total fraction of NK cells in relation to other immune cells.

### Systemic treatment shifts the balance of cytotoxic and immunomodulatory NK-cell subsets in lesional AD skin

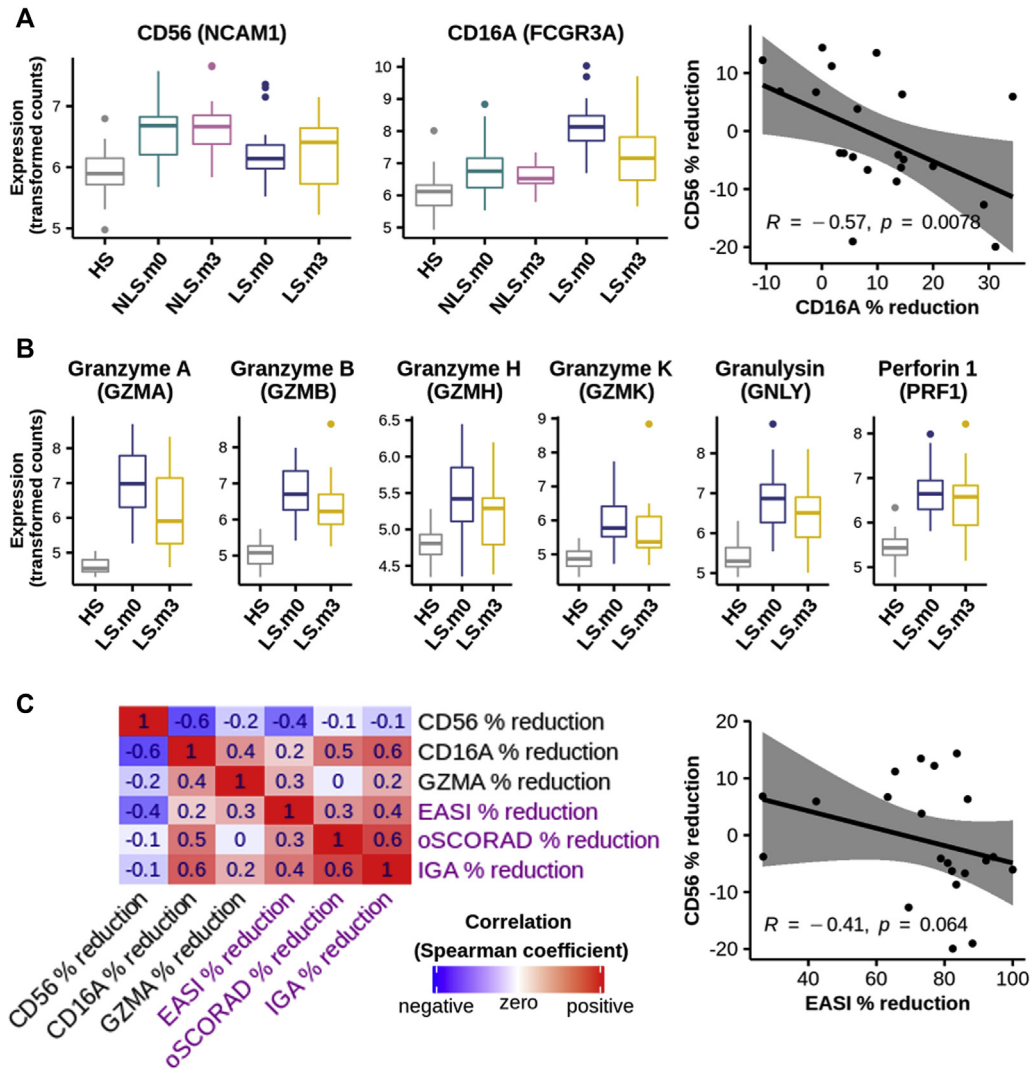
Most of what is known about NK cells is derived from studies on peripheral blood, where additional surface markers, such as CD16A, indicate different functional subtypes, such as CD56<sup>dim</sup> CD16<sup>+</sup> cells, representing 90% of peripheral blood NK cells and cytotoxic effector cells, and CD56<sup>bright</sup> CD16<sup>-</sup>, representing 10%

of peripheral NK cells and predominantly the tissue-resident NK cells that have rather immunomodulatory functions.<sup>1,15,16</sup> We found that both CD56 and CD16A expression was increased in LS and NLS as compared with HS (Fig 4, A). After treatment with both dupilumab and cyclosporine, CD56 had increased in LS, leading to a yet stronger deviation from HS, whereas CD16A had decreased in LS. In NLS, both CD56 and CD16A expression was largely unchanged. The inverse alteration of CD56 and CD16A could indicate a shift in CD56<sup>dim</sup> CD16<sup>+</sup> NK cells toward CD56<sup>bright</sup> CD16<sup>-</sup> NK cells, which might represent tissue-resident NK cells.<sup>1,15,16</sup> However, it has to be considered that apart from NK cells, CD56 and CD16A are expressed by other cells such as NK T cells (CD56) and monocytes (CD16A), and that thus these shifts cannot reliably be attributed to only NK-cell subpopulations.<sup>18,22</sup> Furthermore, additional markers known to be highly expressed by tissue-resident NK cells showed differential changes under treatment, and thus it cannot be ruled out that circulating CD56<sup>bright</sup> NK cells entered the skin.<sup>16</sup> However, CD56 and CD16A expression changes correlated inversely



**FIG 3.** *In silico* quantification of activated and resting NK cells. **A**, Left: Sum of estimated fraction of activated and resting NK cells in HS (n = 31) and baseline nonlesional (NLS.m0, n = 54) and lesional (LS.m0, n = 57) skin. Middle: Estimated fraction of activated NK cells among 22 immune-cell subtypes. Right: Estimated fraction of resting NK cells. **B**, Spearman correlation coefficients for the fraction of activated and resting NK cells in baseline LS (left) and NLS (right), and EASI, oSCORAD, and IGA at baseline. **C**, Left: Sum of estimated fraction of activated and resting NK cells in HS (n = 31), baseline nonlesional (NLS.m0, n = 17) and lesional (LS.m0, n = 21) skin, and nonlesional and lesional skin 3 months after the start of treatment with dupilumab (NLS.3m, n = 17 and LS.3m, n = 21). Middle: Estimated fraction of activated NK cells. Right: Estimated fraction of resting NK cells. **D**, Spearman correlation coefficients for the difference ( $\Delta$ : fraction posttreatment minus fraction baseline) of the fraction of activated and resting NK cells in LS (left) and NLS (right), and the reduction in EASI, oSCORAD, and IGA under treatment with dupilumab. EASI, Eczema Area and Severity Index; IGA, Investigator Global Assessment; oSCORAD, objective scoring of AD. \* $P < .1$  and \*\* $P < .05$  by Wilcoxon test.





**FIG 4.** NK-cell transcriptomic signatures under treatment with dupilumab. **A**, Expression of CD56 and CD16A in HS, baseline nonlesional (NLS.m0) and lesional (LS.m0) skin, and NLS and LS 3 months after the start of treatment with dupilumab (NLS.3m and LS.3m). Right: Reduction in CD16A expression in relation to reduction in CD56 expression in LS under treatment with dupilumab. R: Spearman correlation coefficient. **B**, Expression of cytotoxic effector molecules (granzymes, granulysin, and perforin 1) in HS, baseline LS (LS.m0), as well as LS 3 months after the start of treatment with dupilumab (LS.m3). Gene expression is stated in variance stabilizing transformed sequencing counts. **C**, Spearman correlation coefficients for the reduction in CD56, CD16A, and granzyme A (GZMA) expression in LS and the reduction in EASI, oSCORAD, and IGA under treatment with dupilumab. Right: Reduction in CD56 expression in relation to reduction of EASI in LS under treatment with dupilumab. EASI, Eczema Area and Severity Index; IGA, Investigator Global Assessment; oSCORAD, objective scoring of AD; R, Spearman correlation coefficient.

(Spearman  $\rho = -0.57$ ), indicating that the changes originate from the same cell type (Fig 4, A). Furthermore, the expression of cytotoxic granzymes, particularly of granzyme A, which was shown to be highly expressed by NK cells, showed a decrease under treatment (Fig 4, B).<sup>17,18</sup> Interestingly, the magnitude of CD56, CD16A, and granzyme A shifts was related to the reduction in disease activity under treatment as measured by the Eczema Area and Severity Index (Fig 4, C). Together, these findings may indicate a shift from cytotoxic CD56<sup>dim</sup> CD16<sup>+</sup> NK cells toward immunomodulatory CD56<sup>bright</sup> CD16<sup>-</sup> NK cells under therapy. Based on their chemokine expression repertoire, CD56<sup>dim</sup> NK cells preferentially migrate to acute inflammatory sites, whereas

CD56<sup>bright</sup> NK cells might home to sites of chronic inflammation.<sup>22,23</sup> Thus, an expansion of CD56<sup>bright</sup> NK cells under treatment as suggested by our data might be a consequence of the remission of the inflammation.

Our data indicate (1) an accumulation of NK cells in AD skin, being in line with our immunostaining results, which is quantitatively persistent under systemic treatment as the global NK-cell-related expression signature remains upregulated, (2) a dysbalance of resting and activated NK cells by digital cytometry that appears to revert under systemic therapy with, however, persistently upregulated NK-cell inhibitory receptors, and (3) an increase in the NK-cell lineage marker CD56 together with a

decrease in CD16A and cytotoxic granzymes associated with clinical improvement under therapy, indicating a shift from cytotoxic CD56<sup>dim</sup> CD16<sup>+</sup> NK cells toward immunomodulatory CD56<sup>bright</sup> CD16<sup>-</sup> NK cells. Although these observations strongly support an involvement of NK cells in the inflammatory cascade in AD, they are not readily compatible with the hypothesis of NK-cell activation and cytotoxic activity as a counterregulatory mechanism to type 2 inflammation in the skin.<sup>7</sup> Clearly, more insight into the context-dependent function of cytotoxic cells is needed to better understand the role of NK cells in AD and to work out whether they exert disease-promoting or disease-regulatory effects. Such insight, however, cannot be obtained by the investigation of transcriptomic signatures in a mixed tissue and at 1 or 2 time points only. Analysis on both blood and skin and on the single-cell level will be needed.

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### Key messages

- AD lesions show an accumulation of NK cells and increased NK-cell-related gene expression signature as well as a dysbalance of resting and activated NK cells.
- Systemic treatment with either cyclosporine or dupilumab appears to normalize proportions of resting and activated NK cells and cause a shift from cytotoxic to immunomodulatory NK cells; however, there remains an elevated NK-cell transcriptional signature despite clinical improvement.

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## METHODS

### Patient characteristics and skin samples

To perform an in-depth characterization of NK-cell-related transcriptome signatures, we used skin transcriptome data from patients with AD, which were described in detail in Möbus et al.<sup>E1</sup> Briefly, adult patients with moderate to severe AD from the TREATgermany registry<sup>E2</sup> recruited between July 2017 and February 2019 were included in this study. Intrapersonal lesional and non-lesional skin punch biopsies (4 mm) were collected at baseline (pretreatment, m0) from 57 patients and 3 months (m3) after the initiation of anti-IL-4R $\alpha$  dupilumab (n = 21 patients) and cyclosporine (n = 8 patients). Nonlesional samples were taken at least 5 cm from the active lesion. Samples were stored in RNA later according to the manufacturer's manual until further processing. In addition, RNA from biopsies of 31 healthy volunteers with no history of atopic or chronic inflammatory disease included in a previous skin transcriptome study<sup>E3</sup> was available for this study.

### RNA sequencing

Briefly, total RNA was isolated from skin using the mirVanaTM miRNA Isolation Kit (Lifetechnologies, Carlsbad, Calif), and RNA libraries were prepared using the Illumina Truseq Stranded mRNA protocol including poly-A enrichment. Libraries were sequenced on the HiSeq4000 with 2x75bp. Raw mRNA sequencing data were processed using Cutadapt,<sup>E4</sup> Tophat2,<sup>E5</sup> together with Bowtie 2,<sup>E6</sup> Samtools,<sup>E7</sup> and HTSeq.<sup>E8</sup> The detailed workflow was described recently.<sup>E1</sup>

### Immunofluorescence staining of skin biopsy specimens

Immunofluorescence staining was performed on sections of paraformaldehyde-fixed, paraffin-embedded skin biopsies of lesional and non-lesional AD skin (n = 3 patients), as well as healthy control skin (n = 3 individuals). To detect NK cells, a polyclonal anti-human CD56 antibody (rabbit) was used. Double fluorescence staining was carried out with a monoclonal anti-human CD3 antibody (rat) in addition to CD56. Antibody binding was detected with  $\alpha$ -rabbit Alexa Fluor 488-conjugated and  $\alpha$ -rat Alexa Fluor 594-conjugated secondary antibodies. Nuclear staining was performed with 4'-6-diamidino-2-phenylindole, dihydrochloride (blue). For a quantification of CD3<sup>+</sup>CD56<sup>+</sup> and CD3<sup>-</sup>CD56<sup>+</sup> cells, double-positive and single CD56-positive cells were counted (Fig 1). We calculated the ratio of CD3<sup>+</sup>CD56<sup>+</sup> and CD3<sup>-</sup>CD56<sup>+</sup> cells by dividing the number of double-positive cells by the number of single CD56-positive cells. For a negative control, tissue section was incubated with the secondary antibodies alone (Fig E1).

