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Molecular characterization of atopic dermatitis
a meta-analysis

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Molecular characterization of atopic dermatitis

- A meta-analysis

David Adrian Ewald

Kongens Lyngby 2016

Preface

This thesis is submitted as a requirement for obtaining a Ph.D. degree in Bioinformatics and Systems Biology from the Department of Systems Biology at the Technical University of Denmark (DTU Systems Biology).

Kongens Lyngby, July 22, 2016

David Adrian Ewald

Acknowledgements

Abstract

Atopic dermatitis is a common inflammatory skin disease, affecting 20-30% of children and 3-4% of adults. The disease has a big adverse impact on the patient's everyday life, caused by a variety of factors ranging from the esthetic aspects to the lack of sleep caused by nightly itch. The prevalence of atopic dermatitis is worldwide increasing, but treatment options for patients with moderate to severe disease are limited, and the molecular disease mechanisms are still under debate.

In this work I seek to define the molecular basis of atopic dermatitis by harvesting and combining publicly available and novel gene expression data, as well as evaluating disease models on the basis of these findings.

The thesis consists of two parts, where the first gives a general overview of the biological and computational aspects of my work, and the second presents my findings through three studies.

The first study is a microarray meta-analysis of publicly available gene expression data of lesional versus non-lesional atopic dermatitis skin samples. In this study I applied a random effects model to combine the effect sizes of the published studies, to estimate a combined effect size and to gain sufficient statistical power to detect even subtle changes in gene expression; this resulted in a robust disease transcriptome. The second study presents a more exhaustive approach, using a laser capture micro dissection technique to separate atopic dermatitis skin biopsies into their two main compartments (epidermis/dermis), with subsequent comparative transcriptomics analyses. We find that this approach is substantially more sensitive than analysis of full-thickness biopsies in terms of detecting compartment specific genes and generally low expressed genes.

The third study compares the global gene expression of six mouse models to my previous findings, to enable ranking of the models according to their molecular overlap with atopic dermatitis. We suggest that not one model is superior, but that the choice of model should depend on the disease aspects to be covered.

Altogether, the work in this thesis adds substantially to the molecular characterization and understanding of atopic dermatitis.

Dansk resumé

Atopisk dermatitis er en meget udbredt inflammatorisk hudlidelse, som rammer 20-30% af alle børn og 3-4% voksne. Lidelsen influerer negativt på patienternes hverdag, lige fra de æstetiske aspekter til kronisk søvnmangel pågrund af kløe. Prævalensen af atopisk dermatitis er steget på verdensplan gennem de sidste mange år, men behandlings mulighederne for moderat til svært ramte patienter er begrænsede, og sygdomsmekanismerne er stadig under videnskabelig debat.

Intentionen med denne afhandling er at definere den molekylære basis af atopisk dermatitis, ved at kombinere publicerede og nye ekspressions data, samt at evaluere eksisterende dyremodeller.

Afhandlingen består af to dele, hvor den første giver et overblik over den biologiske og bioinformatiske baggrund for mit arbejde, og den anden del præsenterer mine studier og artikler.

I det første studie har jeg gennemført en mikroarray meta-analyse af publicerede gen-ekspressions datasæt af afficeret of ikke-afficeret atopisk dermatitis hud biopsier. Her har jeg anvendt en "random effects model" for at kombinere datasættene, estimere deres kombinerede effekt størrelse, og for at øge den statistiske power for dermed at muliggøre påvisningen af meget små ændringer i gen-ekspressionen. Dette har resulteret i et robust transkriptom.

I det andet studie har vi anvendt laser-mikrodissektion for at separere hud biopsierne fra atopisk dermatitis patienter i de to hovedlag (epidermis/dermis), efterfulgt af komparativ ekspressionsanalyse. Denne fremgangsmåde er mere sensitiv end analyse af helhuds-biopsier, specielt med hensyn til lagspecifikke og generelt lavt udtrykte gener.

I det tredje studie evaluerer jeg gen-ekspressionen i seks muse-modeller i forhold til mine første resultater, for at rangordne modellerne i forhold til deres molekylære overlap med atopisk dermatitis. Vi finder i dette studie, at der ikke er én "perfekt" model, men at man burde vælge modellen efter de sygdomsaspekter man vil belyse.

Sammenlagt bidrager mit arbejde og denne afhandling væsentligt til den molekylære karakterisering og forståelse af atopisk dermatitis.

Contents

Abbreviations

Short	Long
ACD	Allergic Contact Dermatitis
AD	Atopic Dermatitis
AE	Atopic Eczema
ANOVA	Analysis Of Variance
APC	Antigen-Presenting Cells
CsA	Cyclosporin A
DC	Dendritic Cells
dDC	Dermal Dendritic Cells
DE	Differential Expression
DEG	Differentially Expressed Gene(s)
EDC	Epidermal Differentiation Complex
ES	Enrichment Score
FCH	Fold Change
FEM	Fixed Effects Model
GEO	Gene Expression Omnibus
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GSVA	Gene Set Variation Analysis
GWAS	Genome Wide Association Study
Ig	Immunoglobulin
IgE	Immunoglobulin E
ILC	Innate Lymphoid Cells
KEGG	Kyoto Encyclopedia of Genes And Genomes
LC	Langerhans Cells
LDA	Low-Density Array
LN	Lymph Nodes
logFCH	log2-Fold-Change

LS	Lesional
MIAME	Minimum Information About a Microarray Experiments
NGS	Next Generation Sequencing
NK	Natural Killer Cells
NL	Non-Lesional
NN	Normal (healthy control)
PCA	Principal Component Analysis
PRR	Pattern Recognition Receptors
PSO	Psoriasis
PUVA	Psoralen in Combination with Ultraviolet Radiation of Long Wavelengths
PVCA	Principal Component Variance Analysis
QC	Quality control
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction
REM	Random Effects Model
RNAseq	RNA Sequencing
SALT	Skin Associated Lymphoid Tissues
SCORAD	SCORing Atopic Dermatitis Index
TCR	T-Cell Antigen Receptor
TEWL	Transepidermal Water Loss
TLR	Toll-Like Receptor
UVB	Ultraviolet Radiation Of Short Wavelengths
WGCNA	Weighted Gene Co-Expression Analysis

Manuscripts

During my Ph.D. I performed a variety of analyses resulting in a number of papers and manuscripts. Three of these papers are enclosed for the evaluation of my thesis.

1. **D. A. Ewald**, D. Malajian, J. G. Krueger, C. T. Workman, T. Wang, S. Tian, T. Litman, E. Guttman-Yassky, and M. Suárez-Fariñas, Meta-analysis derived atopic dermatitis (MADAD) transcriptome defines a robust AD signature highlighting the involvement of atherosclerosis and lipid metabolism pathways, BMC Med. Genomics, vol. 8, no. 1, p. 60, 2015. doi:10.1186/s12920-015-0133-x

2. H. Esaki *, **D. A. Ewald ***, B. Ungar, M. Rozenblit, X. Zheng, H. Xu, Y. D. Estrada, X. Peng, H. Mitsui, T. Litman, M. Suárez-Fariñas, J. G. Krueger, and E. Guttman-Yassky, Identification of novel immune and barrier genes in atopic dermatitis by means of laser capture microdissection., J. Allergy Clin. Immunol., vol. 135, no. 1, pp. 153 63, Jan. 2015. doi:10.1016/j.jaci.2014.10.037

3. **D. A. Ewald ***, S. Noda * , O. Margeaux, T. Litman , S. Nakajima, X. Li , H. Xu , C.T. Workman, P. Scheipers, N. Svitacheva, T. Labuda, J.G. Krueger, M. Suárez-Fariñas, K. Kabashima, E. Guttman-Yassky (Second Revision July 2016). Major differences between human atopic dermatitis and murine models as determined by global transcriptomic profiling. Journal of Allergy and Clinical Immunology, Manuscript Number: JACI-D-16-00667R1

*These authors contributed equally to the respective study.

Not included in this work:

1. M. Su rez-Fari as, B. Ungar, J. Correa da Rosa, **D. A. Ewald**, M. Rozenblit, J. Gonzalez, H. Xu, X. Zheng, X. Peng, Y. D. Estrada, S. R. Dillon, J. G. Krueger, and E. Guttman-Yassky, RNA sequencing atopic dermatitis transcriptome profiling provides insights into novel disease mechanisms with potential therapeutic implications, J. Allergy Clin. Immunol., vol. 135, no. 5, pp. 1218 1227, May 2015. doi:10.1016/j.jaci.2015.03.003

CHAPTER 1

Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease, which has been attributed to a complex interplay of skin barrier disruption and abnormal immune responsiveness. The main clinical feature, from a patient's point of view, is severe pruritus (itch), which has been reported to have a significant impact on the patient s quality of life, especially negatively affecting sleep. The prevalence of AD has increased steadily over the past decades. Therefore the patient individual disease impact might soon grow to a general economic problem, when work efficiency and quality is lowered due to, for instance, lack of good sleep. The ongoing (and increasing) efforts to understand the disease mechanisms therefore make great sense not only from a scientific and patient centric point of view, but also in a larger economic or socioeconomic perspective.

In the following I will introduce various aspects of the disease starting with a general overview of the affected organ (the skin) as well as methods to unravel the molecular nature of the disease, like high-throughput laboratory and computational methods.

1.1   The Human Skin

The human skin is the largest organ of the human organism, spanning 1.5-2 m^2 with a total weight of up to 10 kg. Generally the human skin separates the inside of the human body from the outside environment. It has a wide and diverse variety of functions, ranging from protecting the body from loss of liquid, over sensory, communication, and metabolic functions, to providing an efficient immune barrier to keep the outside world out of the body. [1, 2, 3, 4]
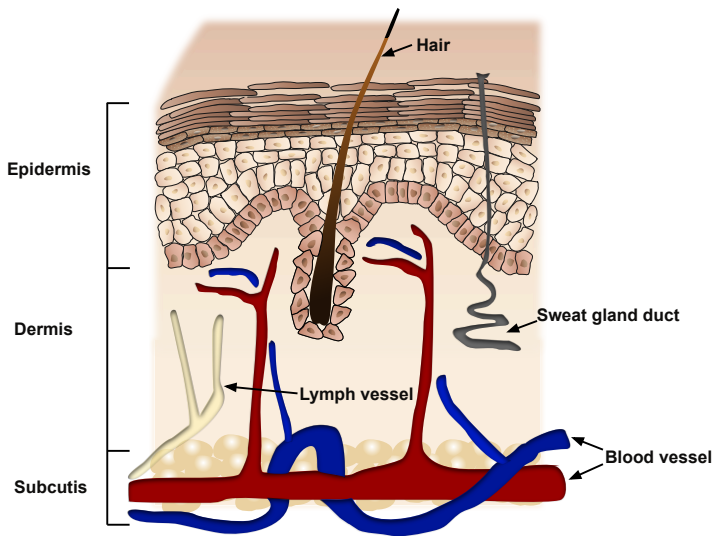
Figure 1.1: Conceptual overview of the human skin.

Overall the human skin consist of three main layers (see Figure 1.1):

- **Epidermis**, the outer barrier layer

- **Dermis**, or Corium, the middle layer

- **Subcutis**, the innermost (or under-skin) layer

Together, the dermis and epidermis are often referred to as the skin or cutis.

The **epidermis** consists of mainly keratinocytes, lipids, a few melanocytes, Langerhans and Merkel cells. Its most prominent function is to protect the human body from loss of liquid, and environmental intruders; it keeps the inside in, and the outside out. [5, 6, 2, 4, 7, 8]
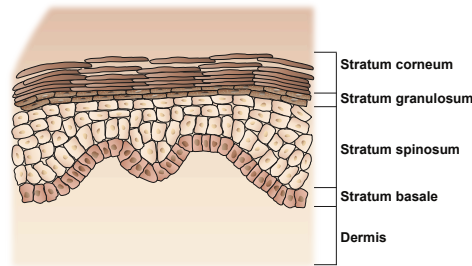
Figure 1.2: Conceptual overview of the epidermal layers.

The keratinocytes in the epidermis are generally organized in four layers (from innermost to outermost), the stratum basale (the single cell basal layer), the stratum spinosum (the middle layer), the stratum granulosum (the corneocyte precursor layer), and the stratum corneum (the corneocyte layer). [2, 4] Figure 1.2 gives a conceptual overview of the epidermal layers.