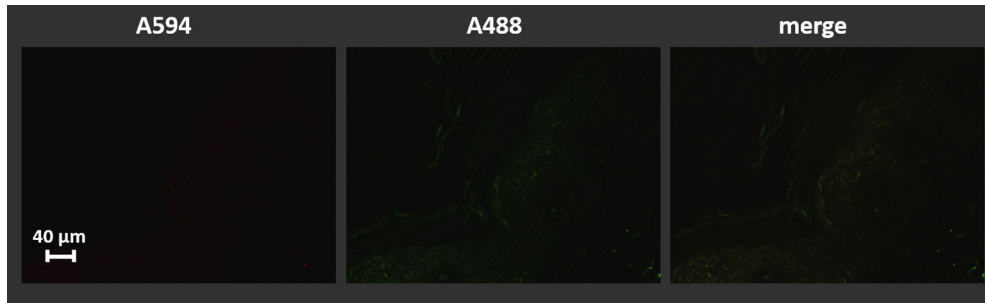
### Statistical analysis

A set of genes related to NK cells was curated on the basis of fulfilling both of the following criteria: (1) Cluster-specific genes for naive or exposed NK cells based on a recent single-cell RNA-Sequencing study.<sup>E9</sup> (2) Gene expression not detected in the skin cell lines HaCaT (keratinocyte cell line), SK-MEL-30 (metastatic malignant melanoma cell line), WM-115 (malignant melanoma cell line), and A-431 (epidermoid carcinoma cell line) according

to the human protein atlas database.<sup>E10</sup> Differential expression analysis was conducted using DESeq2 Bioconductor package (version 1.24.0).<sup>E11</sup> Analysis was performed using the parametric Wald test and independent filtering of the results. Log2 fold change estimates were corrected using DESeq2 inbuilt log2 fold change shrinkage function with the *apeglm* method.<sup>E12</sup> CIBERSORTx is a machine learning method that infers cell-type-specific gene expression profiles from bulk tissues without the need of physical cell isolation ("digital cytometry").<sup>E13</sup> Here, we applied it to impute leucocyte cell fractions from skin RNA-Sequencing data using the gene signature file LM22, which is implemented in the software and defines 22 immune cell subtypes.<sup>E14</sup> The LM22 matrix contains 547 genes that distinguish 22 human hematopoietic cell phenotypes including resting and activated NK cells. We used size factor normalized counts by DESeq2 software as input.<sup>E11</sup> Applied CIBERSORTx settings included batch correction in B-mode and disabled quantile normalization according to the recommendations for RNA-Sequencing data. For descriptive statistics and visualization of results, we used variance stabilizing transformed counts by DESeq2 software and R (version 3.6.1).<sup>E11</sup>

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**FIG E1.** Negative control of immunofluorescence staining. The skin section (HS) was incubated with the secondary antibodies alone to check unspecific binding. Microscopy images were taken in 20 $\times$ . A594:  $\alpha$ -rat Alexa Fluor 594-conjugated secondary antibody; A488:  $\alpha$ -rabbit Alexa Fluor 488-conjugated secondary antibody. Epidermis is oriented to the top left corner.



### **3 Unpublished manuscript**

## **Blood endotypes of atopic dermatitis with potential therapeutic implications (in preparation for submission)**

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## **Background**

Blood transcriptome studies in atopic dermatitis (AD) are rare, although evidence exists for systemic inflammation beyond the eczematous lesions of the skin. Studies based on real-world data of adult patients who undergo systemic treatment as well as studies based on RNA sequencing are missing on the blood level so far.

## **Objectives**

To explore the whole-blood transcriptome and the impact of systemic treatment in patients of the TREATgermany registry.

## **Methods**

PAXgene blood samples of 60 moderate-to-severe AD patients including 49 patients with samples before and 12 weeks after the start of systemic treatment with anti-IL4RA dupilumab, as well as samples from an independent case-control cohort including 31 patients with AD and 43 healthy controls were subjected to mRNA sequencing. Patient clustering, differential expression, correlation and co-expression network analysis were conducted.

## **Results**

Patients clustered in an eosinophil-low and eosinophil-high endotype. The eosinophil-high endotype showed a marked upregulation of eosinophil transcriptomic signatures including elevated levels of IL5RA, CCL23, IL34, and HRH4 as compared to both patients of the eosinophil-low endotype as well as healthy controls. On the phenotype level, both endotypes showed no marked differences, neither in AD activity nor in the prevalence of atopic comorbidities. The eosinophil-high endotype was characterised by a marked transcriptomic dysregulation as compared to healthy controls, and the disease activity as well as the intensity of itch positively correlated with eosinophil signatures centred to IL5 signalling. In contrast, the eosinophil-low endotype showed a weak degree of transcriptomic dysregulation and the disease activity was not associated with blood gene expression. Clinical improvement under dupilumab was accompanied by a decrease of innate immune response signatures related to neutrophils and monocytes, and an increase of signatures related to lymphocytes involving signatures of B cell activation as well as NK cell related signatures indicating an increase of NK cell abundance and /or functional shift of NK cells under treatment. However, persistent downregulation of IL18RAP, interferon gamma, and granzyme A in the eosinophil-high endotype suggested a residual disturbance of NK cell function despite clinical improvement.

## **Conclusion**

Our study indicates that AD similar to asthma can be distinguished in eosinophilic and non-eosinophilic endotypes. While the endotypes are not dictated by atopic comorbidities and do

not reflect disease severity groups, links between eosinophil expression profiles and disease activity might offer novel and patient-stratified treatment approaches.

## **Abbreviations**

AD: Atopic dermatitis

DEG: Differentially expressed gene

EASI: Eczema Area and Severity Index

FDR: False Discovery Rate

FPKM: Fragments per kilobase per million mapped fragments

IGA: Investigator Global Assessment

LFC: Log<sub>2</sub> fold change

NK cells: Natural killer cells

NRS: Numeric rating score of itch

oSCORAD: objective Scoring of AD

$\rho$ : Spearman's rho correlation coefficient

## Background

Multiple transcriptome studies have identified genes dysregulated in the skin of patients with atopic dermatitis (AD) resulting in the description of robust AD-associated signatures.<sup>1-5</sup> Those signatures reflect epidermal barrier dysfunction, activated itch signalling, and exacerbated type 2 immune responses driven by cytokines such as IL13, IL22 and IL31, proinflammatory mediators such as S100 genes and defensins, and skin alarmins such as TSLP, IL33, and IL25.<sup>3-5</sup> Several studies also highlighted a contribution of type 17 and type 1 responses, as well as of natural killer (NK) cell signalling.<sup>5-9</sup> While involved skin tissue appears to be the most informative source to investigate disease mechanisms, no strong gene signatures and functions designating robust endotypes and leveraging the disease heterogeneity have been robustly identified so far. Further, the limitations associated with tissue biopsies, in particular their invasiveness and sampling bias, would hamper their utility as a potential method of measuring biomarkers both in the clinical setting and within drug trials. In contrast, blood presents the advantage of being easily accessible and amenable to repetitive sampling. Given that abnormalities of the cellular composition like the recently reported reduction of total peripheral blood NK cells along with a loss of mature CD56<sup>dim</sup> NK cells and differential serum protein and metabolome profiles were reported for AD, it is surprising that no study has profiled genome-wide transcript abundance and changes associated with treatment in blood samples from adult patients so far.<sup>8,10-14</sup> One study has investigated global blood transcriptomic signatures in children with AD, albeit this was based on microarray data, which are less sensitive and more biased as compared to RNA sequencing (RNA-Seq) data.<sup>15</sup> Children under the age of 6 years who had a recent AD onset showed elevated blood RNA levels of genes related to eosinophils and T<sub>H</sub>2 cells such as IL5RA, IL1RL1, HRH4, CCR3, SIGLEC8, PRSS33, and CLC, whereas T<sub>H</sub>1 markers IFNG and TNF were decreased in those patients as compared to healthy controls.<sup>15</sup>

The real-world TREATgermany AD registry is recruiting patients with moderate-to-severe AD from dermatological routine care and collects rich clinical and lifestyle data as well as multiple types of biosamples such as blood and skin samples on a longitudinal scale.<sup>16</sup> For the current study, we longitudinally profiled whole-blood samples of 60 patients using RNA-Seq before and 3 months after initiation of treatment with the anti-IL4RA antibody dupilumab. Further, whole-blood samples from an independent case-control cohort including 31 patients with AD as well as 43 healthy controls without atopic disease history were analysed.

## Methods

### Patient characteristics and blood samples

Adult patients with moderate-to-severe AD from the TREATgermany registry recruited between August 2017 and March 2019 at five study centres, who agreed to participate in the additional and optional bioanalytics module under a protocol approved by the Medical Faculty of the Christian-Albrechts-University, Kiel, Germany (B 261/16) and the responsible local ethics committees at the other participating sites, were included in this study.<sup>16</sup> PAXgene blood samples were collected from 60 patients prior and 3 months after the initiation of a systemic therapy. In addition, PAXgene blood samples of 31 patients with AD as well as 43 age- and sex-matched healthy controls included in a previous skin transcriptome study under an Institutional Review Board-approved protocol (A110/12) were available for this study.<sup>3</sup> In both cohorts, disease activity was assessed using established instruments such as the Eczema Area and Severity Index (EASI), the Investigator Global Assessment (IGA), the objective Scoring of AD (oSCORAD), and the itch numeric rating score (NRS).

### RNA sequencing

Blood samples were stored at -80°C immediately after shipment until RNA isolation. Total RNA was isolated from PAXgene blood RNA tubes (PreAnalytiX GmbH, Hombrechtikon Switzerland) using the PAXgene Blood miRNA Kit (PreAnalytiX GmbH, Hombrechtikon Switzerland) following the manufacturer's specifications automated on the QIAcube (QIAGEN, Hilden, Germany). Quality control on concentration and integrity of the isolated RNA was performed with a Fluorometer (Quant-iT™, ThermoFisher, Waltham, MA) and a TapeStation 4200 (RNA Screen Tape, Agilent, Santa Clara, CA) following the manufacturer's instructions. RNA libraries were prepared for sequencing using the Illumina TruSeq Stranded mRNA protocol including poly-A enrichment. Post library quality control included evaluation of library concentrations and fragment sizes (100-400bp) on a LabChip GX (PerkinElmer Inc., Waltham, MA). All libraries were index-barcoded enabling multiplexed sequencing. The TREATgermany samples and the samples from the case-control cohort were randomised and sequenced together in a total of two sequencing runs. Samples were sequenced in pools of 37-40 samples per lane of an Illumina S4 flow cell on a NovaSeq 6000 machine with 2x100bp, producing on average 55 million paired-end reads per sample according to the manufacturer's protocol (Illumina, San Diego, CA).

### RNA sequencing data processing

The quality of the sequencing data was assessed using FastQC (version 0.11.5) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Illumina standard adapters were trimmed using Cutadapt (version 1.15) (parameters: --minimum-length=35 --quality-

cutoff=20 --overlap=3 --adapter=AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT).<sup>17</sup> Paired reads were mapped to the human reference genome (GRCh38, Ensembl release 99) using Tophat2 (version 2.1.1) and Bowtie 2 (version 2.3.2) (parameters: --transcriptome-index yes --library-type fr-firststrand --b2-very-sensitive).<sup>18,19</sup> Unmapped reads have been removed (parameters: view -h -F 4), remaining mapped reads have been sorted (parameters: sort -n) using Samtools (version 1.5).<sup>20</sup> Number of reads for each gene was counted using HTSeq (version 0.10.0) (parameters: --order name --stranded reverse --mode union --minaaqual 20) and annotated according to the Ensembl release 99 GTF file ([ftp://ftp.ensembl.org/pub/release-99/gtf/homo\\_sapiens/Homo\\_sapiens.GRCh38.99.gtf.gz](ftp://ftp.ensembl.org/pub/release-99/gtf/homo_sapiens/Homo_sapiens.GRCh38.99.gtf.gz)).<sup>21</sup>

### **Statistical analysis**

In order to identify outliers, i.e. samples that vary exceptionally from the other samples, we used the following approach. After excluding low-count genes, principle component analysis (PCA) was performed for each condition (pre- (m0) and post-treatment (m3), AD-case-control, and healthy). Principle components (PCs) covering significant variance as determined by the Tracy-Widom test as implement in the LEA Bioconductor package (version 3.2.0) were considered for outlier detection.<sup>22</sup> Samples were defined as outliers if they deviated from the mean by more than three times the standard deviation for at least one significant PC. Outliers were excluded from all analyses. In order to identify potential batch effects due to technical variation, PCA and the QCnormSE R package (version 0.99.2, <https://rdrr.io/github/szymczak-lab/QCnormSE/>) were used. We detected batch effects between TREATgermany and the case-control cohort as well as between different shipping times within TREATgermany, and applied the ComBat\_seq function (Bioconductor sva package, version 3.38.0) to remove both batch effects from the data.<sup>23,24</sup> We performed all downstream analysis using the count data with minimal but necessary correction for according batch effects.