The basal layer (**stratum basale**) consists of a single layer of keratinocytes, which steadily grow and reproduce to migrate into the prickle cell layer (**stratum spinosum**). This stratum spinosum consist of 8-10 layers of keratinocytes, with small thorn like outgrows (spinosus) connecting the cells to maintain structural stability of the epidermis. The granular layer (**stratum granulosum**) consists of 3-5 layers of keratinocytes, which gradually lose their nuclei and secrete lipids, while migrating to the outermost horny or cornified layer (**stratum corneum**). The stratum corneum consists of 25-30 layers of keratinocytes that have undergone full differentiation (cornification), i.e. lost their nuclei and are filled with keratin to become flattened corneocytes. These corneocyte layers are organized in a cellular matrix with lipids in lamellar bilayers in-between the cells, providing amongst other functions an efficient hydrophobic seal. [9, 10, 11, 2, 3, 8]

Within this main epidermal structure, the bone marrow-derived antigen-presenting Langerhans cells constitute a first line of defense; the Merkel cells are driving the out-

most sensory function of the skin; while the Melanocytes synthesize melanin, defining the skin color and protecting the basal keratinocytes nuclei from UV radiation. [12, 13, 2, 14, 15]
Some inflammatory diseases like atopic dermatitis or psoriasis show a noticeably thicker epidermis, caused by keratinocyte hyperproliferation. [16, 17]

The **dermis** or **corium** is the comparably thick skin layer right underneath the epidermis. It consists mainly of a connective tissue matrix, fibroblasts, blood and lymph vessels, sensory nerve endings, sweat glands, hair follicles, and immune cells. The main function of the dermis is to generally structure the skin allowing for efficient response to injuries, and providing nutrition and anchoring to the epidermis. [16, 2, 3]

The dermis is composed of two main layers:

- the **papillary dermis**, which with its wave like structure is in close contact with the epidermis.

- the **reticular dermis**, which is the larger, though less tightly structured, sub-layer.

The **papillary dermis** with its wave like structure reaches into the epidermis. It consists of small collections of compact connective tissue fibrils and fibers, and a considerably large amount of fast-proliferating fibroblastic cells. This connective tissue matrix contains a high density of nerve endings and tiny blood vessels amongst other functions supporting nutrient transport to the epidermis. [16, 2, 3]

The **reticular dermis** structure is defined by much larger fibrils and fibers organized in big woven bundles, with increasing size towards the subcutis. This structure adds the flexibility to the skin, which defines its ability to respond to mechanical stress by temporary deformation. This layer of the dermis contains less densely packed larger blood vessels and lymph channels, sweat glands and hair follicles.

The main cell type in the dermis is the fibroblast. These cells are responsible for producing connective tissue proteins, and respond to certain immune mediators. [18, 19, 20] Other prominent dermal cell types are the immune system related monocytes, macrophages and dermal dendrocytes. [21, 13, 16] The **subcutis**, or **hypodermis**, right below the reticular dermis is a less structured adipose tissue-rich layer, which

serves the human body as buffer zone to protect against otherwise harmful impact and temperatures. The adipose tissue of this deepest layer of the skin also stores energy by accumulating lipids in fat cells. [16, 2, 22, 3]

1.1.1   Lipids and The Barrier Function of The Skin

The main barrier functions of the skin are regulation of transepidermal water loss (TEWL), protection from environmental intruders (e.g. ultraviolet light, chemicals, and harmful microorganisms), and mechanical insults; it keeps the inside in, and the outside out. If it wasn't for the sophisticated interplay between the corneocytes and the various lipids in the **stratum corneum**, we would not even be capable of living on dry land or in further consequence living at all.[23, 24, 25]

The **stratum corneum** is comprised of mainly *corneocytes* and *lipid bilayers* (lamellar bilayers/membranes). Its structure is often referred to as the *bricks and mortar* model (associating it to a solid stone wall), where the bricks are the corneocytes and the mortar are the *lipid bilayers.*

The major protein component of these lipid-corneocyte layers is *keratin*, which is expressed extensively during the last differentiation stage of the *keratinocytes. Keratins* add stability and elasticity to the **stratum corneum**, by the formation of larger keratin filaments, a process which is mainly driven by filaggrin (FLG, a filament associated protein of the S100 family). [26, 25]

The majority of **stratum corneum** extracellular lipid precursors are metabolized in *keratinocytes*, where they, upon increased cellular differentiation, accumulate in the *lamellar bodies* together with certain lipid post-processing enzymes. These oval shaped *lamellar bodies*, which are bilayer membrane organelles, form increasingly in the **stratum granulosum** followed by secretion into extracellular space in the upper **stratum granulosum** and **stratum corneum**. Proper formation of the *lamellar bodies* has been shown to depend on the ABCA12 transporter protein (involved in lipid homeostasis). [27, 24] Secretion of the lipid precursors and enzymes takes place by fusion of the *lamellar bodies* with the keratinocyte cell membranes. This secretory process is significantly influenced by changes in calcium concentration; a reduction in calcium concentration in the **stratum granulosum** triggers the secretory process. [23, 10, 28, 24, 29, 30] Upon secretion the precursor lipids are post-processed into the three major groups of final extracellular barrier lipids: ceramides, free fatty acids, and cholesterols. This conversion takes place in extracellular space mainly by co-secreted

enzymes, where β-glucocerebrosidase and sphingomyelinase produce ceramides from glucosylceramides and sphingomyelin respectively, and phospholipase releases free fatty acids from phospholipids. The optimal enzymatic activity of this post-processing depends on an acidic pH ($\sim$5) of the stratum corneum. [28, 24, 6, 26, 30]

As the final **stratum corneum** lipids organized in *lipid bilayers* comprise a major component of the physical barrier function of the epidermis, changes in their composition and proportion have been linked to certain conditions with an altered barrier function. Atopic dermatitis, for example, shows a changed ceramide profile as compared to normal, where an increased amount of short-chain ceramides has been linked to an altered barrier function. [23, 31, 32] This shift in the **epidermal ceramide** population has been linked to an increased expression of IFN-γ, which in turn led to a decreased expression of elongation-of-very-long-chain-fatty-acids proteins (ELOVLs) and ceramide synthases (CerS), resulting in a further decreased level of ceramides with long-chain fatty acids, which might be the mechanism underlying barrier alteration in atopic dermatitis and psoriasis. [32, 33]

In addition to the obvious physical barrier support, some free fatty acids in the upper epidermis show robust antimicrobial activity (e.g. linoleic acid), together with antimicrobial peptides (like cathelicidins, e.g. LL37; and defensins, e.g. hBD1-4) and an acidic pH ($\sim$5) contributing significantly to the non-specific (innate) immune system of the epidermis. [5, 34, 35]

1.1.2 Immunity of The Skin

The human skin protects the body from harmful intruders by a complex interplay between the physical barrier, the innate and the adaptive immune system. [36, 37, 38, 39] The main functionality of the skin immune system is tightly linked to the functional compositions and locations of its compartments.

The **epidermis** is the very first line of defense, both by the physical barrier of the **stratum corneum**, the secreted antimicrobial substances, and the recruitment of circulating immune cells. The main immune system related cells in the **epidermis** are the keratinocytes, Langerhans cells, and CD8^{+} T cells. The **dermis** due to its incorporated blood and lymph vessels is key in transport, but also recruitment of circulating immune cells. It is populated by a wider variety of immune cells, including CD4^{+} T cells, dermal dendritic cells, mast cells, and mononuclear phagocytes. [36, 37, 40, 38] Figure 1.3 gives a conceptual overview of the immune cells of the
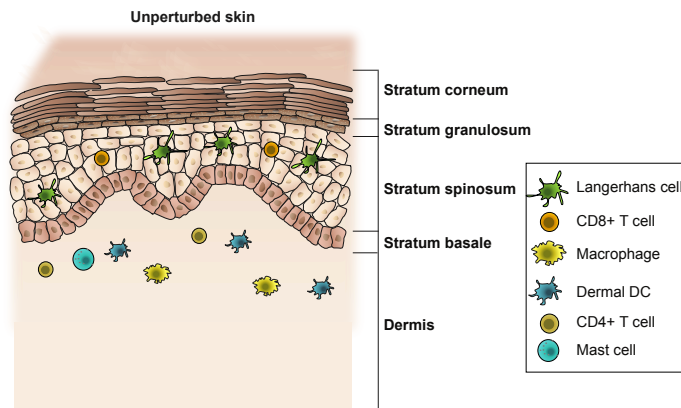
Figure 1.3: Conceptual sketch of the main immune cells of the unperturbed skin.

healthy skin.

Apart from the physical barrier of the skin, its **innate (non-specific) immune system** defends the organism by detecting potentially dangerous intruders with a variety of pattern recognition receptors (PRR), which recognize molecular structures evolutionary conserved or shared by the majority common pathogens. In the recent decades the toll-like receptors (TLR) have become the most well known class of PRRs in the innate immune system. [36, 41, 42, 43]

Important cells of the **innate immune system** are, monocytes/macrophages, neutrophils, eosinophils, basophils, mast cells, natural killer (NK) cells, and the keratinocytes.

The **monocytes/macrophages** belong to the group of phagocytes. They engulf and degrade intruders, and release antimicrobial enzymes. Upon immune response phagocytes produce numerous cytokines, like TNF-α, IL-1, IL-6, IL-8, IL-10 and IL-12, triggering adaptive immune system reactions. Furthermore the survival and activity of monocytes/macrophages can be controlled or influenced by CD4$^+$ T cells. [21, 36, 38]

The **neutrophils** are short-lived (2-3 days) circulating granulocytes, which invade inflamed skin, where they phagocytose and kill bacteria. Neutrophils are attracted to sites of infection predominantly by the cytokines produced in activated macrophages. [44, 38]

The **eosinophils**, like the neutrophils, also belong to the granulocytes, but are tar-

geting parasite worms (like helminths). Their production is stimulated by Th2 cell cytokines (like IL-3, IL-5, GM-CSF), while their proliferation is mainly driven by CCL-11 (Eotaxin-1) and CCL-5 (RANTES). The key cytokines produced and secreted by eosinophils are IL-12, IL-13, IL-16, TGF-b1, promoting a switch from Th2 to Th1 immune response. Increased amounts of epidermal eosinophils are correlated with AD disease severity, and eosinophil products might be used as blood and urine markers of disease severity and treatment response. [45, 46, 47]

The **basophils** are another group of granulocytes, circulating in the blood, and migrating to sites of inflammation, secreting both cytotoxic and immune regulating substances. Activation of the FcϵRI, a high affinity surface receptor for immunoglobulin E (IgE), results in secretion of IL-4 (causing differentiation of macrophages), and histamines. All this links basophils to allergic or inflammatory skin diseases such as AD. [48, 49, 38]

The **mast cells** are resident granulocytes of the dermis in unperturbed skin. They share key features, like the expression of FcϵRI receptor for IgE, with basophils, and are also involved in the immediate immune response by secretion of granule content (e.g. histamine and tryptase) and cytokines (e.g. IL-1, IL-2, IL-5, IL-6, IL-8, IL-9, IL-13, and TNF) production and secretion upon activation. Mast cells have been linked to AD, where stimulation with the cytokine thymic stromal lymphopoietin (TSLP) may play an important role. [49, 38, 50]

The **natural killer (NK) cells** are a group of lymphocytes contributing to the innate immune system (innate lymphoid cells/ILC) by their cytotoxic and cytokine producing abilities. NK cells recognize altered cells, and, upon activation, contribute to regulation of the adaptive immune response. Activation of NK cells is mediated by certain cytokines (e.g. IL-2, IL-4, IL-12, IL-18, IL-23), which in turn leads to the secretion of cytokines like TNF-α, TGF-β, IFN-γ, IL-5, IL-10, IL-13, IL-22. NK cells can be attracted to the site of infection by mast cells. Especially the IL-22 producing NK cell subset (NK-22) have been proposed to play a role in AD, but this group has been recently proposed to be classified as ILC3 and not the ILC1 to which the NK cells belong.[36, 51, 52, 38, 53]

The **keratinocytes** are, as outlined in the previous sections, main contributors to the physical barrier via their proliferation and the secretion of barrier function promoting lipids and proteins. Apart from these important barrier functions, keratinocytes contribute to the innate immune functions of the skin, as they produce various inflammatory, activating, inhibiting and modulating cytokines (e.g. IL-1, IL-6, IL-7, IL-10,

IL-12, IL-15, IL-18, IL-23, TGF-β, TNF-α, GM-CSF). Keratinocytes are involved in T-cell activation, inhibition, and modulation; in Langerhans cell modulation and general leukocyte trafficking. [36, 5, 43]

The **adaptive (specific) immune system** is the slower responding but more specific ("smarter") part of the overall immune system. In the skin the adaptive immune system is mainly comprised of a group of *antigen-presenting cells (APC)*, which present antigens to the group of lymphocytes, thus triggering a specific response targeting a particular foreign intruder.