For this study, we aimed at identifying and characterising potential AD endotypes, their core gene signatures, and transcriptomic changes associated with systemic treatment with dupilumab. For the identification of patient subgroups, we first compiled a set of genes with the highest variances in expression in baseline (m0) AD samples denoted by us as AD-specific hypervariable genes. We selected genes that (1) showed a variance in expression  $\geq 1$  in the AD m0 samples, and (2) a variance at least 2 times higher in the AD m0 samples as compared to the healthy control samples. Euclidean distance matrix was computed based on those AD-specific hypervariable genes and agglomerative hierarchical clustering was performed using the ward.D2 linkage criteria. The optimal number of clusters was determined using the elbow-method and the silhouette score as implemented in the factoextra R package (version 1.0.7, <https://github.com/kassambara/factoextra>). Differential expression testing was conducted



using the DESeq2 Bioconductor package (version 1.30.0).<sup>25</sup> Analysis was performed using the parametric Wald test and independent filtering of the results. Differentially expressed genes (DEGs) were defined by a false discovery rate (FDR as defined by Benjamini-Hochberg) < 5% and an absolute log<sub>2</sub> fold change (LFC) > 0.15 corresponding to a 10% alteration. Log fold change estimates were corrected using the DESeq2 inbuilt LFC shrinkage function with the *apeglm* method.<sup>26</sup> Differential expression analysis of patients vs. healthy controls was conducted across both cohorts together after batch correction. Enrichment tests of DEGs were conducted using the over representation analysis of Gene Ontology (GO) biological processes with default parameters as implemented in the Clusterprofiler Bioconductor package (version 3.18.0).<sup>27</sup> Co-expression network analysis was performed using the INfORM software with fragments per kilobase per million mapped fragments (FPKM) of the DEGs as input.<sup>28</sup> We used default INfORM settings except that we omitted the mrnetb algorithm to speed up the computation of the network. For the calculation of FPKM values, the sizefactor normalised counts were additionally normalised for gene length using the *fpm* function implemented in DESeq2.<sup>25</sup> For the network analysis of the eosinophil-high endotype vs. healthy controls, we included DEGs with an absolute LFC > 0.5 to speed up computation and for better visualisation. For gene-gene or gene-variable correlation analysis, we performed Spearman rank-based correlation using normalised and transformed sequencing counts. We used the Human Protein Atlas in order to explore cell type specificity of gene expression.<sup>29</sup> For descriptive statistics and visualisation, normalised (DESeq2 size factor normalisation) and transformed (log<sub>2</sub> transformation) gene counts were used.<sup>25</sup> Differences in clinical variables were tested using the  $\chi^2$  test for categorial and the Wilcoxon signed-rank test for numeric variables and ordinal categorial variables. All analyses have been performed using R (version 4.0.3) and Bioconductor (version 3.12).

## Results

After quality control, whole-blood transcriptome data were available from 56 AD patients at baseline from the TREATgermany registry. Forty-six of those patients had received treatment with dupilumab and month 3 transcriptome data were available for 42 of those patients. From the AD case-control cohort, whole-blood transcriptome data was available from 31 patients and 40 healthy controls. The characteristics of the study participants are shown in table 1.

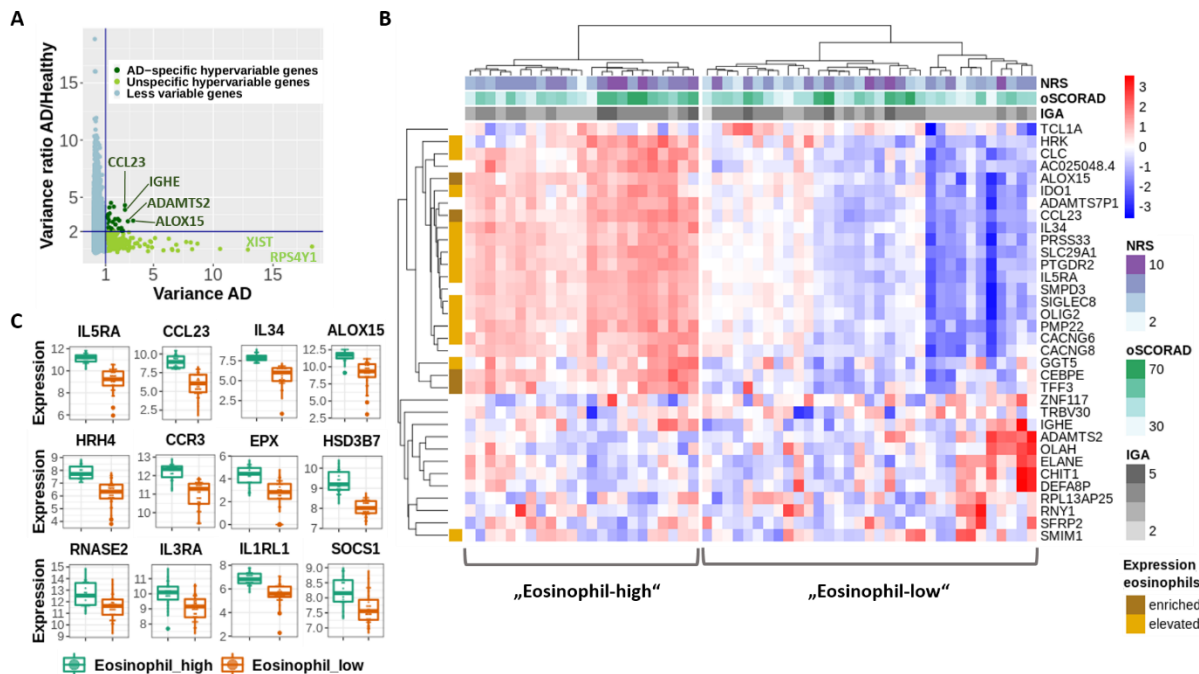
**TABLE 1. Patients and healthy subject basis characteristics**

Characteristic	Patients (n=56) TREATgermany	Patients (n=31) Case-control cohort	Controls (n=40) Case-control cohort
<b>Female sex (%)</b>	16 (28.6)	15 (48.4)	21 (52.5)
<b>Age, mean <math>\pm</math> SD</b>	44.4 $\pm$ 14.2	31.6 $\pm$ 10.3	32.4 $\pm$ 11.4
<b>BMI, mean <math>\pm</math> SD</b>	26.8 $\pm$ 4.6	26.5 $\pm$ 6.8	24.0 $\pm$ 4.2
<b>oSCORAD</b>	44.1 $\pm$ 13.4	30.1 $\pm$ 10.8	-
<b>EASI, mean <math>\pm</math> SD</b>	20.7 $\pm$ 11.0	-	-
<b>IGA, mean <math>\pm</math> SD</b>	3.7 $\pm$ 0.7	2.5 $\pm$ 0.9	-
<b>Pruritus 10-point scale, mean <math>\pm</math> SD</b>	6.3 $\pm$ 2.3	-	-
<b>Age of onset, mean <math>\pm</math> SD (min, max)</b>	9.4 $\pm$ 17.7 (0,77)	4.5 $\pm$ 5.7 (0,22)	-
<b>Diagnosis asthma (%)</b>	36 (64.3)	18 (58.1)	0 (0)
<b>Diagnosis rhinitis (%)</b>	41 (73.2)	17 (54.8)	0 (0)

### **AD patients cluster in an eosinophil-high and eosinophil-low blood endotype with itch and disease activity being associated with signatures in the eosinophil-high cluster**

In order to identify potential disease endotypes, we first determined AD-specific hypervariable genes. We defined AD-specific hypervariable genes as follows: 1) genes showing a variance  $\geq 1$  at baseline (this corresponded to the top 1.4% variable genes), and 2) genes showing a variance at least 2 times higher in AD as compared to healthy controls. We identified 34 AD-specific hypervariable genes with ALOX15, ADAMTS2, CCL23, and IGHE showing the highest variance (Fig 1, A). Based on the expression of these 34 genes we performed a hierarchical clustering of the 56 m0 TREATgermany patient samples. We determined the optimal number of clusters using the elbow method and the silhouette score, which both identified a number of 2 clusters as optimum (Supplementary Fig S1). The two clusters showed striking differences in the expression levels in particular of genes highly specific for eosinophils such as IL5RA, CCL23, IL34, SIGLEC8, SLC29A1, PRSS33, and PTGDR2 (Fig 1, B). We therefore termed the two identified clusters “eosinophil-high” and “eosinophil-low” endotype. Transcriptome-wide differential expression analysis between the two endotypes showed a total of 548 genes with significant differential expression and confirmed the upregulated expression of IL5RA, CCL23, IL34, and ALOX15 in the eosinophil-high endotype as compared to the eosinophil-low

endotype (Fig 1, C). In addition, further genes with specific expression in eosinophils and/or basophils such as HRH4, CCR3, EPX, HSD3B7, RNASE2, IL3RA, IL1RL1, and SOCS1 were upregulated in the eosinophil-high endotype (Fig 1, C, supplementary Table S1).

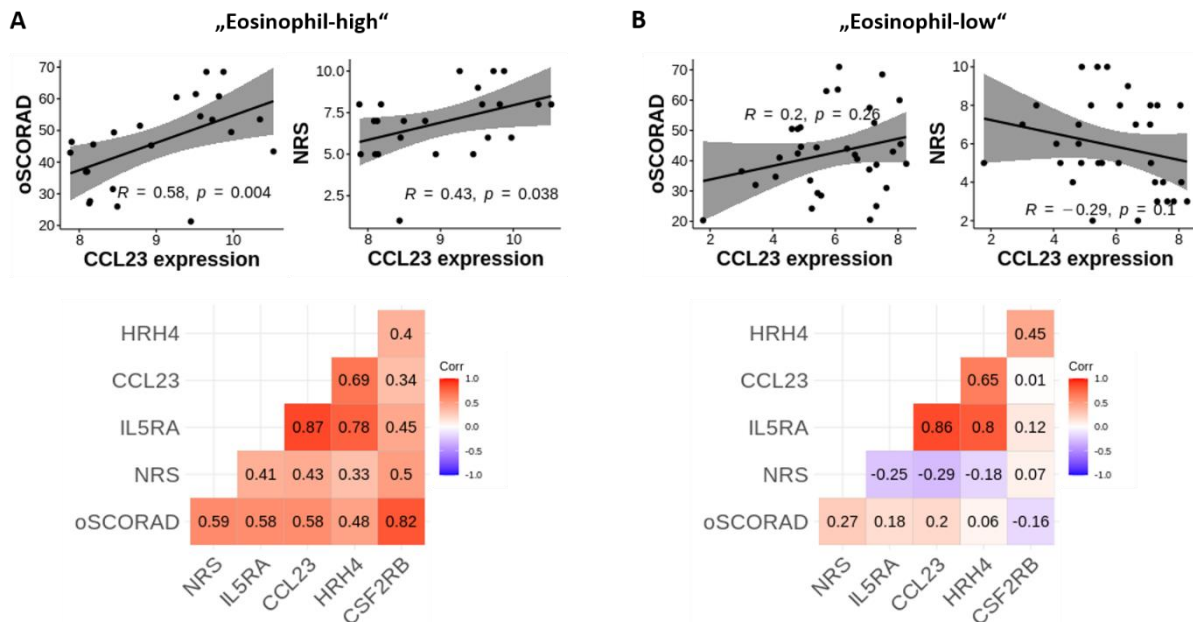


**Figure 1: Clustering of TREATgermany m0 AD samples.** **A**, Selection of 34 AD-specific hypervariable genes was performed based on two criteria: 1) a variance >1 in the m0 AD samples; 2) a variance at least 2 times higher in the m0 AD samples as in the healthy control samples (ratio: variance AD / variance healthy controls >2). **B**, Hierarchical clustering of 56 m0 AD samples based on the expression of 34 AD-specific hypervariable genes grouped the patients in an eosinophil-high and an eosinophil-low endotype. The vertical yellowish colour bar shows the cell-type specificity for eosinophils according to the Human Protein Atlas. Horizontal colour bars show the NRS, oSCORAD, and IGA of the patients. The heatmap colour code represents row z scores. **C**, Differential expression of genes related to eosinophils and basophils between the two endotypes within the 56 m0 AD samples. Expression is stated in transformed sequencing counts on the log2 scale.

We next examined whether the endotypes differed in terms of clinical characteristics. Both endotypes did not differ significantly for the tested clinical variables (supplementary Table S2). The NRS was slightly higher in the eosinophil-high endotype (median NRS: 7) as compared to the eosinophil-low endotype (median NRS: 5) (p-value 0.08 by Wilcoxon test).

Next, we examined the correlation of NRS and AD activity with gene expression in both endotypes. In the eosinophil-high endotype, oSCORAD and itch NRS were positively correlated with the expression levels of CCL23, IL5RA, and HRH4 (Fig 2, A), whereas in the eosinophil-low endotype, the gene expression showed a markedly weaker (CCL23) or no correlation at all (HRH4) with the oSCORAD, and an inverse correlation with the NRS (Fig 2, B). Likewise, while on the transcriptome-wide level no significant correlation was detected between gene expression and oSCORAD in the eosinophil-low endotype, 222 genes

(Benjamini-Hochberg adjusted  $p$ -value  $< 0.05$ ) were correlated in the eosinophil-high endotype. Of note, CSF2RB, which together with IL5RA encodes the IL5 receptor heterodimer, was among the top correlated genes (Spearman correlation coefficient  $\rho$ : 0.82) further supporting the correlation of the oSCORAD with IL5RA (supplementary Table S3 and S4).

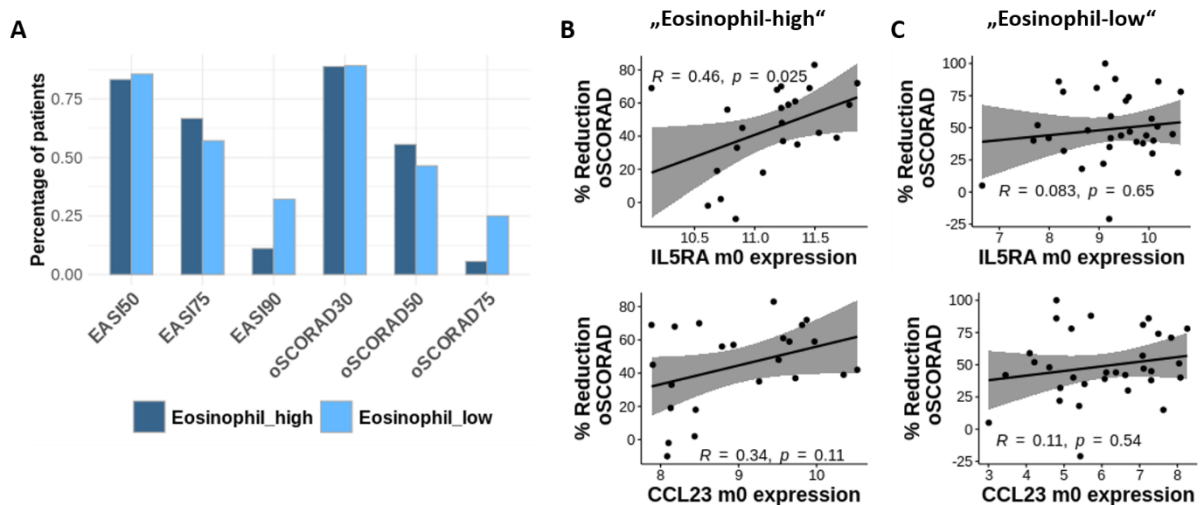


**Figure 2: Correlation of NRS and oSCORAD with gene expression in both endotypes.** **A**, In the eosinophil-high endotype, CCL23 (scatter plot) as well as IL5RA, CSF2RB, and HRH4 (heatmap) gene expression positively correlated with the oSCORAD and the itch NRS. **B**, In the eosinophil-low endotype, CCL23, IL5RA, CSF2RB, and HRH4 gene expression showed only a weak positive correlation (CCL23), no correlation (HRH4) or an inverse correlation (CSF2RB) with the oSCORAD, and an inverse correlation with the itch NRS. Colour code of the heatmaps represents Spearman correlation coefficient ( $\rho$ ), the same for “R” in the scatter plots.

In order to validate our observations, we performed a clustering based on the 34 identified AD-specific hypervariable genes of the AD patients in an independent case-control cohort. In accordance with our observations in the TREATgermany cohort, patients clustered in an eosinophil-high ( $n=23$  patients) and eosinophil-low endotype ( $n=8$  patients) (Supplementary Fig S2). As shown for the TREATgermany cohort, the oSCORAD positively correlated with the expression of CCL23, IL5RA, IL34, and HRH4 in the eosinophil-high endotype, whereas in the eosinophil-low endotype, the correlation was markedly weaker (Supplementary Fig S3). The NRS was not available for this cohort. Patients in the eosinophil-high endotype showed a higher disease activity as measured by the IGA (mean: 2.7; median: 3) and oSCORAD (mean 32.3; median: 32) as compared to patients in the eosinophil-low endotype (IGA: mean: 1.9; median: 2; oSCORAD: mean: 23.5; median: 24.75) ( $p$ -value: IGA 0.08 by Chi Square test; oSCORAD 0.07 by Wilcoxon test). However, across both cohorts, the disease activity was not different between the eosinophil-high and eosinophil-low endotype.

## Eosinophil-high and -low endotypes differ in treatment response to dupilumab

The 56 patients from TREATgermany included 46 patients who commenced a therapy with anti-IL4RA dupilumab and had a follow-up visit 3 months after initiation. In order to examine whether patients with a baseline eosinophil-high and eosinophil-low expression profile, respectively, show differences in their therapy responsiveness, we first compared dupilumab response rates after 3 months of therapy between both endotypes, and second compared correlation of response rates with baseline (m0) gene expression between both endotypes. Among the eosinophil-low endotype, 32% and 25% of patients treated with dupilumab reached a 90% reduction of the EASI (EASI90) and 75% reduction of the oSCORAD (oSCORAD75), respectively, while in the eosinophil-high endotype, only 11% and 5.5% of patients reached these endpoints, indicating that the eosinophil-low endotype responds better to dupilumab (Fig 3, A). In the eosinophil-high endotype, but not in the eosinophil-low endotype, the therapy response rate was positively correlated with the expression of IL5RA and CCL23 (Fig 3, B and C).

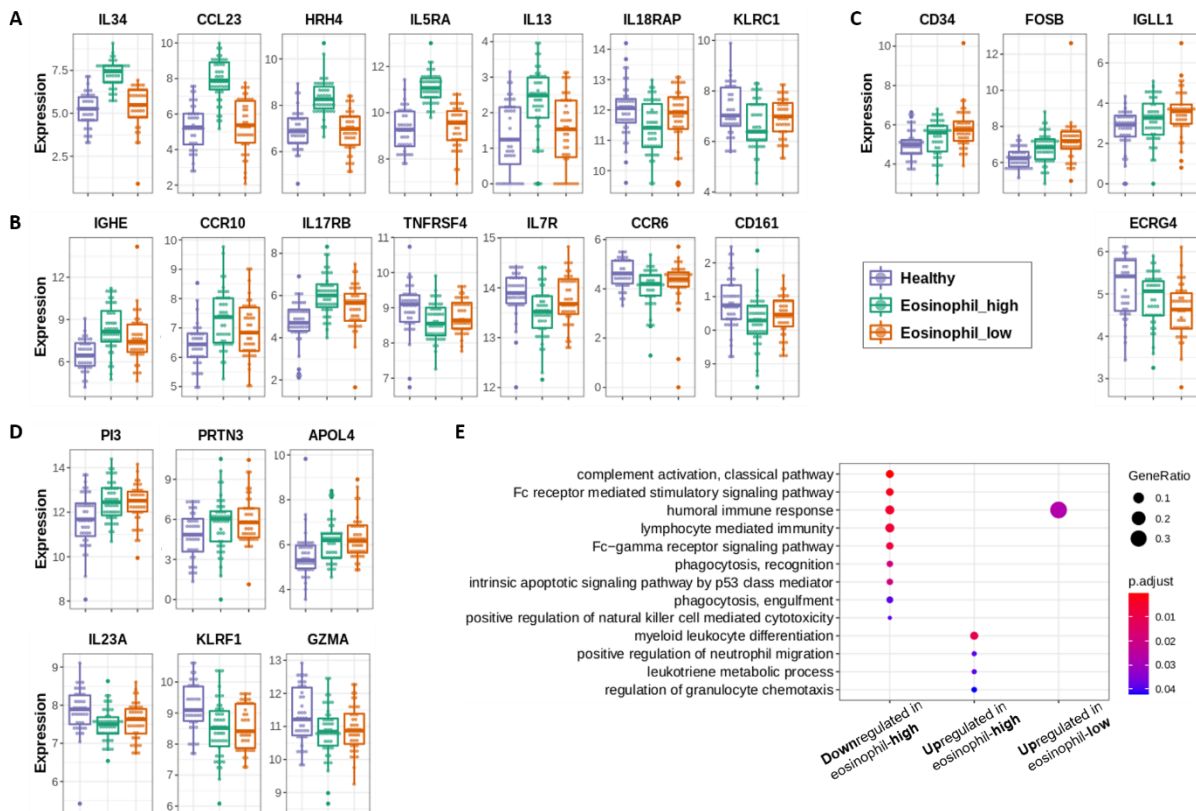


**Figure 3: Response to dupilumab in both endotypes.** **A**, Percentage of patients who reached EASI50 (EASI reduction  $\geq 50\%$  within 3 months of treatment with dupilumab), EASI75, EASI90, oSCORAD30, oSCORAD50, and oSCORAD75, respectively, in both clusters. **B**, Correlation of the reduction of the oSCORAD within 3 months of treatment with dupilumab with the baseline (m0) expression of IL5RA and CCL23 in the eosinophil-high endotype. **C**, Correlation of the reduction of the oSCORAD within 3 months of treatment with dupilumab with the m0 expression of IL5RA and CCL23 in the eosinophil-low endotype. The formula indicates the Spearman correlation coefficient ( $\rho=R$ ) as well as the p-value.

## The eosinophil-high endotype reveals a high degree of systemic dysregulation as compared to healthy states

Transcriptome-wide differential expression analysis revealed 1,002 differentially expressed genes (DEGs) for the eosinophil-high endotype with 562 genes showing an upregulation and

440 genes showing a downregulation as compared to healthy controls (supplementary Table S5). For the eosinophil-low endotype, 33 DEGs were detected with 19 genes showing an upregulation and 14 genes showing a downregulation as compared to healthy controls (supplementary Table S6). Thus, the number of significantly dysregulated genes was substantially higher in the eosinophil-high cluster. Among the top upregulated genes in the eosinophil-high endotype were those genes that also differentiated the two endotypes such as IL34, CCL23, IL5RA, and HRH4 as well the key type-2 cytokine IL13. In contrast, those genes showed no upregulation in the eosinophil-low endotype indicating that eosinophil blood expression profiles of patients from the eosinophil-low endotype resemble those of healthy individuals (Fig 4, A). Of note, the CSF2RB gene, which showed a strong correlation with the oSCORAD in the eosinophil-high endotype, showed no dysregulation as compared to healthy controls. The genes downregulated in the eosinophil-high but not eosinophil-low endotype comprised IL18RAP and KLRC1 (NKG2A), both of which have a high specificity for NK cells (Fig 4, A). Several genes primarily expressed by lymphoid cell lines such as T cells, B cells, and NK cells showed a considerably stronger dysregulation in the eosinophil-high endotype including the upregulated genes IGHE, CCR10, IL17RB as well as the downregulated genes TNFRSF4 (OX40), IL7R, CCR6, and KLRB1 (CD161) (Fig 4, B). In contrast, genes such as CD34, FOSB, IGLL1 and ECRG4 showed a stronger degree of dysregulation in the eosinophil-low endotype (Fig 4, C). Genes that showed a comparable dysregulation as compared to healthy results comprised e.g. the neutrophil-related PI3 (elafin) and PRTN3, and APOL4 (upregulated) as well as IL23A and the NK cell related genes KLRF1 encoding an activating receptor on NK cells as well as GZMA (downregulated) (Fig 4, D). On the functional level, genes downregulated in the eosinophil-high endotype were enriched for lymphocyte mediated immunity, humoral immune responses and B-cell related processes, as well as for NK cell mediated cytotoxicity supporting our observations on the single-gene level with downregulations of TNFRSF4, IL7R, CCR6, IL23A (B and T lymphocytes) and IL18RAP, KLRC1, and KLRF1 (NK cells) (Fig 4, E). In contrast, upregulated genes in the eosinophil-high endotype were enriched for processes related to neutrophil differentiation, as also reflected on the single-gene level by upregulations of the neutrophil-related genes PI3 (elafin) and PRTN3. As the degree of systemic dysregulation was substantially lower in the eosinophil-low endotype, there was an inherently low power to detect enriched gene groups. We further examined the expression of selected genes known to be dysregulated in the skin of patients with AD. Except for IL13, which showed significant upregulation in the eosinophil-high endotype, no other type-2-marker including IL4, IL4RA, IL13RA1, IL31RA and TSLP showed a relevant dysregulation as compared to healthy controls (Supplementary Fig S4). The T<sub>H</sub>1 cell differentiation marker IFNG showed a slight (non-significant) downregulation in both endotypes, while other T<sub>H</sub>1 markers showed no marked dysregulation.



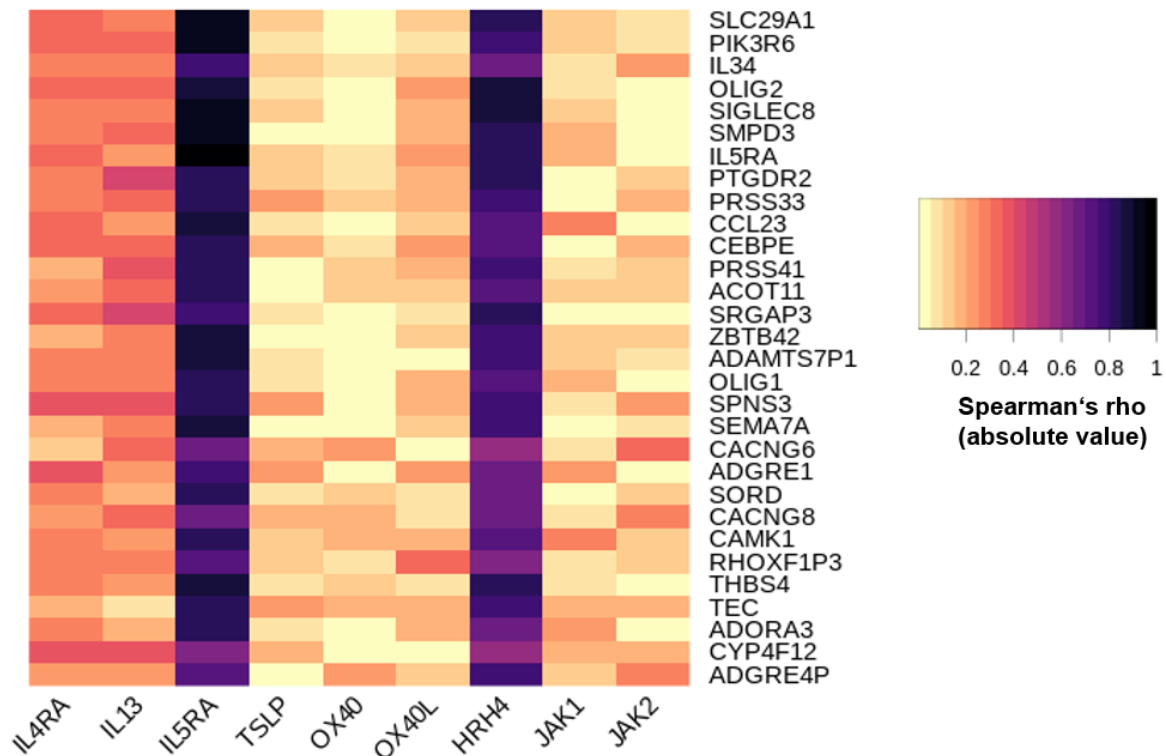
**Figure 4: Dysregulation in AD endotypes as compared to healthy controls.** **A**, Expression of genes that were dysregulated solely in the eosinophil-high endotype as compared to healthy controls. Gene expression is stated in transformed sequencing counts on the log2 scale. **B**, Genes that showed a higher degree of dysregulation in the eosinophil-high endotype as compared to the eosinophil-low endotype. **C**, Genes that showed a higher degree of dysregulation in the eosinophil-low endotype as compared to the eosinophil-high endotype. **D**, Genes that showed a comparable dysregulation in both endotypes as compared to healthy controls. **E**, Gene ontology (GO) gene set enrichment analysis with the differentially expressed genes (DEGs) detected in both endotypes as compared to healthy controls. For this analysis, DEGs were separated in up- and downregulated genes, respectively. There were no significantly enriched terms identified for the downregulated genes in the eosinophil-low endotype. Colour code shows the p-value of the enrichment. The dot size indicates the gene ratio, i.e. the number of genes matching the GO term divided by the total number of DEGs.