The **adaptive immune cells** are mainly comprised of the antigen presenting dermal dendritic and Langerhans cells, as well as the lymphocyte B- and T-cell groups.

The **Langerhans cells (LC)** are the main players of initiating an adaptive immune response in the skin. LCs are an antigen-presenting subset of dendritic cells (DC) resident in the epidermis, which upon perturbation of the skin emigrate into the lymphatic system to stimulate and recruit naïve T-cells to the site of infection, via the MHCII surface complex and secretory cytokines. Furthermore LCs secrete chemokines (like CCR-2 and CCR-6) to attract more LCs, upon perturbation. LCs attach to keratinocytes by E-cadherin mediated adhesion, and are distinguishable from the majority (but not the small subset of Langerin$^+$ DCs) of other resident skin cells by the expression of the Ca2$^+$-dependent lectin Langerin (CD-207) and their tennis rack shaped Birbeck granules. Langerhans cells have been described to be overrepresented in atopic dermatitis lesional skin as compared to normal skin. [36, 12, 54, 43]

The **dermal dendritic cells (dDC)** are resident APCs of the dermis. These APCs differ from the LCs by the absence of the Birbeck granules. dDCs can be subdivided into two major classes based on several cell surface markers (Cluster of Differentiation Genes/CDs), but the main classes are the dDC Langerin+ and the dDC Langerin-, where the Langerin expressing dDCs have been described relatively recently, and are considered the smaller subset. It should be noted that Langerin expression in the past has been linked to LCs only. [55] Generally dDCs function comparable to, but quicker than, LCs in the sense, that they, upon perturbation of the skin, migrate to the lymph nodes (LN) and activate T-cells via the MHCII surface complex and secretory cytokines. [36, 56, 57]

The **B-cells** are lymphocytes that play a major role in the humoral immune response, preventing the spread of infections through specific antibody production. Generally

B-cells differentiate into plasma cells upon antigen encounter, essentially resulting in the secretion of immunoglobulins (Ig) and subsequent neutralization of intruders. These B-lymphocytes have been linked to allergies and inflammatory skin diseases like atopic dermatitis via Ig secretion. [58, 38] A recent study by Czarnowicki and co-workers in 2016, showed an increase in B-cell count in AD versus psoriasis patients and uninvolved skin and blood samples. [59]

B-cells are found mainly in the dermis upon infection, but recent studies have suggested the presence of very few B-cells also in healthy skin. The exact mechanism of B-cell migration to the site of infection is not completely understood, but certain ligand-receptor pairs (CCL-3, CCL-4, and CCL-5 with CCR-5; CCL-20 with CCR-6; CXCL-10 and CXCL-11 with CXCR-3) have been suggested to play a role in B-cell homing and migration. Furthermore several adhesion molecules (ICAM-1, VCAM-1, CD-47) have been proposed to be involved in the transmigration of B-cells. [36, 58, 37, 38]

The **T-cells** are the group of lymphocytes that are involved in the specific immune response. In an early development stage they leave the bone marrow as progenitor cells, and undergo maturation in the thymus, after which they relocate to the secondary lymphoid tissue where they reside in a mature naïve state.

The naïve T-cells are activated by antigen presenting DCs to differentiate into effector T-cells, that help to fight foreign intruders. Antigen recognition by T-cells happens through their T-cell antigen receptor (TCR), a heterodimer surface protein complex encoded by genes with a highly variable region, which enables antigen specific recognition. The TCR heterodimer consists of either $\alpha\beta$ - or $\gamma\delta$ chains, with the $\alpha\beta$ T-cells having the antigen binding properties, whereas the $\gamma\delta$ T-cells can directly bind pathogenic glycoproteins. The $\alpha\beta$ T lymphocytes are the predominant type of T-cells in the skin. Two main lineages of these naïve $\alpha\beta$ T-cells are important in the skin, the $CD4^+CD8^-$ (CD4 T-cells) and the $CD4^-CD8^+$, (CD8 T-cells) differing in their antigen recognition capabilities. [36, 60, 61, 38, 62]

Activated naïve T-cells proliferate and differentiate to effector T-cells in the secondary lymphoid tissue, and are subsequently attracted to the site of inflammation by chemokines (e.g. CCL-17 and CCL-22 with CCR-4 and CCL-27 with CCR-10). Upon pathogen clearance some of the T-cells differentiate into memory T-cells. Of these memory T-cells the effector memory T-cells are of special importance for the
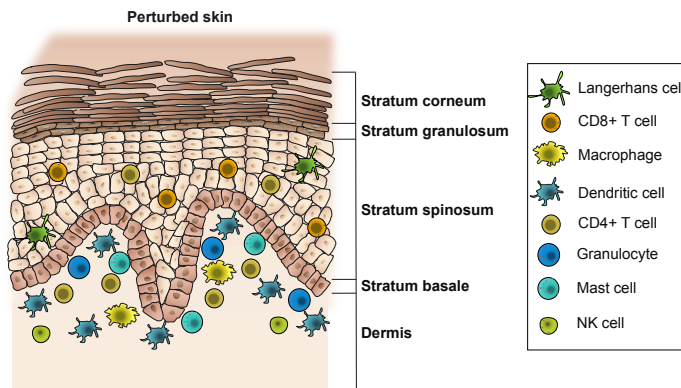
Figure 1.4: Conceptual sketch of the main immune cells of the inflamed skin.

skin, as they home to the both the dermis and epidermis to provide immediate protection against pathogens. The predominant effector memory T-cells in the dermis are of the CD4$^+$CD8$^-$ phenotype, whereas those with the CD4$^-$CD8$^+$ phenotype reside mainly in the epidermis. [63, 60, 37, 61] Figure 1.4 gives a conceptual overview of the most important immune cells present in the skin upon inflammation.

The CD4 and CD8 T-cells can be further subdivided into subsets according to their cytokine and transcription factor expression. Most prominent in the context of inflammatory skin diseases are the *Th1, Th2, Th17* and *Th22* subsets of the CD4 lineage (or correspondingly the *Tc1, Tc2, Tc17,* and *Tc22* for CD8).

Th1 cells express IFNγ to activate macrophages, dendritic cells, CD8 T-cells and NK cells for fighting intracellular intruders. [64, 61, 65]

Th2 cells are characterized by IL-4 expression; they are mainly involved in extracellular pathogen response, and have been linked to inflammatory skin diseases like atopic dermatitis. [66, 67, 61, 68]

Th17 cells are characterized by IL-17 expression; they are involved in the protection against extracellular intruders like fungi and bacteria, and show a major contribution in the pathogenesis of psoriasis. [69, 70, 61]

The *Th22 cells* have been recently characterized to express IL-22 and TNF-α but no IL-17; they are involved in promoting the production of antimicrobial peptides, and are linked to inflammatory skin diseases like psoriasis and atopic dermatitis via the IL-22 expression. [71, 72, 73, 74]

1.1.2.1 Skin Associated Lymphoid Tissues (SALT)

In the early eighties a general model of the skin immunity, termed Skin Associated Lymphoid Tissues (SALT), was proposed by Dr. Streilein [75]. The main statement in the proposal is that the components of the immune system acting in the skin, are unique to the skin, and comprise the unique skin immune system SALT. The original SALT hypothesis was formulated in four parts: SALT is comprised of (1) a specialized set of antigen presenting cells within the epidermis, Langerhans cells; (2) distinctive populations of recirculating T lymphocytes that preferentially infiltrate the skin, especially the epidermis; (3) keratinocytes that provide an anatomically distinct environment for these lymphoreticular cells and secrete into that environment immune-regulatory molecules that can profoundly affect the immune recognition and differentiation; and (4) a set of draining peripheral lymph nodes, integrating this multicellular system, that contain, along with the dermis, blood vessels with endothelial cells whose surfaces capture lymphocytes passing through the blood. [75]
The main model of a specialized skin immune system holds true and has been conceptually generally accepted in the field, even though with a few factual updates, e.g. APCs including dDCs. [76]

1.1.3 Common Inflammatory Skin Conditions

As the skin is one of the largest organs and the outer most layer of defense of the human organism, it is under constant attack by the outside (and to some degree inside) world, and therefore vulnerable to a variety of diseases. Many of the common skin diseases are linked to harmful intruders, barrier dysfunction and autoimmune reactions. In the following I will introduce some of the most common inflammatory skin conditions, namely **Allergic Contact Dermatitis (ACD), Hand Eczema, Pruritus, Psoriasis, Atopic Dermatitis**.

Allergic Contact Dermatitis describes immune reactions of the skin caused by direct contact to substances, and is classified as a delayed-type hypersensitivity.
Common allergens causing **ACD** are fragrance, rubber, and metals (especially nickel). These allergens are, upon entry into the skin, mistakenly recognized as harmful by the immune system and an inflammatory immune response is triggered, if the skin has been in contact with the substances before (i.e. sensitized). The clinical manifestation and symptoms of the disease range from smaller red spots on the skin to

severe itchy rashes and blisters. [77] Generally, ACD has been considered a Th1/Th2 polarized inflammatory disease. Recent studies have defined the distinct molecular profiles linked to the various allergen dependent ACD subtypes, suggesting a pronounced Th1/Th17 and Th22 component in nickel ACD, and a predominant Th2 polarization in rubber and fragrance ACD. [78, 72, 77, 79] Furthermore, recently a pilot study suggested the regulatory importance of certain micoRNAs in ACD. [80] The most common treatment options for ACD are removal of the irritant, topical corticosteroid creams or ointments, and in server cases systemic corticosteroids.

Hand Eczema is an inflammatory skin disease found on all parts of the hands, from the wrist to the fingertips. The severity of the disease ranges from reddish spots on the affected areas of the skin, to itching blisters, scaly areas and even fissurae. This non-contagious condition is not trivial to diagnose, as it often co-occurs with bacterial or fungal infections, and due to its clinical similarity to inflammatory skin conditions like psoriasis and atopic dermatitis. [81, 82] Recent findings suggest the atopic dermatitis as the single most common risk factor for hand eczema, [83] while a link to filaggrin (FLG) mutations has been proposed for chronic appearance of the disease. [84]
The current treatment options for hand eczema are numerous, ranging from topical treatments by emollients, calcineurin inhibitors, and steroids; over physical therapies by UVB or PUVA; to systemic treatments with mainly corticosteroids, ciclosporine, or methotrexate. [85, 81]

Pruritus or itch is the most common symptom on the skin, usually appearing due to dermatological (e.g. psoriasis, atopic dermatitis, or fungal infections) or systemic conditions (e.g. hepatitis, asthma, food, drugs, cancer), but has also been attributed to psychogenic factors. It is commonly classified into three subgroups 1) on diseased skin, 2) on non-diseased skin, 3) chronic. [86, 87] Even though the exact pathophysiological mechanisms underlying this distressing symptom are yet to be established, it has been linked to histamine, serotonin (5-HT), acetylcholine, prostaglandins, and mechanical factors like heat and trans epidermal water loss (TEWL). [88] Pruritus is increased at night (nocturnal pruritus), which has been attributed especially to heat, TEWL, and low level of naturally occurring systemic corticosteroids. [89]
Common treatment options of pruritus, apart from treating the causative dermatologic or systemic condition, span a wide variety of topical and systemic substances,

like topically applied corticosteroids, antihistamines, menthol, capsaicin, local anes-
thetics, salicylic acid, and cannabinoids; or the systemically applied antihistamines,
antidepressants or opioid agonists/antagonists. [86, 90]