## Co-expression network and drug-target interaction analysis in the eosinophil-high endotype

Co-expression network analysis including a ranking of the genes by the combined influence of network centrality properties and differential expression scores identified SLC29A1, PIK3R6, and IL34 as the top 3 central genes in the eosinophil-high endotype (Fig 5). Other highly ranked genes included SIGLEC8, IL5RA, PTGDR2, PRSS33, as well as CCL23 (supplementary Table S7). We correlated the top 30 central genes of the consensus network with 9 specific target proteins of monoclonal antibodies or small-molecule biologics under development for AD (IL4RA, IL13, IL5RA, TSLP, TNFRSF4 (OX40), TNFSF4 (OX40L), HRH4, JAK1, and JAK2). Further target proteins such as IL5, IL22, and IL31 showed too low gene expression to be



included into this analysis. The strongest correlation with the top central genes was seen for IL5RA and HRH4 (mean  $\rho$  IL5RA = 0.83; mean  $\rho$  HRH4 = 0.76). IL5RA was a central gene itself explaining the high correlation of the top central genes with it. Clearly weaker correlations were seen with IL13 (mean  $\rho$  = 0.28) and IL4RA (mean  $\rho$  = 0.27), and very weak correlations only with OX40L (mean  $\rho$  = 0.15), JAK1 (mean  $\rho$  = 0.11), JAK2 (mean  $\rho$  = 0.11), TSLP (mean  $\rho$  = 0.1), and OX40 (mean  $\rho$  = 0.05).



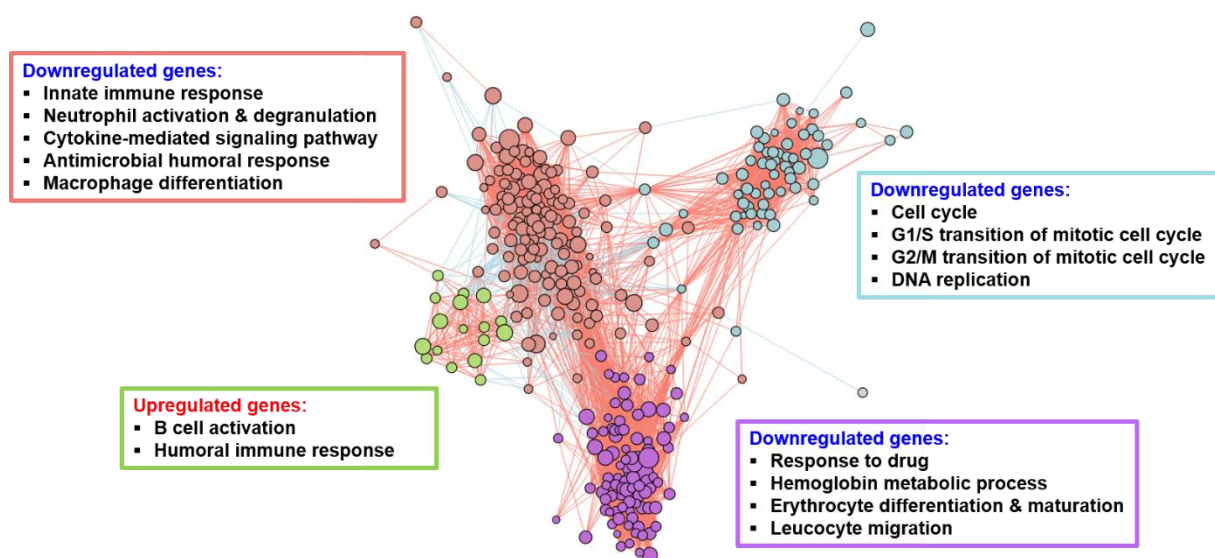
**Figure 5: Central genes in a co-expression network of the eosinophil-high endotype and their relation to (potential) therapeutic targets.** The top 30 central genes from the co-expression network analysis and their correlation with genes that can be blocked by targeted treatments that have been or are currently in clinical trials of AD. Colour code of the heatmap represents Spearman  $r$  ( $\rho$ ) (absolute value). The genes were ranked (top-down) by the combined influence of network centrality properties and differential expression scores.

### Blood-transcriptome changes under dupilumab treatment indicate a downregulation of neutrophil signatures and an activation of B cells and elevation of NK cells

Baseline as well as 3 months blood transcriptome datasets were available from 42 patients who received dupilumab treatment. The mean oSCORAD and EASI reduction was 51% and 74%, respectively. Patient-wise differential expression testing showed expression changes for 347 genes (306 genes downregulated, 41 genes upregulated, supplementary Table S8). The most pronounced changes were seen for the IGHE gene encoding the constant region of the IgE heavy chain (LFC: -1.8, 70% reduction) as well as KLRF1 (Nkp80) (LFC 0.44, 36%



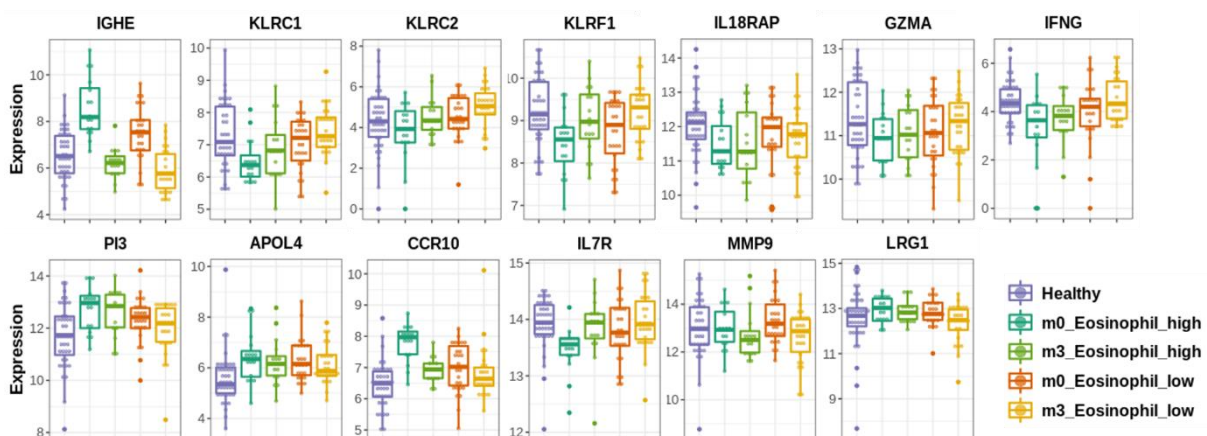
increase) and KLRC2 (NKG2C (LFC 0.40, 32% increase), both of which encode activating NK cell receptors. Co-expression network and network community analysis based on the DEGs m3 vs. m0 identified four modules of highly inter-correlated genes (Fig 6). The largest module consisting mainly of downregulated genes was enriched for innate immune response processes such as neutrophil activation and macrophage differentiation and included genes such as LILRA2, IL1RN, MMP9, PRTN3, OSM, BCL3, ORM1, and S100A11 indicating that inflammatory activities of innate immune cells decreased under dupilumab treatment (supplementary Table S9). Two additional modules consisting of downregulated genes were enriched for processes related to mitotic cell cycle as well as general metabolic processes and GO terms such as “drug response” (supplementary Table S10 and S11). The smallest module consisted of upregulated genes only and was enriched for B cell activation and humoral immune response by genes such as BANK1, FCRL1, MS4A1, and BLNK indicating an activation of B cells under treatment with dupilumab (supplementary Table S12).



**Figure 6: Changes under dupilumab on co-expression and functional group level.** Co-expression network was computed based on the differentially expressed genes (DEGs) from the comparison m3 vs. m0. Community detection identified four modules of genes within the network. Functional gene set enrichment analysis using Gene ontologies (GO terms) was carried out for each module.

Of the 42 patients treated with dupilumab, 16 and 26 patients were assigned to the eosinophil-high and eosinophil-low endotype, respectively. In the eosinophil-high endotype, 177 genes differentially expressed 3 months after treatment start were identified, with 40 genes showing an upregulation and 137 showing a downregulation (supplementary Table S13). In the eosinophil-low endotype, only 12 DEGs were identified, and all of them showed a downregulation after 3 months of treatment as compared to baseline (supplementary Table S14). The IGHE gene showed the highest expression fold change under dupilumab in both

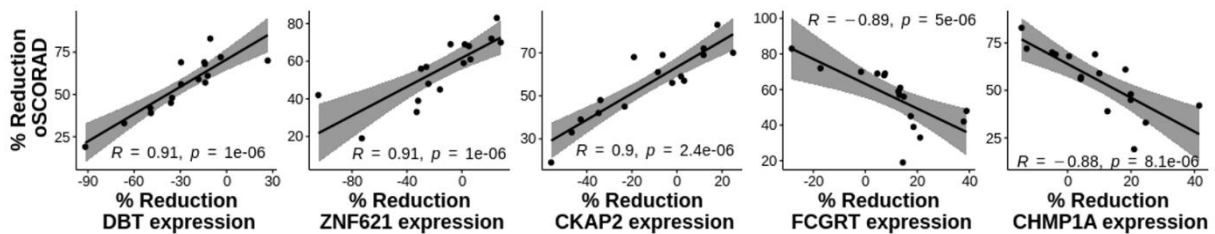
endotypes, with complete normalisation to healthy control levels within 3 months of treatment (Fig 7). The genes KLRC1, KLRC2 and KLRF1 encoding an inhibitory and two activating receptors primarily expressed by NK cells, respectively, showed an upregulation under treatment in both endotypes indicating a normalisation of NK cell transcriptomic profiles with clinical improvement. However, the IL18RAP, GZMA and IFNG expression remained downregulated in the eosinophil-high endotype as compared to healthy controls suggesting a residual disturbance of IFNG production by NK and T<sub>H</sub>1 cells. Baseline dysregulated genes such as PI3 and APOL4 showed constantly upregulated expression levels in both endotypes after 3 months of treatment, indicating a resilient sub-inflammatory signature in the blood of AD despite clinical improvement. Expression of the T cell related genes IL7R and CCR10 showed a baseline dysregulation especially in the eosinophil-high endotype, and within 3 months of treatment, the expression of IL7R and CCR10 markedly normalised in the eosinophil-high endotype. Of note, the majority of genes that showed particularly strong upregulation at baseline in the eosinophil-high endotype such as IL5RA, IL34, CCL23, HRH4, and IL13 showed little or no changes under therapy with dupilumab and no correlation with the responsiveness indicating that the endotype structure is largely stable (Supplementary Fig S5). Overall, the eosinophil-high endotype showed considerably more expression changes under dupilumab treatment, while few genes related to neutrophils and monocytes such as MMP9 and LRG1 showed a stronger alteration in the eosinophil-low as compared to the eosinophil-high endotype (Fig 7).



**Figure 7: Transcriptomic changes under dupilumab in both endotypes.** Gene expression of selected genes in healthy controls, AD patients at baseline (m0) as well as AD patients 3 months after the start of treatment with dupilumab (m3), separated in the eosinophil-high and eosinophil-low endotype, respectively. Gene expression is stated in transformed sequencing counts on the log<sub>2</sub> scale.

Last, we examined which transcriptional alterations under treatment correlated with the clinical improvement of AD as measured by the oSCORAD. In the eosinophil-high endotype, we

detected a significant (after Benjamini-Hochberg correction for multiple testing) correlation of the expression changes of the five genes DBT, ZNF621, CKAP2, FCGRT, and CHMP1A with the reduction of the oSCORAD within 3 months of treatment with dupilumab (Fig 8, supplementary Table S15). While DBT, ZNF621, and CKAP2 showed a positive correlation, i.e. a high reduction of the expression level was associated with a high reduction of disease activity, FCGRT and CHMP1A showed a negative correlation. The gene expression of DBT, ZNF621, CKAP2, and CHMP1A has a low immune cell and tissue specificity according to the Human Protein Atlas, allowing no prediction on the functional and cellular level. FCGRT shows a high specificity for neutrophils and an enhanced expression in the blood as compared to other tissues. For the eosinophil-low endpoint, we did not detect any significant correlations with reduction of the oSCORAD after correction for multiple testing (supplementary Table S16).