Psoriasis (PSO) is a chronic inflammatory autoimmune disease of the skin. It ap-
pears most commonly on the elbows, knees, hands and scalp, but can also affect many
other parts of the skin and in severe cases nearly the whole body surface. The clini-
cal manifestation is the formation of inflamed, scaly, itching red lesions (or plaques),
which are a direct result of the interplay between a T-cell and dendritic-cell driven au-
toimmune response and epidermal keratinocyte hyper-proliferation. PSO is currently
the best-understood inflammatory skin disease, and is regarded to be a disease with
pronounced T17 and Th1, and some Th22 involvement, i.e. involving IL-17, IFN-γ
and IL-22 producing lymphocytes respectively. The general disease model states that
the release of the cytokine IL-23 by DCs triggers the activation of T17, Th1, and Th22
cells, which in turn produce/release the afore mentioned specific cytokines, resulting
in increased keratinocyte activation and subsequently hyperproliferation. [91, 69, 74]
Common classic therapies include both topical and systemic treatments. In most
cases, topical treatment with the vitamin D derivative calcipotriol, the glucocorticoid
betamethason, or a combination of the two yields good therapeutic results. In se-
vere cases the use of systemic immunosuppressants (like methotrexate or retinoids,
including the calcineurin inhibitor cyclosporine) or physical therapies like UVB or
PUVA treatment are recommended. Generally, moisturizing skin care should be con-
ducted routinely. Current research is focusing on targeting the IL-23/T17 involving
pathways. [91, 69, 74, 92]

1.1.3.1   Atopic Dermatitis

Atopic Dermatitis (AD) or atopic eczema (AE) is the most common chronic in-
flammatory skin disease, with an approximate prevalence of 15-25% in children and
3-4% in adults, and a marked increase in lifetime prevalence over the last decades.
AD has been linked to other atopic diseases like hay fever and asthma, as many
children affected by AD later in life develop either of these atopic diseases; which
is referred to as "the atopic march". The clinical manifestations and symptoms of
the disease are red, inflamed and itchy skin, and blisters with oozing and crusting.
It is common that parts of the skin, where no inflammation has occurred recently,

are dry and rough. Even though rashes may occur anywhere on the skin, the most common areas for adults are the inside of elbows and knees, whereas in children the rashes predominantly occur on the face, hands and feet. For AD currently no specific pathognomonic biomarkers exist. [93, 94, 95]

AD has been linked to both a contribution of immune cells as well as epidermal barrier defects. The main genetic risk factor has been attributed to various mutations in the FLG gene, which encodes filaggrin, a protein responsible for epidermal structure that also serves as a precursor for the natural moisturizing factor (NMF). Note however, that only approximately 20% of the AD patients carry FLG mutations, and that up to 60% of the carriers will not develop atopic dermatitis. [17, 96, 97] In addition to the FLG mutation it is well accepted that multiple other dysfunctions of the epidermal barrier play an important role in the disease; among the most prominent are epidermal hyper-proliferation (i.e. marked thickening and change of structure of the epidermis) and change of the epidermal lipid composition. [98, 32] Apart from, or in addition to, the barrier defects, AD is known to be an inflammatory disease linked to immune dysregulation, with a marked Th2/Th22 polarization and some Th17 contribution.

It is still under discussion which factor is the main driver of the disease, the barrier dysfunction ("outside-in" model) or the immune abnormalities ("inside-out" model). This is a "chicken or the egg causality dilemma". The true answer is difficult to determine due to the multi-factorial nature of the disease, and most likely it is the complex interplay of these two main aspects, where one is dependent on the other, and one can trigger the other. [54, 99, 100, 95] The most common treatment options for atopic dermatitis are topical and systemic corticosteroids, systemic immunosuppressants (e.g. the calcineurin inhibitors cyclosporine), moisturizing skin care, and physical therapies like UVB or PUVA treatment. [99, 93, 101]

Atopic dermatitis is, as briefly reviewed above, a skin disease driven by a complex interplay between skin barrier disruption and abnormal immune responsiveness. In the recent decade, great effort has been put into unraveling the disease mechanisms, by detecting possible **genetic risk factors** and investigating its **transcriptome** to describe and understand the **barrier and immune aspects** of the disease.

It has long been known that parental inheritance is one important risk factor of AD.

This has been attributed to both epigenetic and genetic factors, which led to multiple genome wide association studies (GWAS) of AD, investigating possible genetic causes of the disease. [102, 103, 104, 101] The most prominent genetic risk factor for AD is a variety of mutations in the FLG gene, located within the epidermal differentiation complex (EDC) on chromosome 1 (1p.21), where multiple other important barrier function genes are also located (e.g. S100 s, FLG2, IVL, LOR). [105, 106, 107, 108] Other genetic risk factors have since been described by large consortium efforts and GWAS meta-analyses that found significant disease association with SNPs within close proximity to epidermal differentiation and proliferation genes and genes within the cytokine cluster and proposed a number of new AD risk loci. [109, 110, 111]

Another important field of studies is the characterization of the **AD transcriptome**, i.e. quantifying mRNA levels to reveal which genes are differentially expressed in biopsies of diseased tissue (lesional/LS) versus non-diseased or normal control tissue (non-lesional/NL, normal/NN).
Early transcriptomics studies of AD revealed up-regulation of key immune related genes like CCL18 in LS versus both NL and NN skin, down-regulation of important barrier genes (FLG, LCE2B, LOR, CDSN) in LS versus NN skin, and increased expression of the water channel AQP3. [112, 113] A later study of LS versus NL epidermis (obtained by epidermal shave) from AD patients, confirmed some of the previous immune and barrier related findings, and suggested an inverse correlation between the expression of Th2 related biomarkers to barrier genes CLDNs 1 and 23 (i.e. down-regulation of the barrier genes is correlated with up-regulation of Th2 related genes). [114] Further work highlighted the progressive activation of the Th2/Th22 axis of the disease from the acute to the chronic state. [73] Some of the early transcriptional findings led to investigations of the transcriptional effects of classical AD treatments, namely UVB phototherapy and betamethasone, in LS skin. [115, 116] Tintle and co-workers (2011) showed, that UVB phototherapy significantly reverses the transcriptional levels from LS to those of NL skin; especially immune genes (amongst others the Th2-associated products CCL13, CCL18, CCL26 and IL10) appeared to be down-regulated after UVB treatment. [116] In line with these results, betamethasone, a potent anti-inflammatory glucocorticoid, appeared to significantly reduce mRNA levels in LS AD samples to NL state, besides immune genes, noticeably recovering FLG and LOR expression levels. Recent studies investigated the transcriptional effects of other AD treatment options, including the broad immunosuppressant cyclosporine A

(CsA) and the IL4R antagonist dupilumab. [117, 118, 119, 99, 120] CsA significantly improved the clinical manifestation of AD, and recovered especially activated inflammatory pathways to NL levels. [119] The more specific immune modulator dupilumab (a monoclonal antibody), which prevents IL4 and IL13 signaling by binding to the alpha subunit of the interleukin-4 receptor, significantly improved the molecular signature of LS AD samples, especially down-regulation of the important Th2 biomarker CCL17 points towards the importance of the Th2 axis of the disease. [117, 118]

In the context of detecting transcriptional treatment responses, obviously it is important to posses a distinct set of molecular biomarkers and to understand the molecular basis of the disease subsets. Important efforts have been made on investigating and defining AD specific biomarkers; a noteworthy study by Suárez-Fariñas and co-workers in 2011 described the expressional difference between uninvolved samples from AD patients (NL) and normal control samples (NN), defining the "molecular scar" of AD. [121] This study therefore suggests that the treatment effect in AD may be defined as the normalization towards the untreated NL expression levels, rather than those of normal control samples.

Recently, a disease classifier on the basis of the expression levels of two genes (NOS2 and CCL27) has been established to distinguish between psoriasis and atopic dermatitis. Interestingly, even-though this study included only patients affected by both PSO and AD, to rule out the high interindividual variability, the researchers were able to reproduce commonly accepted aspects of each diseases transcription profile, when contrasting disease specific samples of each patient. [122]

Typically transcription studies in AD are carried out on patients with moderate to severe disease (i.e. SCORing Atopic Dermatitis [SCORAD] $\geqslant$ 25). The most recent thorough study in the field by Martel and co-workers (2016), focused on the important group of mildly affected patients versus normal controls (SCORAD < 25) with distinction between intrinsic and extrinsic patients (i.e. patients with low versus high serum IgE levels), and in comparison to PSO. [123, 124] Overall, this study found that differential expression of genes involved in inflammation of moderate-severe AD was also detected in mild AD, but most barrier related DEGs found in moderate-severe AD did not appear in mildly affected patients. Furthermore the intrinsic type of AD had a greater overlap of DEGs with PSO than the extrinsic type. [123] Other interesting work by Rodríguez (2014) aimed at linking the gene expression in AD skin samples to corresponding epigenetics analysis. [125] In this study it was observed that over-expression of AD related S100s (S100A2, A7, A8, A9, and S100A7A) in LS samples,

correlated with DNA hypermethylation of a single CpG site within S100A5. Furthermore, overexpression of KRT6A and KRT6B correlated with decreased methylation of a single CpG site in KRT6A. [125] The findings of this integrated experimental design are very promising in order to more thoroughly elucidating the molecular basis of AD, and should be established as a standard method not only in basic research but also in clinical trials.

Both the transcriptomics and GWAS analyses introduced above have, apart from the immune related factors, provided support for the established **barrier disruption elements of AD** on the molecular level. [23, 24, 126, 127] While much of the genetics focus has been on FLG mutations, expression analysis identified dysregulation of a broader set of barrier and cornification genes (e.g. FLG, IVL, CDSN, and LOR). [128, 107, 96] Recently, the Bouwstra lab in the Netherlands pointed out the altered lipid composition and organization in AD and the destructive effects on the epidermal barrier function. [32, 129, 130] The researchers showed that especially the distribution of free fatty acids (FFA) and ceramides (CER), the main class of lipids in the stratum corneum, is shifted to the short-chained subgroups in lesional skin. [32, 130] These findings are in line with the general observations of an impaired barrier in AD. [126, 131]

Historically, and at least since the identification of IgE and the corresponding Fc-ϵ receptor in the 1970s, AD has been considered an inflammatory skin disease with marked **immune aspects**. [132, 46, 133, 134] A recent major milestone in the characterization of the **immune aspects of AD** was the detection of IL22$^+$ T cells (Th22), and their overrepresentation in inflamed skin. [71] The importance of the Th22 cells in AD was further established by transcriptional studies, showing a progressive activation of the Th2/Th22 axis from the acute to the chronic state of the disease. [72, 73] As is the case for the mutations in FLG linked to barrier defects, which are only found in approximately 20% of the AD patients, the immune aspects of the disease are not unified neither. Therefore, the disease is commonly stratified with respect to the serum IgE levels, where **intrinsic AD** patients are defined as those with no specific or at least very low levels of IgE for common environmental food allergens, while **extrinsic AD** patients are those with high corresponding IgE levels. [100] A recent flow cytometric analysis of a set of intrinsic/extrinsic stratified AD blood samples highlighted the importance of this IgE based stratification when investigating T

cell populations. [135]

The overall model of the immune aspects of AD is still under discussion, especially in the context of the barrier dysfunction of the disease. But the current model defines the two IgE based disease subtypes (intrinsic/extrinsic), and states that AD is a disease with a marked Th2/Th22 component. [136, 95]

The complexity and the multi-factorial nature of AD, pose a great challenge to the development of **disease models** for studying treatment effects and mechanisms. The most common model organisms for studying aspects of AD are murine rodents. Generally three main types of murine disease models exist: **1) Induced models**, where the mice are epicutaneously (EC) sensitized to allergens like ovalbumin (OVA) or oxazolone (OXA) ; **2) Transgenic mutant models**, where certain supposedly disease related genes like FLG are manipulated; **3) Spontaneous mutant models** that spontaneously develop mutations resulting in a disease-like phenotype. [137, 138] Even though many of the murine models, as for example the OXA sensitized, [139] are currently used in drug-development, one of the to date most promising models is the spontaneous NC/Nga mutant. [140, 141]

1.1.4   Atopic Dermatitis Expression Datasets

In this work I aim at describing the transcriptional aspects of atopic dermatitis, by
the expression analysis pipeline and meta-analytical model described in the following
sections, making use of publicly available and in-house expression data.

Table 1.1.4 presents publicly available AD related expression data sets, from both the
NCBI Gene Expression Omnibus (GEO) and the EMBL-EBI ArrayExpress repos-
itories. These datasets comprise a wide variety of experimental and technological
setups, including lesional (LS), non-lesional (NL), and healthy control (NN) samples
from simple disease assessment studies, compartment specific and treatment studies.
[142, 143]

Overall, 10 studies include both LS and NL samples, 13 studies include LS and NN
samples, and 4 studies include all three tissue types. Of the LS+NL studies five
where carried out on the *Affymetrix Human Genome U133 Plus 2.0 Array* (GPL570),
where one (GSE27887) was re-analyzed data from a previous study (GSE32924). Two
of the LS+NL studies (E-MTAB-729, GSE60709, GSE5667) where carried out on
different chip types, *Illumina HumanHT-12 v3.0 Expression BeadChip* (A-MEXP-
1171,GPL6947), *Affymetrix Human Genome U133A/B Array* (GPL96/GPL97) re-
spectively. The remaining three LS+NL where either epidermis only (GSE60709) or
from co-affected AD+PSO patients (GSE57225).

Seven studies investigated the expression levels in various cell specific setups (GSE13709,
GSE22611, GSE27533, GSE20706, GSE48310, GSE38039, GSE48586).

One recent study (GSE75890) carried out on the *Affymetrix Human Gene 2.1 ST Ar-
ray* (GPL17692), investigating AD lesions from extrinsic and intrinsic patients versus
NN and psoriasis samples.

   In the following I will introduce a set of bioinformatics tools that can be applied
to unravel the transcriptomic nature of both human and murine skin samples, in the
context of disease understanding and molecular marker definition.

Author	Year	GSE/eMTAB	Sample size	LS	NL	NN	Platform	PMID
Beck[†]	2014	GSE59294	23	16	7	0	GPL570	25482871
Khattri[†]	2014	GSE58558	35	18	17	0	GPL570	24786238
Gittler[†]	2012	GSE36842	29	7	7	15	GPL570	22951056
Suárez-Fariñas[†]	2011	GSE32924	33	13	12	8	GPL570	21388663
Han	2012	GSE35582	3	2	0	1	GPL9052	22564738
Rebane	2012	E-MTAB-728	6	3	0	3	A-MEXP-1171	22445417
De Benedetto	2011	GSE26952	12	5	0	7	GPL2700	21163515
Winge	2011	E-MTAB-768	20	15	0	5	A-AFFY-141	22164253
Guttman-Yassky	2009	GSE16161	18	9	0	9	GPL570	20004782
Olsson	2006	GSE6012	20	10	0	10	GPL96	16918518
Rebane	2012	E-MTAB-729	10	3	3	4	A-MEXP-1171	22445417
Plager	2010	GSE5667	17	6	6	5	GPL96, GPL97	17181634
Tintle	2011	GSE27887	17	9	8	0	GPL570	21762976
Rodríguez	2014	GSE60709	33	12	7	14	GPL6947	24739813
Jensen	2012	GSE32473	30	30			GPL570	22142306
de Jongh	2005	GSE6601	6	6	0	0	GPL4695	16354186
Yoshikawa	2013	GSE13709	48				GPL7687, GPL7688	23385231
Billmann-Born	2011	GSE22611	27				GPL570	21335489
Gutowska-Owsiak	2012	GSE27533	6				GPL10558	
Hirakawa	2011	GSE20706	9	6	3	0	GPL6480	21411736
Chisaguano	2013	GSE48310	124	20		104	GPL17344	
Cohen	2012	GSE38039	6				GPL6244	22936986
Titova	2013	GSE48586	12				GPL13938	
Quaranta	2014	GSE57225	62	23	17		GPL14550	25009230
Dhingra	2014	GSE60028	47				GPL570	24768652
Wong	2012	GSE32245	54				GPL9258, GPL9551	22235269
Rettew	2013	E-MTAB-62	5372				A-AFFY-33	23552467
Martel *	2016	GSE75890	22	14		8	GPL17692	26841714

Table 1.1: Overview of publicly available atopic dermatitis related expression datasets, sorted by relevance for this work, and publication date. (* added after preparation of meta-analysis ; † included in meta-analysis.).

1.2   Gene Expression Analysis

Gene expression analysis essentially describes the technologies capable of quantifying the expression of genes. This is commonly achieved by measuring the levels of mRNA transcribed from the template DNA in the tissue of interest. When a global gene expression analysis is performed - i.e. when one measures all (or at least: the known) mRNAs - this is called transcriptomics, quantifying the transcriptome. [144, 145, 146] Of the various technologies, available for gene expression analysis, the most common are quantitative reverse transcription polymerase chain reaction (qRT-PCR), microarrays ("chips"), and RNA sequencing (RNAseq).

qRT-PCR is based on three steps, 1) reverse transcription of mRNA to cDNA, 2) amplification of the cDNA by PCR, 3) quantification of the PCR products at each thermal cycle (each cycle comprising a denaturation, an annealing, and an elongation step). This technology is considered the most sensitive of the three, but is not of high-throughput nature, even-though it can be applied in semi-high-throughput manner by low-density arrays (LDAs). [147, 148, 149]

The **microarray** technology is a true, and the first, high-throughput technology for measuring gene expression. It is based on probes, sequence fragments that match their target genes, which are fixed (spotted) in a matrix like pattern on a glass chip. Essentially fluorescently labeled mRNA or cDNA fragments are added onto that microarray chip, and if hybridization between a pre-fixed probe and its corresponding gene fragment has happened, this will result in fluorescence signals upon laser excitation. That way, the analysis of the expression levels of many thousands of genes can be parallelized. [150, 151, 152, 153, 146]

The most recent technology, **RNAseq**, is based on reverse-transcription and subsequent high-throughput sequencing by a next generation sequencing (NGS) technique. The main difference compared to the qRT-PCR and microarrays is, that in the best cases, the only prior knowledge necessary about the investigated transcriptome, is the reference genome of the organism. [154, 155, 156, 157, 158]

Historically, it has been possible to measures single gene expression for a couple of decades, using manual and cumbersome northern blotting, but the main breakthrough in transcriptomics, however came with the introduction of standardized commercialized microarrays in the 1990's, which enabled researchers to measure gene transcription in a high-throughput manner. The commercialization and standardiza-

tion of the microarray chips, along with the introduction of public expression data
repositories (especially the NCBI Gene Expression Omnibus[GEO] and the EMBL-
EBI ArrayExpress) and the *Minimum Information About a Microarray Experiment*
(MIAME) reporting standard, have made it possible to reuse and combine expression
data from different research groups. [142, 144, 143] The reuse of publicly available
data has greatly contributed to research transparency (an important matter in the
times of high-throughput data analysis), and has also led to great new findings in
"old" data. [144] Correspondingly the combination of publicly available data on the
same topic from different research groups, can lead to increased statistical power, and
thereby enable the detection of more subtle changes in expression profiles of different
tissues or conditions. [159, 144, 160]

In this work I focus on evaluating the transcriptional profile of atopic dermatitis,
making use of in-house and publicly available data. Therefore I will here introduce
the various technological and bioinformatical aspects of microarray expression analy-
sis, including relevant down-stream analysis methods.

1.2.1   Microarray Expression Analysis

The essence of the microarray technology, as outlined above, is the fixation of comple-
mentary known sequences (probes or features) on a surface ("chip"), with subsequent
annealing of the fluorescently labeled target sequences (e.g. reverse transcribed mR-
NA/cDNA), and finally, fluorescence excitation and signal detection. [151, 161, 162]
Various adaptations of the microarray technology exist, primarily varying in the pro-
duction method, the probe design, and the labeling of the target sequences. The main
differentiations in the **production methods**, whether the probes are spotted onto
the surface, or synthesized directly on the surface; with the latter being the preferred
method today. [163, 146] Proper **design of the probes** is the key element in this
technology, where the primary difference is the sequence length (usually 25-60 bases)
and the thermodynamically optimal probe selection (with respect to the hybridiza-
tion behaviour); but also other fundamental design properties of the chip, defining if
it is an array measuring e.g. gene expression, with special features like splice junction
detection. In order to increase the specificity and sensitivity it is common practice,
that a single gene is covered by multiple probes (located on different areas of the
chip), which span different regions on that gene. This collection of probes is referred

to as a **probe-set**. [164, 146, 165]
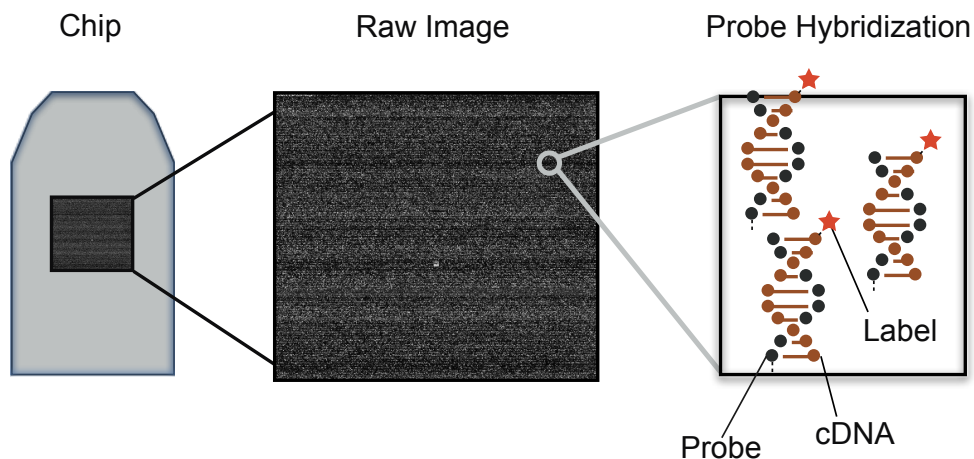
Figure 1.5: Sketch of the microarray technology.

Finally, another interesting distinction is the choice of **fluorescence labeling** and hybridization, where competitive hybridization and two-color labeling versus single hybridization and single-color labeling have been commonly applied. For **two-color labeling**, two samples, between which an expression contrast is to be measured, are prepared in parallel being labeled with distinct fluorescence dyes (usually Cy3 and Cy5, resulting in different wavelength/colors), and hybridized on to the same chip. The measure of differential expression in this case, is the direct intensity ratio between the two detected colors, for each probe-set. **One-color labeling** is carried out with one chip per sample using only one fluorescence dye. Figure 1.5 gives a simple conceptual overview of the single-color labeling microarray technology, from chip, over raw image, to hybridized probes. [151, 163, 166, 146]

In this work I focus on one-color arrays from *Affymetrix Inc.*, mainly the human expression array *Human Genome U133 Plus 2.0* (HGU133-plus2). The measured fluorescence signals (*array image*) of these chip types essentially result in one intensity data file (*CEL file*) per sample, which is the starting point for computational expression analysis pipelines, including summarization, normalization, differential expression analysis, and subsequent downstream-analysis. [167, 168, 169, 170, 171, 172]

1.2.2   Quality Control, Background Adjustment, Normalization and Summarization

Microarray expression data generation involves many, often manual, preparation steps, ranging from sampling, processing and mRNA extraction over labeling and hybridization to scanning, analysis and interpretation. At all these processing and anlysis steps both **random unsystematic variation** (noise) and/or systematic **batch effects** can be introduced. Naturally the actual non-biological sources of variation can be manifold, and should be carefully accounted for in an analysis pipeline. [173, 170, 174] An overview of the microarray data analysis pipeline applied in this work is given in figure 1.6.

Various methods and software packages for microarray data **quality control** (QC) and assessment exist, but I suggest, to always first take a quick look at the log-scaled array images. [175] Visual inspection of the array images often easily reveals regional bias on a chip (spatial artifacts), which is often biased target-probe hybridization, possibly caused by dust, air bubbles, non-uniform washing, temperature or liquid flow rate. These spatial artifacts can also be systematically detected and adjusted for by the *Harshlight/R* package proposed by Suárez-Fariñas and co-workers in 2005. [176, 177]
Other diagnostic QC plots in this context are: [178, 173]

- Sample-wise **boxplots** of the log-intensity distributions.

- **MA-plots** of the gene expression change (M) versus the average gene expression (A). For single color arrays expression change of one array is often contrasted against the gene-wise medians for all chips in the experiment.

- **Principal Component Analysis** (PCA) plots of the first few principal components, in order to get a glance on possible outliers, or systematic biases.