**Figure 8: Correlation of gene expression changes and response rates in the eosinophil-high endpoint.** Correlation of the reduction of the oSCORAD within 3 months of treatment with dupilumab with the reduction of gene expression in the 16 patients of the eosinophil-high endpoint. Both reduction of oSCORAD and expression were calculated as  $100 \cdot (1 - (m_3/m_0))$  i.e. a negative reduction means an increase of gene expression or oSCORAD under treatment. The five genes showed a significant correlation after correction for multiple testing. The formula indicates the Spearman correlation coefficient ( $\rho=R$ ) as well as the p-value.

## Discussion

To our knowledge this is the first sequencing-based transcriptome-wide study of blood from adult patients with AD. Our analysis provides evidence for the systemic nature of AD, however, based on a hierarchical clustering approach, we identified two AD blood transcriptomic endotypes, one of which was characterised by elevated eosinophil signatures and a high degree of systemic dysregulation, and a second that showed no marked eosinophil related signature and a low degree of systemic dysregulation. We found a notable overlap of genes showing a marked difference in expression between the two endotypes and genes that have been recently shown to be upregulated in the blood of children with recent AD onset as compared to healthy controls including CCL23, IL5RA, IDO1, HRH4, CCR3, CLC, SIGLEC8, ALOX15, PTGDR2, and PRSS33.<sup>15</sup> Interestingly, only patients of the eosinophil-high endotype showed an upregulation of the above mentioned genes, while patients from the eosinophil-low endotype showed comparable expression levels with healthy controls. Any conclusion about existence of eosinophil endotypes in the study cohort of Brunner *et al.* remain purely speculative, since only a minor proportion of DEGs do overlap and since both study designs are not readily comparable concerning age and disease onset.

Noteworthy, the proportion of patients diagnosed with asthma and/or rhinitis as well as the prevalence of sensitisations was not different between the two endotypes. This is contradictory to the findings of Lemonnier *et al.*, who reported an upregulation of CLC, EMR4P, IL5RA, FRRS1, HRH4, SLC29A1, SIGLEC8, and IL1RL1 in the group of patients who suffered from at least two atopic diseases, i.e. asthma, allergic rhinitis, and atopic dermatitis, but not in patients with atopic dermatitis alone.<sup>30</sup> While all of those 8 genes were significantly upregulated in the eosinophil-high endotype, they showed no upregulation in patients of the eosinophil-low endotype. According to our approach, the eosinophil signature is not related to the presence of atopic comorbidities, i.e. the endotypes were not dictated by the co-occurrence of asthma or rhinitis. Concordantly to our findings, eosinophilic and non-eosinophilic subtypes have been described for asthma indicating that eosinophilic profiles might present an intrinsic endotype of both AD and asthma.<sup>31</sup> Severe as well as moderate disease manifestations have been described for both eosinophilic and non-eosinophilic asthma, and likewise, in our study, AD patients of both endotypes on average showed a comparable disease activity.<sup>31</sup> Nonetheless, we demonstrated an interrelation of blood endotype profiles with AD disease activity. In the eosinophil-high endotype, high expression levels of genes encoding members of the IL5 signalling pathway as well as other eosinophil-related genes such as CCL23 and HRH4 were associated with both higher disease activity and a higher intensity of itch.

While IL5RA was strongly upregulated in patients of the eosinophil-high endotype as compared to healthy controls, CSF2RB (encoding the IL5 receptor beta subunit) showed no elevated expression levels, but a remarkably strong positive correlation with the disease activity. This

suggests that it might be relevant to consider not only dysregulated molecules as biomarkers, but also molecules that show normal levels. Thus, dysregulation might not be a necessity to detect links between gene expression and clinical characteristics. Therefore, it is intriguing that besides the low level of systemic dysregulation in patients of the eosinophil-low endotype, we did neither detect any correlation of gene expression and disease activity in this endotype. We conclude that the eosinophil-low endotype might comprise a certain group of patients who lack a systemic component related to AD. Instead we postulate that the phenotype of this group of patients might be driven by extrinsic factors such as the skin microbiota, cutaneous neuroimmune interactions, and skin-specific regulatory patterns, rather than by intrinsic and systemic dysregulation of immune profiles.

According to the inferred co-expression network of the transcriptomic dysregulation in the eosinophil-high endotype, IL5RA was among the top central genes implying that interfering with IL5RA signalling might strongly affect multiple other dysregulated genes that are under mutual regulatory control. The same applies for the histamine receptor H4 (HRH4) and the prostaglandin D2 receptor 2 (PTGDR2/CRTH2), which were both central in the dysregulation in eosinophil-high patients. While the anti-IL5RA antibody benralizumab is currently in a phase IIa study in AD (HILLIER Study, NCT04605094), a study on anti-IL5 mepolizumab showed only moderate effects in patients with AD.<sup>32</sup> While anti-IL5 mepolizumab and anti-IL5RA benralizumab have been trialled for eosinophilic asthma with certain thresholds of eosinophilia as inclusion criterion, the missing stratification of AD patients for their eosinophilic profile in clinical studies of mepolizumab (anti-IL5), adriforant (anti-HRH4), fevipiprant (anti-PTGDR2), and timapiprant (anti-PTGDR2) might impact their power of detecting therapeutic effects as suggested by our results.<sup>32-37</sup> Patients belonging to the eosinophil-low endotype might not profit from anti-IL5, anti-HRH4 and anti-PTGDR2 therapy, as other factors than systemic eosinophil-related pathways are probably associated with their cutaneous inflammation.

Albeit dupilumab resulted in a comparable clinical improvement of AD in both endotypes, patients from the eosinophil-low endotype were more often super-responders (EASI90) than patients from the eosinophil-high endotype. While with the growing number of available targeted therapies for AD, a response beyond reaching the usual primary endpoint EASI50 or EASI75 should be the goal of stratified medicine, it might be necessary to consider the individual patients' molecular expression profiles in clinical studies instead of a retrospective identification of drug endotypes in observational studies.<sup>38,39</sup> Large consortia exploring massive amounts of patients' omics data as well as clinical data such as BIOMAP will hopefully help stratifying patients with AD, which will support clinical trial design.

Besides the elevated levels of genes related to eosinophils in the eosinophil-high endotype, we observed further dysregulations associated with AD such as signatures reflecting elevated neutrophil as well as inflammatory and innate immune processes reflected by upregulation of

genes such as PI3 and PRTN3. In contrast, signatures related to lymphoid cells in particular NK and related cytotoxic killer cells were diminished in AD reflected by a downregulation of genes such as KLRF1, GZMA, KLRC1, IL18RAP, and IFNG. KLRF1, which showed the strongest downregulation, encodes NKp80, an activating receptor stimulating NK cell cytotoxicity and cytokine release.<sup>40</sup> The downregulation of the NK cell specific genes IL18RAP and GZMA as well as IFNG, which are involved in stimulation and cytotoxicity of NK cells, thus is in line with the downregulation of the activating receptor NKp80.<sup>29,41</sup> Of note, NKp80 is a marker of NK cell maturity with NKp80(-) cells representing a more immature stage, while NKp80(+) NK cells produce IFNG and are cytotoxic suggesting that circulating NKp80(+) NK cells are reduced in AD.<sup>42</sup> Our observations are consistent with recent reports about decreased peripheral NK cell numbers in patients with AD, and further emphasise a selective reduction of certain killer cell subpopulations, as other NK cell markers such as CD56, CD16A (FCGR3A), CD94 (KLRD1), and NKG7 showed no downregulation in our data.<sup>8</sup> We further showed that blood transcriptomic signatures of AD do not readily reflect AD-specific skin transcriptomic signatures, but elevated eosinophil and T<sub>H</sub>2 blood profiles might precede the type 2 inflammation in the skin. As the skin is the site where inflammation and defence against microbes takes place, genes encoding alarmins and anti-microbial proteins such as TSLP, defensins and S100 proteins are exceptionally upregulated in this organ, while this appears not to be the case in the periphery.<sup>5</sup>

Treatment with dupilumab resulted in a shift of the blood transcriptome that indicated a decrease of innate inflammatory processes based on neutrophils and monocytes, and a concomitant increase of B cell activation and NK cell abundance and/or a functional shift of NK cells. Interestingly, the eosinophil-related endotype structure was largely retained despite clinical improvement, further supporting a potential benefit of targeted anti-IL5 treatment for long-term disease control in patients showing strong eosinophilic signatures. Intriguingly, the NK cell abundance appeared to normalise within 3 months of treatment with dupilumab, however, a persistent downregulation of GZMA, IL18RAP as well as IFNG in the eosinophil-high endotype might reflect a residual systemic dysregulation based on a lack of IFNG production by NK cells and/or T<sub>H</sub>1 cells, and a disturbed cytotoxic effector function of killer cells.<sup>29,40</sup> Cell-based and transcriptomic studies on the single-cell level are urgently needed in order to elucidate the role of NK cells in the pathophysiology of AD.

Our study has several strengths such as the use of sensitive and transcriptome-wide RNA sequencing, standardised sample collection and comprehensive clinical data recording in the TREATgermany registry, as well as the use of unsupervised but simple analytical approaches. While the use of PAXgene whole-blood instead of only the PBMC fraction is limiting the analytical depth of immune cell signatures due to high abundant transcripts from erythrocytes and reticulocytes, we compensated this issue by an exceptional coverage depth of 55 Mio

reads per sample. Further, whole-blood sequencing has advantages above PBMC sequencing such as the capturing of all blood cell populations including granulocytes as well as a lower variability and higher sensitivity, which is relevant for multi-centre studies.<sup>43–45</sup> Our study also revealed some limitations of whole-blood RNA sequencing such as the low or missing coverage of relevant immune mediators such as interleukins, which often were at the detection limit in our data. As known from qPCR-based and protein-based studies, several interleukins and chemokines are upregulated in the blood of patients with AD such as IL4 and CCL17 (TARC).<sup>10,46</sup> Thus, a combined approach of whole-transcriptome RNA-sequencing together with a capturing approach for candidate gene/protein panels may be advantageous in future studies. Further, our study is limited by a comparable small sample size with view on blood-based studies. With the growing number of patients' PAXgene samples becoming available within TREATgermany and other consortia, large-scale studies and metanalysis approaches should decipher blood endotype profiles in AD.

In summary, our study provides novel insight into blood transcriptomic signatures in AD. Within this study we not only accomplished the successful discovery of two novel eosinophil-related endotypes that might promote new treatment approaches with existing targeted therapies, but also identified a selective and persistent disturbance of NK cell signatures, thereby highlighting a potential yet underestimated disease mechanism.

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## Supplementary tables

Tables S1, and S3-S16 are available in digital format only

**Table S1: Differential expression analysis: eosinophil-high vs. eosinophil-low endotype**

see separate excel file

**Table S2. Patient characteristics of the eosinophil-high and eosinophil-low endotype**

Characteristic	Eosinophil-high (n = 23)	Eosinophil-low (n = 33)	p-value
Age (mean (SD))	44.09 (13.72)	44.67 (14.67)	0.65 <sup>†</sup>
Sex = male (%)	16 (69.6)	24 (72.7)	1 <sup>#</sup>
BMI (mean (SD))	28.03 (5.72)	25.96 (3.51)	0.15 <sup>†</sup>
oSCORAD (mean (SD))	46.21 (13.51)	42.60 (13.31)	0.23 <sup>†</sup>
EASI (mean (SD))	22.35 (12.01)	19.61 (10.35)	0.44 <sup>†</sup>
IGA (mean (SD))	3.74 (0.69)	3.61 (0.70)	0.96 <sup>#</sup>
NRS (mean (SD))	6.91 (2.09)	5.88 (2.34)	0.08 <sup>†</sup>
Age of AD onset (mean (SD))	8.48 (17.64)	9.94 (17.94)	0.59 <sup>†</sup>
Diagnosis Asthma (%)			0.25 <sup>#</sup>
no	6 (26.1)	14 (42.4)	
yes	17 (73.9)	19 (57.6)	
Diagnosis Rhinitis (%)			1 <sup>#</sup>
no	6 (26.1)	9 (27.3)	
yes	17 (73.9)	24 (72.7)	
Asthma / rhinitis (%)			0.61 <sup>#</sup>
none	4 (17.4)	7 (21.2)	
one	4 (17.4)	9 (27.3)	
both	15 (65.2)	17 (51.5)	
Asthma and/or rhinitis (%)			0.74 <sup>#</sup>
no	4 (17.4)	7 (21.2)	
yes	19 (82.6)	26 (78.8)	
Sensitisation mould (%)			1 <sup>#</sup>
no	8 (34.8)	13 (40.6)	
yes	7 (30.4)	13 (40.6)	
Sensitisation pollen (%)			0.7 <sup>#</sup>
no	4 (17.4)	4 (12.5)	
yes	18 (78.3)	27 (84.4)	
Sensitisation dust mite (%)			1 <sup>#</sup>
no	3 (13.0)	5 (15.6)	
yes	19 (82.6)	25 (78.1)	
Sensitisation food (%)			0.12 <sup>#</sup>
no	6 (26.1)	17 (53.1)	
yes	11 (47.8)	10 (31.2)	
No. of sensitisations (%)			0.29 <sup>#</sup>
0	0 (0.0)	2 (8.3)	
1	4 (33.3)	3 (12.5)	
2	4 (33.3)	8 (33.3)	
3	0 (0.0)	5 (20.8)	
4	4 (33.3)	6 (25.0)	

<sup>#</sup>: by Chi square test; <sup>†</sup>: by Wilcoxon test

**Table S3: Spearman correlation of genes expression and oSCORAD in the eosinophil-high endotype**

see separate excel file

**Table S4: Spearman correlation of genes expression and oSCORAD in the eosinophil-low endotype**

see separate excel file

**Table S5: Differential expression analysis: eosinophil-high endotype vs. healthy controls**

see separate excel file

**Table S6: Differential expression analysis: eosinophil-low endotype vs. healthy controls**

see separate excel file

**Table S7: Rank table from the co-expression network analysis based on DEGs of eosinophil-high endotype vs. healthy controls**

see separate excel file

**Table S8: Differential expression analysis: m3 dupilumab vs. m0 all patients**

see separate excel file

**Table S9: Enrichment analysis of the red module: m3 dupilumab vs. m0 all patients network analysis**

see separate excel file

**Table S10: Enrichment analysis of the blue module: m3 dupilumab vs. m0 all patients network analysis**

see separate excel file

**Table S11: Enrichment analysis of the violet module: m3 dupilumab vs. m0 all patients network analysis**

see separate excel file

**Table S12: Enrichment analysis of the green module: m3 dupilumab vs. m0 all patients network analysis**

see separate excel file

**Table S13: Differential expression analysis: m3 dupilumab vs. m0 eosinophil-high endotype**

see separate excel file

**Table S14: Differential expression analysis: m3 dupilumab vs. m0 eosinophil-low endotype**

see separate excel file

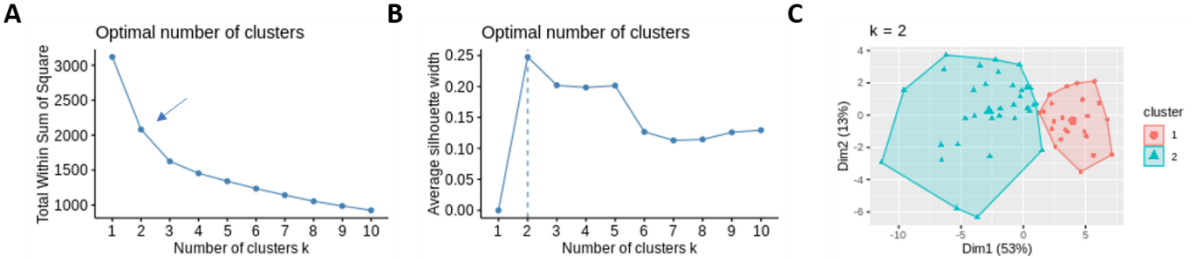
**Table S15: Spearman correlation of genes expression change and reduction of oSCORAD under dupilumab in the eosinophil-high endotype**

see separate excel file

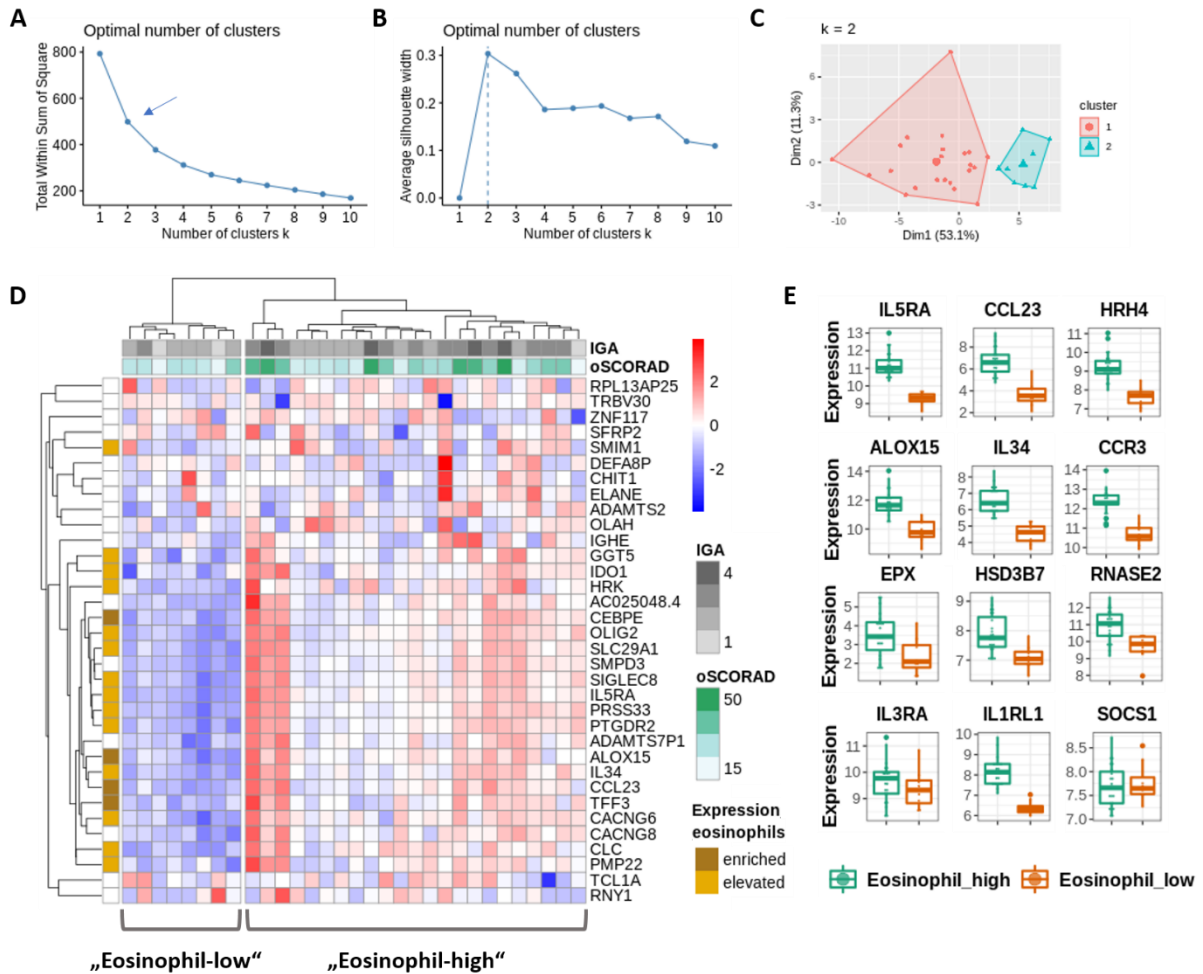
**Table S16: Spearman correlation of genes expression change and reduction of oSCORAD under dupilumab in the eosinophil-low endotype**

see separate excel file

# Supplementary figures

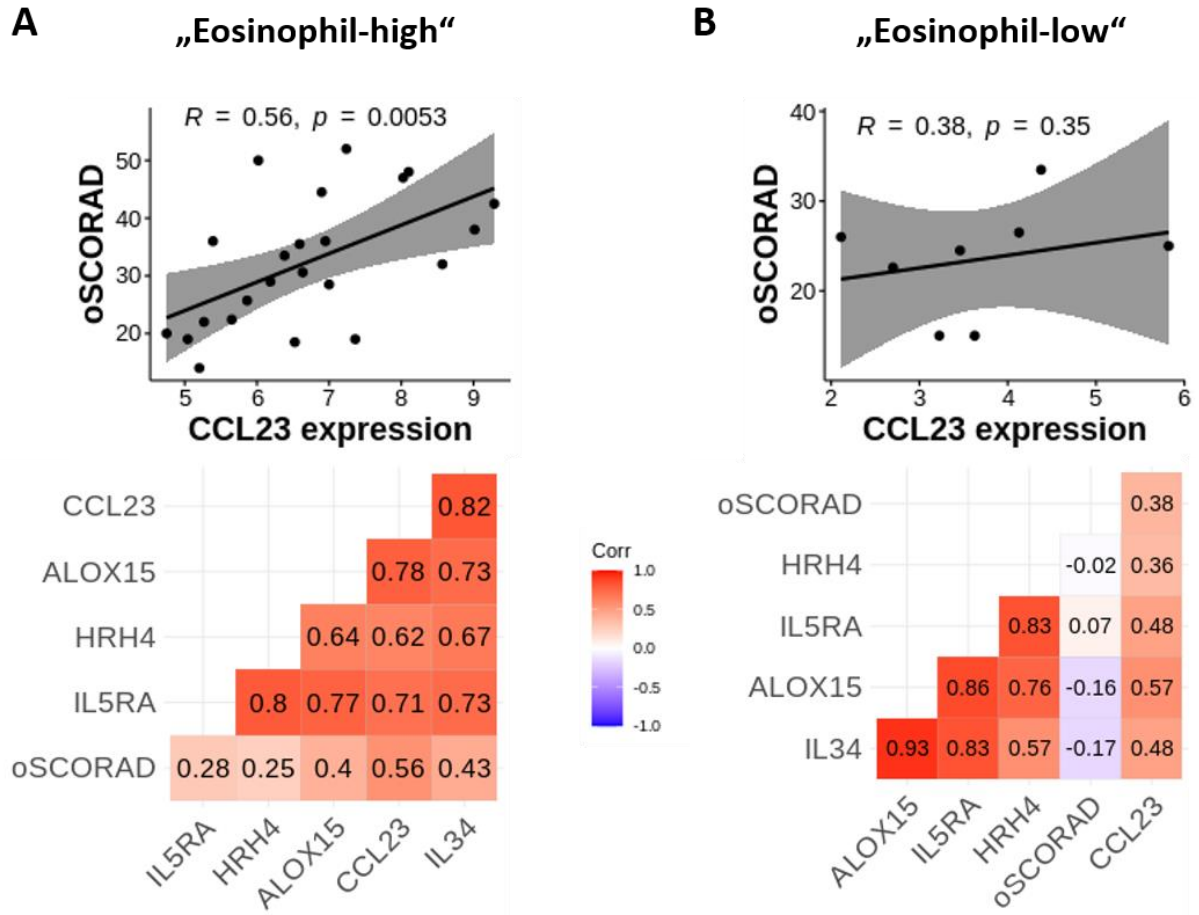


**Figure S1: Determination of the optimal number of clusters of the TREATgermany samples. A,** The elbow method applied on the hierarchical clustering of 56 TREATgermany patient samples. The arrow shows the “elbow” indicating the optimal number of clusters minimising the total intra-cluster variation. **B,** The average silhouette method to determine the quality of a clustering with a high average silhouette width indicating a good clustering. **C,** Visualisation of the clustering by the first two principle components with 2 clusters.

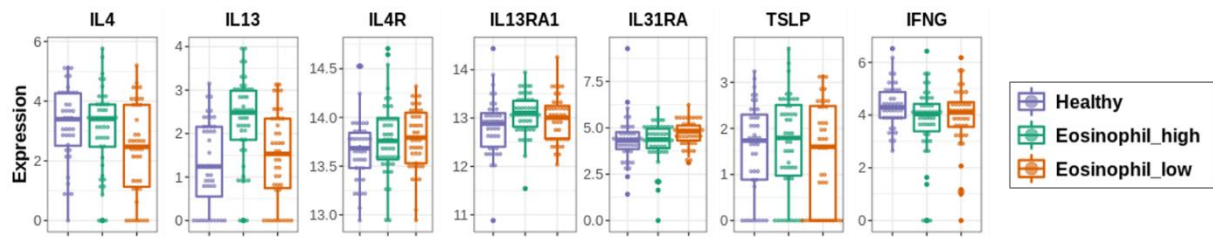


**Figure S2: Clustering of the patients of the case-control cohort.** **A**, The elbow methods applied on the hierarchical clustering of 31 patient samples from the independent case-control cohort. The arrow shows the “elbow” indicating the optimal number of clusters minimising the total intra-cluster variation. **B**, The average silhouette method to determine the quality of a clustering with a high average silhouette width indicating a good clustering. **C**, Visualisation of the clustering by the first two principle components with 2 clusters. **D**, Hierarchical clustering of 31 AD samples based on the expression of 34 AD-specific hypervariable genes (as identified by the TREATgermany cohort) grouped the patients in an eosinophil-high and an eosinophil-low endotype. The vertical yellowish colour bar shows the cell-type specificity for eosinophils according to the Human Protein Atlas. Horizontal colour bars show the oSCORAD and IGA of the patients. The heatmap colour code represents row z scores. **E**, Differential expression of genes related to eosinophils and basophils between the two endotype. Expression is stated in transformed sequencing counts on the log<sub>2</sub> scale.

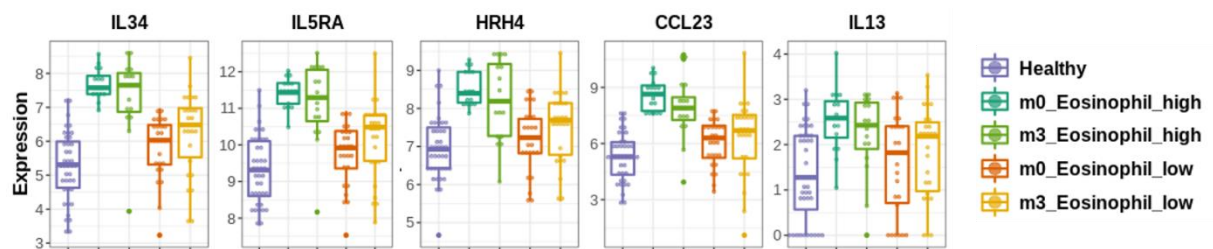




**Figure S3: Correlation of oSCORAD with gene expression in both endotypes. A,** In the eosinophil-high endotype, CCL23 (scatter plot) as well as IL5RA, IL34, ALOX15 and HRH4 (heatmap) gene expression positively correlated with the oSCORAD. **B,** In the eosinophil-low endotype, CCL23, HRH4, and IL5RA showed a markedly weaker correlation with the oSCORAD as compared to the eosinophil-high endotype, while ALOX15 and IL34 showed an inverse correlation with the oSCORAD.