The actual preprocessing pipeline of microarray data includes, 1) **background correction**, 2) **between arrays normalization** and 3) **probe level summarization**. **Background correction** is a crucial first step towards proper data analysis; it essentially estimates and removes backgrounds noise to enable better overall sensitivity. The commonly applied algorithms for doing so (RMA and GCRMA) are implemented in ways, which keep the background-adjusted signals positive and in GCRMA even
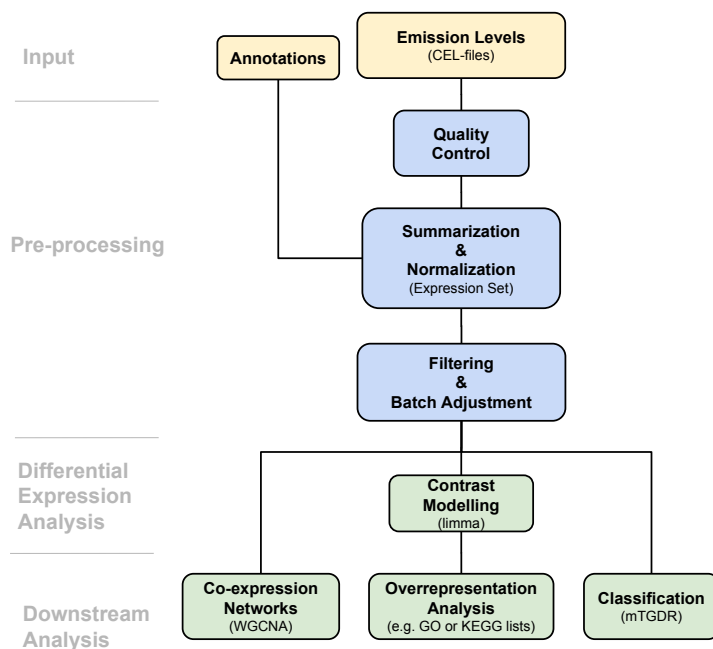
Figure 1.6: Overview of the microarray data analysis pipeline applied in the context
of this work.

take into account probe sequence information. [179] **Between arrays normaliza-
tion** is carried out to bring together the distributions of the background-adjusted
expression values of all arrays in an experiment. The most common normalization
method to achieve this is the quantile normalization, implemented in the RMA and
GCRMA algorithms. Generally, the normalization methods assume that only a very
small proportion of the genes are significantly different between the investigated con-
ditions. The final preprocessing step is **probe level summarization**, which in
essence is combining the intensity measures of single probes into predefined groups
(probe-sets). The three preprocessing steps are commonly implemented together in
a single software package (e.g. MAS5.0, RMA, GCRMA, fRMA), varying slightly in

the different steps. [180, 181, 179]

1.2.3 Controlling Batch Effects

When comparing microarray expression data of the same type but from different batches, systematic non-biological variations between the batches (batch-effects) are frequently observed. The batches and subsequently the batch-effects arise mainly due to differences in preparation or run-time, location (e.g. laboratory), or responsible technician. Adjustment for these batch-effects is an important, if not to say mandatory, step in the microarray data-analysis pipeline. [182, 169] Multiple algorithms to perform batch-effect adjustment have been proposed, differing in the underlying statistical methods. The most common methods are essentially based on either support vector machines (SVM), mean-centering by gene-wise analysis of variance, singular value decomposition, scaling by the group-wise geometric mean, or gene-wise empirical Bayes location and scale adjustment. [182, 169, 183]

These systematic batch-effects are most commonly accessed by PCA or principal component variance analysis (PVCA) of the normalized and summarized expression data. [184, 182, 185]

Chen and co-workers in 2011 found, that the empirical Bayes method implemented in the **ComBat** algorithm performed best in terms of sensitivity, specificity, and overall batch-effect adjustment. [182, 186] Essentially ComBat is a location (mean) and scale (variance) batch adjustment (L/S method), where the location and scale parameters for each batch are estimated using an empirical base method, that basically assumes batch-effects to be comparable between probe-sets, and therefore (for each batch) "lends" information between probe-sets. [186] ComBat is part of the R/Bioconductor package SVA. [187, 188, 189]

1.2.4 Differential Expression Analysis

In this work I applied microarray expression analysis to quantify differential gene expression between groups of skin samples, mainly human lesional (LS) versus non-lesional (NL) atopic dermatitis samples.

Differential expression (DE) between two groups of samples is usually reported by an effect size and a significance measure for each gene. Effect size in this context is defined as the gene-wise (or probe-set-wise) mean difference between the prepro-

cessed log2-transformed intensities of the two groups, denoted the log2-Fold-Change (logFCH). The significance measure is most commonly based on a t-statistic (or often a moderated t-statistic) for calculating a P-value using the Student's t-distribution. These P-values are commonly adjusted for multiple testing by the False Discovery Rate (FDR) procedure proposed by Benjamini and Hochberg to account for the many genes tested in parallel.[150, 190, 191, 192]

Differential expression analysis can be carried out using various statistical methods, ranging from simple t-tests, over analysis of variance (ANOVA), to more advanced linear models with shrinkage methods (as implemented in the R/Bioconductor *limma* package). [191, 193, 171] It has been shown that the linear model implementation in the *limma* package performs superior, especially for small sample sizes, due to the build in ability to "borrow" information across genes or probe-sets in order to shrink the observed variance. [191]

Historically, in dermatological expression analyses experiments, differentially expressed genes (DEGs) have been defined with the arbitrary cut-offs fold-change (FCH) $\geqslant 2$ and adjusted p-value $\leqslant 0.05$. For comparability with the published data I herein use the same definitions, but it should be noted, that these cut-offs are arbitrary and not dynamic with respect to the study specific sample size.

1.2.5   Meta-Analysis

The microarray technologies have evolved and been widely used for differential expression analysis throughout the past decade(s). This has led to the establishment of public expression databases (especially the NCBI Gene Expression Omnibus/GEO and the EMBL-EBI ArrayExpress), which have enabled the research community to compare and combine expression datasets from different laboratories and batches. [142, 194, 144, 143]

Strikingly, even-though investigating comparable RNA samples on the same platform, the results often show a pronounced laboratory effect, which limits direct comparison. [194, 195, 196, 197] The laboratory effects are manifold, and the batch-effect adjustment methods described in the previous sections are often insufficient to correct for this. Therefore, meta-analysis methods have been established, to combine the expression data of different laboratories after laboratory-wise differential expression analysis has been carried out in a uniform annotation and pre-processing pipeline. This enables the generation of more robust sets of differentially expressed genes, which serve

as a solid starting point for disease classification and various downstream analyses. [198, 199, 200, 195]

In the following sections I will give an overview of common microarray meta-analytical methods, and thoroughly introduce the Random Effects Model (REM), applied in this work.

1.2.5.1   Overview of Models and Algorithms

Three main types of microarray meta-analysis methods exist, 1) **Combining P-values**, 2) **Combining rank statistics**, and 3) **Combining effects**.

A variety of meta-analysis methods combining p-values exist. The most classic, and oldest, of this class of methods was proposed by Sir Ronald Aylmer Fisher in 1925, and is based on summing the log-transformed p-values (P) of each study (k):

$$\chi^2_{2k} = -2 \sum_{i=1}^{k} log(P_i) \tag{1.1}$$

Based on Fishers first introduction of this concept, more suitable methods have been developed specifically for combining microarray datasets. [199, 160] Song et al. recently suggested the *rth Ordered P-value* method (rOP) for combining p-values of multiple microarray studies, by testing whether a gene or probe-set is significantly differentially expressed in the majority (but not all) of the studies, making use of a single ordered p-value. [201] This method has been further improved by Li and Gosh, combining all p-values by adding a weighting that corresponds to their respective order. [202]

Another approach of defining a consensus set of differentially expressed genes across multiple microarray datasets is **combining rank statistics**. The most prominent method of this class is the *rank product method* (RankProd), initially introduced by Breitling et al. (2004). [203] *RankProd* is based on simply combining the gene or probe-set wise ranks (in terms of DE evidence) of the included datasets as their products, and is most suitable for detection of consensus DE genes or probe-sets if DE in at least one of the included datasets. [199, 204]

Especially the p-values methods are both powerful and suitable for a robust combination of multiple microarray studies. However, in the biological context it is often preferable to gain information of the effect size, i.e. a quantification of the contrast

between two groups (logFCH). This is possible when datasets with two distinct conditions are investigated, in our context LS versus NL skin samples. To gain information of the gene or probe-set wise effect sizes across datasets, meta-analysis methods **combining effects sizes** have been developed. Two main methods for doing so are generally applied, namely the *Random Effects Model* (REM) and the *Fixed Effects Model* (FEM); where the later is a special case of the REM assuming no difference other than sampling error between the datasets. [200] In essence the REM estimates the consensus effect sizes for each gene or probe-set making use of DerSimonian and Liards point estimate of the between study variance. [200, 205, 206]

As is the case for the rOP, the REM is well suited for detection of differentially expressed genes or probe-sets if DE in the majority of the included datasets. [199] An in-depth introduction of the algorithm and statistical basis of the random effects model is given in the following.

1.2.5.2 The Random Effects Model

In this work I applied the Random Effects Model proposed by Choi et al. (2003) to estimate probe-set wise consensus effect sizes for atopic dermatitis microarray studies of lesional and non-lesional full-thickness skin biopsies.

Choi and co-workers define the observed effect size (y_{ji}) and the overall mean effect size (μ_j) for each probe-set ($j = 1, 2, ..., l$) and study ($i = 1, 2, ..., k$) as:

$$
\begin{aligned}
y_{ji} &= \theta_{ji} + \epsilon_{ji}, & \epsilon_{ji} &\sim N(0, s_{ji}^2) & (1.2) \\
\theta_{ji} &= \mu_j + \delta_{ji}, & \delta_{ji} &\sim N(0, \tau_j^2), & (1.3)
\end{aligned}
$$

with the intra-study variance s_{ji}^2 and the inter-study (between study) variance τ_j^2. In this model each observed mean effects size y_{ji} is defined by the study and probe-set specific mean difference θ_{ji} and its corresponding variance s_{ji}^2, where θ_{ji} is considered to be drawn from a super-population with mean μ_j and variance τ_j^2 . I estimate this overall mean effect size μ_j ,with the definitions established by Cooper and Hedges, and Choi and co-workers: [200, 207]

$$
\hat{\mu}_j(\tau_j^2) = \frac{\sum\limits_{i=1,..,k} (s_{ji}^2 + \tau_j^2)^{-1} y_{ji}}{\sum\limits_{i=1,..,k} (s_{ji}^2 + \tau_j^2)^{-1}}, \tag{1.4}
$$

with its corresponding variance estimate:

$$Var(\hat{\mu}_j(\tau_j^2)) = \frac{1}{\sum\limits_{i=1,..,k}(s_{ji}^2 + \tau_j^2)^{-1}}, \tag{1.5}$$

based on the DerSimonian-Liard (DL) point estimate of τ_j^2, calculated as a methods of moment estimator making use of the expected value for the Q-statistic (Q_j) introduced by Cochran in 1954: [208, 205]

$$\hat{\tau}_j^2 = max\left\{0, \frac{Q - (k - 1)}{S_{j,1} - (S_{j,2}/S_{j,1})}\right\}, \tag{1.6}$$

where $S_{(j,r} = \sum\limits_{i=1,...,k} w_{ji}^r$ and $w_{ji} = s_{ji}^{-2}$, and the Q-statistic following Cochran's definition:

$$Q_j = \sum\limits_{i=1,..,k} w_{ji}(y_{ji} - \hat{\mu}_j), \tag{1.7}$$

ignoring the between study variance for the weighted least squares estimator $\hat{\mu}_j = \sum\limits_{i=1,..,k}(w_{ji}y_{ji})\sum\limits_{i=1,..,k} w_{ji}$. [200, 208]

In order to make this algorithm easily accessible in a pipeline based fashion, e.g. for use with *Biopython* packages, I implemented a *pandas* and *numpy* based version of it in a Python (www.python.org) package, which will be submitted to the *Biopython* consortium. [209, 210]

1.2.6   Downstream Analysis

Differential expression analyses and as in this work, subsequent meta-analyses, generally produce lists of gene wise contrasts and corresponding significance measures between two tissue types. An initial assessment of these lists is the ranking by effect sizes and/or significance measures, followed by a qualitative investigation of the interesting, i.e. most significantly respectively over- or under-expressed, genes. As this approach, even-though thorough, is tedious for the up to thousands of interesting genes, great effort has been put into the development of methods for systematic and informed grouping of the expression analysis results. Generally, these methods work on either the pre-processed sample wise expression values or the lists of differentially expressed genes (DEGs). In the following I will give a conceptual overview

of overrepresentation, gene set enrichment, co-expression networks and classification methods. **Overrepresentation analyses** and **gene set enrichment methods** are commonly applied on the final list of DEGs and essentially determine whether certain predefined sets of interesting genes are significantly overrepresented or enriched in the DEGs list; **co-expression network analyses** take the pre-processed expression values as input, and aim at defining groups of genes, that show similar expression patterns across all samples in one tissue type; and **classification algorithms** work on pre-processed expression values to select a small number of genes that stably distinguish between two tissue types (e.g. lesional and non-lesional, or diseased and healthy). [211, 212, 213, 214, 215, 216]

1.2.6.1 Overrepresentation Analyses

Overrepresentation analysis in its most simple form seeks to determine whether a set of genes is significantly overrepresented in a list of DEGs. The sets of interesting genes (gene-sets) grouped by biological functional features of interest can be obtained from public sources like the Gene Ontology (GO) consortium, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the comprehensive Molecular Signatures Database (MSigDB). [217, 218, 219]

A classic approach to test for significant overlap between certain gene-sets and a list of DEGs, is to make use of **Fisher's exact test** or the **hypergeometric test** with a 2x2 (contingency) table. [220] This type of approach, even-though beautifully simple, is limited by being based on the list of DEGs, which is defined by arbitrary effect size and significance cut-offs (often absolute logFCH$\geqslant$1 and adjusted P-value$\leqslant$0.05); hence with the natural risk of not taking into account smaller but maybe still biologically important changes in gene expression.

To overcome these limitations, methods considering the contrasts of all measured gene expressions have been developed. In this work I applied the **Gene Set Variation Analysis** (GSVA) algorithm, a Single-Sample based extension to the popular Gene Set Enrichment Analysis (GSEA) method. [221, 215]

GSEA takes the ranked list of genes from an expression analysis as input, and basically determines 1) whether a gene-set is significantly overrepresented in this ranked list and 2) at which end (top or bottom) of the list it is overrepresented. GSEA presents the level of enrichment and its corresponding significance by an enrichment score (ES), which is based on an adapted Kolmogorov-Smirnov test statistic. [220, 215] The

limitations of this method are obviously that it is based on the final contrasts between all samples of two tissue types, not taking into account possible sample wise variations. This is of great interest in the context of this work, as I am investigating the contrasts between LS and NL AD skin samples, which are of paired nature, enabling potential paired testing.

Due to this limitation I applied the *GSVA* method, which essentially transforms a set of gene or probe-set wise expression values, into a set of gene-set wise pseudo expression values (GSVA scores). So, each sample gets a GSVA score for each gene-set of interest, resulting in an expression set based on GSVA scores rather than individual gene or probe-set expression values. Because of this samples wise approach GSVA is considered a single sample (SS) gene-set enrichment method, which can be readily fit into a differential expression analysis based on e.g. *limma*, enabling the investigation of differential expression of gene-sets between two tissue types.

1.2.6.2   Weighted Gene Co-Expression Analysis

Co-expression analysis is a method of analyzing gene expression values, which seeks to simplify the large sets of gene expressions by grouping genes with similar expression values across samples into modules of co-expressed genes. Furthermore, co-expression analysis provides a gene-wise measure of connectivity, for each module defining highly connected genes as hub genes. By defining modules and subsequent hub genes, co-expression analysis reduces the dimensions of a set of expression values, and enables us to efficiently systematically investigate these large datasets even if only one tissue type is available (i.e. differential expression analysis is not possible to be carried out). The core of gene co-expression analysis is the definition of pairwise correlations between gene profiles across samples. This is commonly carried out by calculating the absolute value of Pearson's correlation between two genes, defined as sij = abs(cor(xi,xj)) for each gene i and j across all samples. [222, 223]

An extension of the basic gene co-expression analysis is implemented in the popular **Weighted Gene Co-Expression Analysis** (WGCNA) *R* package. [212] Essentially the *WGCNA* algorithm introduces a weighting, which favors strong correlations and penalizes weak ones, thus making it more robust with respect to the often noisy nature of the initial microarray expression data. The weighted networks produced by WGCNA, are defined by the weighted adjacency matrix:

$$A_{ij} = (|cor(x_i, x_j)|)^{\beta}, \tag{1.8}$$

with the power parameter $\beta \leqslant 1$. In the WGCNA pipeline, these adjacency matrices are finally subject to clustering, to define modules of co-expressed genes, which can be brought into a biological context for instance by the simple gene set enrichment methods introduced in the previous section. [222, 212, 224]

1.2.6.3   Classification by Gene Expression

One of the ultimate goals of investigative dermatology and translational biomedicine in the context of AD research is to evaluate the effect of treatments. On a molecular level the most intuitive treatment evaluation is to investigate whether the expression profiles of skin biopsies from lesional areas are reverted to those of non-lesional areas. As outlined in the previous atopic dermatitis section, the reason for comparing to non-lesional as opposed to normal skin samples is the fact that there is a striking molecular difference (some researchers in the field consider it a "molecular scar") between these two tissue types. [121]

In the context of treatment effect evaluation there are multiple sub-goals; researchers could for instance be interested in being able to predict the outcome of a trial after a shorter period of time than common practice, e.g. predicting the effect of a drug after 2 instead of 12 weeks. In this work I applied an approach that is a step towards this more complex treatment response setup, as I here simply aimed at defining an unbiased distinction (classification) between LS and NL AD samples.

Classification algorithms to achieve these distinctions have been around for a long time, and been applied on expression data (especially in cancer research) over the past decades. These algorithms have become more and more complex in the recent years. [225, 226]

The core concept of an expression value based classifier is, that given a set of expression values for samples of a known class (in this case LS or NL), select enough features (genes or probe-sets) to stably distinguish between the classes. The classifier is commonly build on a subset of the known expression set, and then tested on the remaining subset that was not used to build the classifier, to get an insight into performance in terms of sensitivity and specificity.

Among the more common (and relatively simple) classification algorithms are the linear (*linear discriminant analysis* [LDA], and *nearest centroid*) and the non-linear (*k nearest neighbor* [kNN], *artificial neural network* [ANN] and *support vector machine* [SVM]). [227, 228]

Here I applied the Multi Threshold Gradient Decent Regularization (multi-TGDR) algorithm, an extension of the meta-TGDR algorithm specifically developed for microarray meta-analysis datasets, proposed by Tian and Suárez-Fariñas in 2013. [229, 230]

CHAPTER 2

Manuscripts

2.1 A Robust Atopic Dermatitis Transcriptome

2.1.1 Prelude

As the prevalence of the inflammatory skin disease atopic dermatitis (AD) has been increasing over past decades, dermatological and biomedical research has focused on unraveling the molecular basis of the disease.

The introduction of high-throughput screening methods like cDNA microarrays has enabled the researchers to rapidly screen the expression levels of thousands of genes in parallel. Recently, focus has been on defining the relative expressions between lesional (LS) and non-lesional (NL) biopsies from AD patients. Many journals have established data reporting standards, which require the research to deposit high-throughput data of this nature in publicly available databases like the NCBI Gene Expression Omnibus (GEO) or the EMBL-EBI ArrayExpress, making it possible to mine and re-analyze these data.

In this paper I carried out a systematic review of publicly available microarray expression data of AD LS and NL samples. I pre-processed the resulting datasets with a uniform pre-processing pipeline, carried out individual differential expression analyses, and combined the results (effect sizes) by means of the meta-analytical random effects model (REM).

This study essentially resulted in a robust AD transcriptome, which was able to identify 86 differentially expressed genes that were not discovered by the individual included studies. This AD transcriptome furthermore highlighted the lipid abnormalities and Th2 activation of the disease.

Meta-analysis derived atopic dermatitis (MADAD) transcriptome defines a robust AD signature highlighting the involvement of atherosclerosis and lipid metabolism pathways

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Abstract

Background: Atopic dermatitis (AD) is a common inflammatory skin disease with limited treatment options. Several microarray experiments have been conducted on lesional/LS and non-lesional/NL AD skin to develop a genomic disease phenotype. Although these experiments have shed light on disease pathology, inter-study comparisons reveal large differences in resulting sets of differentially expressed genes (DEGs), limiting the utility of direct comparisons across studies.

Methods: We carried out a meta-analysis combining 4 published AD datasets to define a robust disease profile, termed meta-analysis derived AD (MADAD) transcriptome.

Results: This transcriptome enriches key AD pathways more than the individual studies, and associates AD with novel pathways, such as atherosclerosis signaling (IL-37, selectin E/SELE). We identified wide lipid abnormalities and, for the first time in vivo, correlated Th2 immune activation with downregulation of key epidermal lipids (FA2H, FAR2, ELOVL3), emphasizing the role of cytokines on the barrier disruption in AD. Key AD "classifier genes" discriminate lesional from nonlesional skin, and may evaluate therapeutic responses.

Conclusions: Our meta-analysis provides novel and powerful insights into AD disease pathology, and reinforces the concept of AD as a systemic disease.

Keywords: Atopic dermatitis, Meta-analysis, Transcriptome, Atherosclerosis, Expression analysis

Background

Atopic dermatitis (AD) is the most common inflammatory skin disease (4–7 % prevalence in adults, and ~15 % in children), with a large unmet need for safer and more effective treatments [1–7]. Immune and barrier abnormalities characterize AD, with Th2/Th22 cytokine activation, increased hyperplasia and significant decreases in differentiation markers. These observations have led to two competing pathogenic hypotheses [1, 8, 9], although recent studies

characterizing AD primarily as an immune-driven disease have shown reversal of barrier defects following specific and non-specific therapeutic interventions [10–13].

Genomic expression profiling using gene-arrays and real time (RT)-PCR has been widely used to identify gene alterations in lesional (LS) and non-lesional (NL) AD compared to normal skin to better understand interactions between activation of cytokine pathways and epidermal abnormalities [6, 7, 10, 12, 14, 15]. Similar to other diseases, the AD phenotype/transcriptome can be defined genomically by differentially expressed genes (DEGs) between LS and NL skin [15]. A robust transcriptome was established as a powerful tool in identifying core psoriasis pathogenesis and evaluating the efficacy of targeted therapeutics at transcriptomic level

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[16, 17]. The high rates of placebo effect in AD patients contrasts with a worsening of disease phenotype at the transcriptomic level [10], reinforcing the importance of a robust disease transcriptome against which therapeutic effects can be evaluated [18]. Genomic profiling may also be used to predict therapeutic responses, as in juvenile idiopathic arthritis, in which profiling correctly predicted therapeutic responses at 6 months [19].

Nevertheless, high-throughput genomic analyses are vulnerable to multiple biases, including random noise, biological heterogeneity, and differences in experimental procedures (biopsies, hybridization, etc.), leading to remarkably little overlap between DEGs in similar scale studies [20–22]. A meta-analysis approach that combines microarray data from independent yet similarly designed studies allows one to overcome these variations, ultimately increasing the power and reproducibility of the transcriptome [23–25]. While several meta-analysis methods exist for combining microarray data from independent studies [26], the random-effects model (REM) has been established as one of the most suitable for heterogeneous studies [25, 27, 28].

We applied a REM meta-analysis model including 4 published AD microarray studies (including 97 samples; 54 LS and 43 NL; 41 paired) to determine core pathogenic elements and new disease associated genes [9, 10, 12, 15], resulting in the Meta-Analysis Derived AD (MADAD) disease transcriptome, a robust active disease signature of 595 DEGs, including 86 that were not previously detected by any individual study.

Methods
Sample collection
All samples were collected according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [29]. A total of 28 datasets were detected in Gene Expression Omnibus (GEO), but only datasets including LS and NL skin samples of AD patients were retained. Datasets run on platforms other than the HGU133Plus 2 chip, subject to treatments, or with non-randomly selected NL or LS samples (e.g. FLG homozygous/heterozygous loss of function mutation), and datasets without NL samples were excluded. When overlapping samples were found between datasets, only one copy was kept. Four microarray datasets satisfied the established criteria (GSE32924, GSE36842, GSE58558, GSE59294) [9, 10, 12, 15], including 97 samples (54 LS and 43 NL), which coincidently have been carried out by our group.