**Figure S4: Expression of AD skin candidate genes in the blood.** Expression of genes related to type-2 inflammation, neuroimmune interactions leading to itch, and IFNG (T<sub>H</sub>1 marker) in both identified AD blood endotypes as well as healthy controls. Gene expression is stated in transformed sequencing counts on the log<sub>2</sub> scale.



**Figure S5: Transcriptomic changes of endotype-characteristic genes under dupilumab.** Expression of genes which discriminated both endotypes plotted for healthy controls, AD patients at baseline (m0) as well as AD patients 3 months after the start of treatment with dupilumab (m3), separated in both endotypes. Gene expression is stated in transformed sequencing counts on the log<sub>2</sub> scale.

## 4 Discussion

Though the knowledge about the molecular characteristics of AD is increasing in the era of omics analysis, many questions remain to be answered in order to promote and eventually accomplish stratified medicine. Challenges arise from the high complexity of AD regarding molecular disease mechanisms as well as the heterogeneity of its clinical characteristics. In the scope of this work, I analysed skin and blood transcriptomic data of patients with AD from the TREATgermany registry, in order to characterise AD associated transcriptomic signatures as well as expression changes under systemic treatment and their association with clinical variables.

In the first study, skin transcriptomic data of both lesional and nonlesional samples of patients and healthy control skin were analysed in-depth. Several findings by former studies could be verified by our work, such as the upregulated expression of type 2 inflammatory markers centred to IL13, which is one of the key mediators of the type 2 phenotype in AD skin.<sup>1-4</sup> Further, our study confirmed the inverse correlation of type 2 markers and genes encoding epithelial barrier proteins such as FLG and LOR.<sup>1</sup> In my analysis, I differentiated a stable from a dynamic transcriptomic signature. The stable signature, which was denoted as core signature, was defined as transcriptomic dysregulation that showed a comparable extent in both lesional and nonlesional skin of patients as compared to healthy skin. This dysregulation potentially reflects general features of AD skin, which are not attributable to the current (visible) cutaneous inflammation, but might enable it by leaving the skin vulnerable to excessive immune responses. Intriguingly, the core signature comprised various downregulated structural genes such as genes encoding keratins and kallikreins indicating a disturbed differentiation of the epidermal barrier. This observation endorses the outside-in hypothesis for AD, which postulates that an acquired barrier disturbance precedes the immune dysregulation.<sup>5</sup> Our observation that also nonlesional skin shows signatures of barrier dysfunction indicates that this might be a mechanism by which immune dysbalance is triggered, since a disturbed barrier presents an insufficient shield against antigens and microbes leading to exaggerated cutaneous immune responses and a chronic inflammatory milieu.<sup>6,7</sup>

AD is characterised by persistent and intense pruritus, which drives a self-maintaining itch-scratch cycle further promoting barrier disruption.<sup>7</sup> Interestingly, the core signature identified by our study comprised upregulated genes involved in itch signalling such as TSLP encoding an alarmin which is released by keratinocytes after epithelial barrier disruption, and IL31RA encoding the alpha subunit of the IL31 receptor.<sup>8,9</sup> IL31 is a pruritus- and T<sub>H</sub>2-associated cytokine, and it was shown that IL31 signalling has implications in cutaneous hyperinnervation and growth of sensory nerves as shown *in vitro* and *in vivo*.<sup>10</sup> Moreover, a study showed that subcutaneous injection of IL31 triggered scratching via IL31RA expressed by mouse neurons.<sup>11</sup> Thus, the overexpression of IL31RA in both lesional and nonlesional skin of AD indicates a generally increased potential for innervation of sensory nerves and therefore a susceptibility to pruritus. Recently, the effectiveness of different biologics provided hints on the relevance of itch signalling pathways in the pathogenesis of AD. While treatment with anti-IL4RA dupilumab reduced AD signs such as tissue inflammation and additionally improved pruritus symptoms, nemolizumab blocking IL31RA significantly improved itch in patients with AD in a phase II trial, but had only modest effects on AD signs. This indicates that IL31 signalling is not a causal factor in AD pathogenesis, albeit our study indicates that it might be

an early contributing factor.<sup>8,12</sup> While the overexpression of TSLP in both lesional and nonlesional skin of AD might be caused by a general disturbance of the epidermal barrier in patients with AD, it might also be regulated by environmental factors, as a study showed that a TSLP promoter hypomethylation is associated with both AD and prenatal exposure to tobacco smoke.<sup>13</sup>

While the dysregulation of TSLP and IL31RA could be the result of both an intrinsic dysregulation and of a disturbed skin barrier, our findings indicate that their dysregulation is not exclusively associated with an active (visible) inflammation, but renders unaffected skin susceptible to pruritus independent of an inflammatory milieu. The pathway analysis I conducted confirmed the findings on the single-gene level, as genes belonging to the core signature were enriched for pathways related to the nervous system as well as broad signal transduction pathways such as Rap1 and cAMP signalling pathway. In contrast, the dynamic signature, which comprised genes that showed a progressive dysregulation from healthy to nonlesional to lesional skin, i.e. the highest degree of dysregulation in the lesional skin, was enriched for multiple pathways related to the immune system. On the single-gene level, this was reflected by a progressive upregulation of genes related to T<sub>H</sub>2 differentiation such as IL13 and CCL17, and T<sub>H</sub>17/T<sub>H</sub>22 differentiation such as S100 genes, IL23A, and IL22. Remarkably, the core signature was not enriched for any pathway directly related to the immune system. Therefore, the results of our study indicate that a marked intrinsic immune dysregulation is not part of the AD core signature. Thereby our results are contradictory to the inside-out hypothesis of the aetiology of AD, which in contrast to the outside-in theory postulates that the epidermal barrier disturbance is the result of cutaneous inflammation.<sup>5</sup> Instead, we observed signatures of a disturbed epidermal barrier in both lesional and nonlesional skin without marked signatures of inflammation in nonlesional skin.

Our study further provides insights into the effects of systemic treatment with anti-IL4RA dupilumab and cyclosporine. In a network analysis, I identified IL4RA as one of the most central genes of the lesional dysregulation of gene expression. Nonetheless, after three months of therapy, patients treated with cyclosporine on average showed larger transcriptomic shifts towards healthy conditions as compared to patients treated with dupilumab. The broad immunosuppressant effects of cyclosporine provide a possible explanation for larger transcriptomic alterations within three months of treatment.<sup>14</sup> However, after a treatment period >3 months, normalisation on the transcriptomic level might be similar for both drugs. Long-term longitudinal studies of dupilumab therapy are needed to elucidate this. Despite the lower degree of molecular normalisation, patients treated with dupilumab yielded a comparable clinical improvement as patients treated with cyclosporine, which underscores the power of targeted therapies and the central role of type 2 cytokine signalling in the skin of AD patients.

Although a high extent of dysregulation was reverted after three months of systemic treatment, I observed a substantial residual disease-associated transcriptomic profile. A residual profile of transcriptomic dysregulation after several weeks of therapy was shown by other studies of AD as well.<sup>15,16</sup> The treatment duration was shown to have a marked impact on the extent of the detected residual dysregulation as it was shown that after a longer treatment period, the residual dysregulation was smaller.<sup>15</sup> Possibly, a treatment duration of three months is too short for a complete normalisation of the transcriptomic dysregulation. Alternatively, the detected residual profile might present a subliminal therapy-resistant dysregulation responsible for the reoccurrence of AD after termination of therapy. Long-term studies including post-therapy examinations are needed to address this question.

In a second work, I re-analysed the skin transcriptome data with respect to signatures that are attributable to NK cells and other cytotoxic killer cells. A recent study that showed a marked reduction of total peripheral blood NK cells along with a loss of mature CD56<sup>dim</sup> CD57<sup>+</sup> NK cells, and an elevation of activated NK cell transcriptomic signatures in lesional skin in independent sets of patients with moderate-to-severe AD.<sup>17</sup> Further, Mack *et al.* reported that the promotion of NK cell survival and proliferation by an IL15 superagonist in a murine AD model reduced AD disease severity as well as the number of skin type 2 innate lymphoid cells (ILC2) and eosinophils. Their observations suggest that an NK cell-based immunotherapy might be a promising treatment approach for AD. Based on these findings, it was postulated that in AD, NK cells are recruited from the periphery to the inflamed skin as an endogenous counterregulatory response to type 2 skin inflammation.<sup>18</sup>

Our study demonstrated that NK cells are accumulated in the skin of AD, shown by immunofluorescence staining as well as by elevated NK-cell related transcriptomic signatures. However, in contrast to Mack *et al.*, I detected a diminished abundance of activated NK cells with a concomitantly increased abundance of resting NK cells by *in silico* cytometry. While the study of Mack *et al.* was focused on blood samples and only included skin samples of six patients, our skin transcriptomic study including skin samples of 57 patients as well as healthy control samples clearly had a higher power. Although the dysbalance of activated and resting NK cells appeared to normalise under systemic treatment of AD, the accumulation of NK cells was quantitatively persistent according to the transcriptomic profile after three months of treatment. These observations are not readily compatible with the hypothesis that NK cells are a counterregulatory mechanisms to the cutaneous type 2 inflammation in AD, as type 2-related signatures were markedly diminished after three months of treatment as shown by our first study. Beyond the idea of a counterregulatory mechanism, it was speculated that the release of type 2 cytokines such as IL4 and IL13, and a deficient IFNG release by certain NK cell subsets may contribute to deviating immune responses towards a type 2 pattern in AD.<sup>19-21</sup> While this can only be disentangled by studies on the single-cell level, our results strongly point towards a role of NK cells in the pathophysiology of AD.

Of note, my analysis suggested that there was a qualitative rather than a quantitative shift of NK cells under treatment. The upregulation of the NK cell lineage marker CD56 with a concomitant downregulation of CD16A and genes encoding cytotoxic granzymes suggested a shift from cytotoxic CD56<sup>dim</sup> CD16<sup>+</sup> NK cells towards immunomodulatory CD56<sup>bright</sup> CD16<sup>-</sup> NK cells.<sup>22-24</sup> However, our data were derived from bulk-tissue samples, precluding a final conclusion. As the skin is a tissue with heterogeneous cell populations, gene expression profiles of distinct cell types can only be inferred. Further insight derived from single-cell transcriptomic approaches should disentangle the function of NK cells in the skin of AD, whether they present a disease-promoting or a disease-regulatory population as well as a potential eligibility as a novel target for therapy.

In a blood transcriptomic study, I detected NK cell related signatures that are coherent with the observations of Mack *et al.*, as my results indicated a reduced NK cell related transcriptomic profile in patients with AD. In detail, this was reflected by a downregulated expression of NK cell specific genes such as KLRF1, KLRC1, GZMA, and IL18RAP, and a concomitantly lower expression of IFNG.<sup>25,26</sup> In contrast, genes encoding typical lineage markers of peripheral NK cells such as CD56 and CD16A showed comparable expression levels as healthy controls indicating that only certain NK cell subtypes were reduced rather than the NK cell population in general.<sup>22-24</sup> Intriguingly, the dysregulation of KLRF1 and KLRC1 was largely reverted after three months of therapy with dupilumab, whereas the downregulation of GZMA, IL18RAP, and

IFNG was persistent despite clinical improvement. Since IFNG production in innate-like lymphocyte populations such as NK cells is primarily induced by IL18 and IL12, a diminished IL18 receptor expression, with IL18RAP showing high specificity for NK cells, is a potential causal factor for the observed decreased IFNG blood levels in this analysis.<sup>25,27,28</sup> Of note, a reduced NK cell activity and a defective production of IFNG was reported by several studies of peripheral NK cells in AD.<sup>29–33</sup> Thus, my results gathered from blood transcriptomic data support the afore mentioned hypothesis of a deficient IFNG release by NK cell subsets contributing to the deviation of immune responses towards a type 2 pattern in AD.<sup>19–21</sup> While it remains to be elucidated by single-cell transcriptomic studies if either NK cell abundances are reduced in AD or if existing NK cells are dysfunctional or both, possible explanations for reduced blood NK cell signatures in AD are (1) NK cells are increasingly recruited from the periphery to the inflamed skin, or/and (2) NK cell deficiencies exist due to an impaired cell survival or a disturbed maturation or activation of NK cells in AD. Our findings of accumulated NK cells in the skin of AD support (1), whereas (2) needs to be examined by further functional studies. In conclusion, my analysis of NK cell related signatures revealed persistently accumulated NK cells in the skin of AD, as well as persistent patterns of diminished NK cell numbers or disturbed effector function in the blood of patients with AD after three months of systemic treatment with dupilumab despite clinical improvement.

In order to identify endotypes of patients with AD on the blood transcriptomic level, I performed a cluster analysis based on genes with hypervariable expression. I obtained two clusters that indicated an eosinophilic and a non-eosinophilic endotype, respectively. My analysis showed that the blood endotype profile has implications on AD activity as in the eosinophilic endotype, the eosinophilic signature positively correlated with AD skin scores measuring the severity of the disease. Therefore, those endotype profiles might be relevant for therapeutic decisions. With the growing number of patients recruited to the TREATgermany registry, the availability of biosamples will increase and future analysis will allow a symmetric and integrated analysis of intrapersonal skin and blood transcriptomic data. Further, with growing numbers of patients under systemic treatment, in-depth analyses exploring the responsiveness to systemic therapy will be possible, while the for this work available sample size was a limiting factor for robust therapy-reponse analyses. The inflammatory profile of AD is complex and diverse, and there will not be any one-fit-all therapeutic approach for this disease. Ongoing clinical trials of new targeted therapies in AD will also promote molecular research in AD with the aim to identify biomarkers and to define robust patient and drug endotypes, in order to accomplish the goals of personalised medicine.

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