Pre-processing and expression analysis
Pre-processing and statistical analyses were performed using *R* and *Bioconductor* packages [30, 31]. Raw expression data were combined, summarized, and normalized

using *GCRMA* [32]. Batch effects between datasets were adjusted for by the empirical Bayes method *ComBat/SVA* [32–35]. Agreement of the individual studies raw microarray data was estimated by the Integrated Correlation Coefficient Analysis, which produces the general Integrated Correlation Coefficient (ICC), representing agreement between studies, and can be interpreted in the same way as Pearson correlation coefficient. The ICC was used to eliminate background noise prior to the analysis, by excluding genes with incoherent behavior across studies [36].

For each study, estimation of differences in expression levels of LS vs NL skin was performed using the mixed-effect framework of the *limma* package.

Meta-analysis
The formal *random effects model (REM)*, described by Choi [27]. was applied to estimate the true effect size for each probe (see Additional file 1). These estimation and calculation steps were performed using the package *GeneMeta.* P-values were adjusted for multiple testing using the *Benjamini-Hochberg procedure* [37], with criteria for DEGs of absolute fold change ($|FCH| \geq 2.0$ and a false discovery rate (FDR) ≤ 0.05.

Post-processing
The MADAD transcriptome was subject to multiple downstream analysis methods. Integration-driven discovery/IDD-DEGs were defined as those not identified in any of the included studies. To explore functional annotations, overrepresentation analysis was carried out for BP GO-terms and KEGG pathways (both in DAVID) [38], Ingenuity Pathways (*IPA* – www.ingenuity.com, as described) [25], and on previously reported gene-sets [39].

The normalized LS and NL expression data were subject to Weighted Gene Co-Expression Networks Analysis (WGCNA) to detect clusters (modules) of correlated genes and respective hub genes [40]. These modules were subject to trait correlation and corresponding gene-set overrepresentation analysis.

Meta Threshold Gradient Directed Regularization (MTGDR) method [41, 42] was used to select disease-associated genes while allowing for varied estimates of those genes across different experiments, as previously published [41, 42].

Primers and probes were designed for RT-PCR as previously described (see Additional file 1 and Additional file 2) [43]. *Ribosomal protein, large, P0*/RPLP0 normalized RT-PCR expression data was analyzed using a mixed-effect framework after log2-transformation.

Lipid metabolism genes were defined as genes related to one of the four groups: *Ceramides*, *Free Fatty Acids*, *Sphingolipids*, and *Cholesteryl Esters* in the Gene Cards database (www.genecards.org). We included all genes with

a relevance score ≥ 10 [44, 45]. Pairwise Pearson correlations were calculated between the gene and patient specific deregulations. Multivariate correlations between sets of genes were calculated making use of the gene set specific μ-scores calculated by the *muStat* package [46].

Results
Data collection
Two major repositories (GEO Omnibus and ArrayExpress) were queried to identify studies with expression profiles of LS and NL punch biopsies from AD patients. Four studies (GSE59294, GSE58558, GSE36842, GSE32924; including 97 samples, with 54 LS and 43 NL, 41 paired) met all inclusion criteria following the PRISMA guidelines (see Methods, Additional file 3: Figure E1 and Additional file 4: Table E1) [9, 10, 12, 15]. We only used data from chronic lesions in the analysis. No significant differences in disease severity (as measured by Scoring of AD/SCORAD and Eczema Area and Severity/EASI

indices) or IgE levels were found across patients who all had moderate-to-severe AD (SCORAD > 25; EASI > 12).

Meta-analysis framework
The Venn diagram in Fig. 1a represents the overlap of DEGs identified by the individual studies, including 25 consensus DEGs (see Additional file 5: Table E2). Besides inter-study variation, sources of inconsistency in DEGs include choice of model, annotation, cut-off, and non-uniform pre-processing steps [47]. To combine results of individual studies and to aggregate robust DEGs with reliable effect sizes, we chose a meta-analysis approach [21, 27].

To address these sources of inconsistency, we applied a uniform pre-processing pipeline to the combined datasets, incorporating noise reduction techniques as proposed [23, 25, 36, 47] (see Methods, Additional file 3: Figure E1C, and Additional file 1). This pre-processing increased the average of the pairwise maximum canonical

Fig. 1 a Approximate area proportional Venn diagram with overlaps of the differentially expressed genes of the four individually analyzed datasets under the same threshold (|FCH| ≥ 2, FDR ≤ 0.05). **b** Ingenuity Canonical Pathway overrepresentations compared between this MADAD transcriptome and the included dataset from Khattri et al. 2014 (GSE58558). The bars represent −log10 transformed Benjamini-Hochberg adjusted p-values termed −log(FDR), and the red threshold line indicates the FDR ≤ 0.05 cut-off. **c** Cutaneous localization of the MADAD transcriptome as compared to the epidermis and dermis related genes defined by Esaki et al. [63]

correlations between each pair of studies from 0.76 to 0.85.

To combine heterogeneous effect sizes across studies, a random effects model/REM was chosen over fixed effects model. The use of REM is supported by the sample distribution of the Cochran's Q statistic [27], which departs substantially from the theoretical χ_3^2-distribution under the assumption of homogeneity of study effects. Furthermore, a Kolmogorov-Smirnov-test also rejected the equality of these two distributions ($p = 2.22^{-15}$; D = 0.235) (Additional file 6: Figure E2).

The Meta-analysis-derived AD (MADAD) transcriptome
Applying the meta-analysis approach to 4 studies and 97 samples, we identified a set of 595 DEGs (387 up- and 208 downregulated) using the classical |FCH| ≥ 2 and FDR ≤ 0.05 criteria, representing a robust profile defined as the meta-analysis derived AD (MADAD) transcriptome (see Additional file 7: Table E3 for the entire MADAD DEGs list). Among the highest dysregulated genes are key AD genes, including markers of general inflammation (MMP12), specific T helper activation (e.g. Th2/CCL18, Th1/IFN/CXCL10, Th17/PI3/elafin, Th17/Th22 S100A7/A8/A9), and markers of epidermal proliferation (KRT16, Mki67).

To show the robustness of the MADAD transcriptome versus a single study, we conducted an over-representation analysis (see Methods) of ingenuity canonical pathways (IPA) and of previously published immune and barrier gene-sets [12, 48]. Figure 1b illustrates the comparison of the largest available' dataset (Khattri 2014 – GSE58558) and the MADAD transcriptome; similar results were obtained for the other three included datasets. Overall, the MADAD yields a more significant over-representation of key immune pathways, such as *Granulocyte Adhesion and Diapedesis*, *T Cell Receptor Signaling and differentiation*, *iCOS-iCOSL Signaling*, and *IL-12, IL-17*, and *IL-9 signaling*. Innate pathways (e.g. *Role of Pattern Recognition Receptors in Recognition of Bacteria*), which are associated with AD [49, 50], were also represented (Fig. 1b and Additional file 8: Tables E4 and Additional file 9: Table E5). Interestingly, *Atherosclerosis Signaling* was the second highest IPA pathway, and includes genes previously associated with vascular inflammation, such as IL-37, SERPINA1, S100A8, selectin E/SELE, lipoprotein lipase/LPL, and MMP1/3/9 [51–62]. An equivalent analysis of previously reported immune and barrier gene-sets [12] (Additional file 10: Figure E3A) similarly showed increased sensitivity and representation in the MADAD transcriptome compared to the largest data set, including IFNα, IL-4, immune genes, cytokine-treated keratinocytes and epidermal differentiation gene subsets (Additional file 10: Figure E3A). Thus, the MADAD

transcriptome provides a more robust AD-specific signal, consistent with known disease pathology.

Comparison of the MADAD transcriptome to the recently described epidermal and dermal layer-specific transcriptomes linked 223 and 231 DEGs to the epidermis and dermis, respectively (Fig. 1c, Additional file 7: Table E3) [63]. Of these, 55.3 % of the upregulated genes were epidermal, whereas 67.5 % of the downregulated genes were dermal ($P = 2.6 \times 10^{-5}$, Fisher's exact test), as has been previously noted [63].

Of the top 25 up-regulated MADAD DEGs, 17 encode epidermal components, including key antimicrobial genes (DEFB4A, PI3/elafin, S100A9) (Additional file 7: Table E3). Top 25 up-regulated dermal genes include those related to collagen production (COL4A4, COL6A5) and inflammation (GZMB, OASL) [64, 65]. Among top down-regulated epidermal genes are structural/lipid-related genes (LEP, FABP7, ELOVL3), and genes linked to epidermal differentiation (CLDN8) [66–68]. Several genes associated with the pathogenesis of AD (e.g. IL-22, OX40L and TSLP) and reported in the layer-specific AD transcriptomes [63] were not detected in the MADAD transcriptome, most likely due to low expression levels of cytokines on whole tissue microarrays, a known limitation of microarrays [24, 69], that cannot be overcome by the meta-analysis approach.

Integration-Driven Discovery Genes in the MADAD transcriptome
The MADAD comprises a subset of IDD-DEGs, which were not detected by any of the individual datasets using identical cutoffs [9, 10, 12, 15]. These 86 IDD-DEGs consist of 45 up- and 41 downregulated genes (Table 1). Using IPA and GOterm overrepresentation analyses on the IDD-DEGs, we found in addition to the expected inflammatory processes (e.g. Chemokine Signaling), several pathways related to lipid and fatty acid metabolic processes (Additional file 10: Figure E3B, Additional file 11: Table E6).

We further validated, using RT-PCR, several IDD-DEGs and top MADAD DEGs with plausible biologic relevance to AD, including immune (CCL8, CD1E, IL-37, IL-36G), structural (AQP5, a water channel) and lipid (FAR2, ELOVL3, FA2H) genes, which encode enzymes involved in fatty acid and ceramide metabolism (Fig. 2a). Immune-related genes (e.g. CCL8, LCP2, CD1E) and IL-36G, which was recently associated with psoriasis pathogenesis [70], were increased in AD LS skin. The only gene that showed increased expression in NL vs LS skin was IL-37, a negative immune regulator [71], consistent with past reports [72]. All structural and lipid metabolism genes showed downregulation in LS AD skin (P ≤ 0.05 for all), reinforcing the role of lipids and water channels in preserving barrier function in AD [73, 74].

Table 1 Integration-Driven Discovery (IDD) Genes in the MADAD transcriptome, with indication of compartmental allocation as defined by Esaki et al. [63]

Symbol	Description	logFC	FC	Layer
Up				
COL6A6	collagen, type VI, alpha 6	2,08	4,22	D
CD1B	CD1b molecule	1,84	3,57	D
SPRR1B	small proline-rich protein 1B	1,68	3,20	E
CCL22	chemokine (C-C motif) ligand 22	1,65	3,13	D
MMP9	matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	1,51	2,84	D
IL13RA2	interleukin 13 receptor, alpha 2	1,46	2,74	D
CCL26	chemokine (C-C motif) ligand 26	1,37	2,59	D
SASH3	SAM and SH3 domain containing 3	1,35	2,56	D
IL36RN	interleukin 36 receptor antagonist	1,35	2,55	E
CCL13	chemokine (C-C motif) ligand 13	1,27	2,41	D
KIAA1644	KIAA1644	1,26	2,39	D
IL12RB1	interleukin 12 receptor, beta 1	1,23	2,34	D
XCL2	chemokine (C motif) ligand 2	1,21	2,31	D
CCL5	chemokine (C-C motif) ligand 5	1,21	2,31	D
ADAMDEC1	ADAM-like, decysin 1	1,19	2,29	D
TIFAB	TRAF-interacting protein with forkhead-associated domain, family member B	1,18	2,27	D
P2RY1	purinergic receptor P2Y, G-protein coupled, 1	1,16	2,23	E
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	1,15	2,22	D
FAM124B	family with sequence similarity 124B	1,14	2,21	D
SLAMF8	SLAM family member 8	1,12	2,18	D
CXADR	coxsackie virus and adenovirus receptor	1,12	2,17	E
GPSM3	G-protein signaling modulator 3	1,11	2,16	D
HCK	hemopoietic cell kinase	1,09	2,13	D
LOC100288860	uncharacterized LOC100288860	1,09	2,12	E
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	1,08	2,11	D
CD1E	CD1e molecule	1,07	2,10	D
KLRK1	killer cell lectin-like receptor subfamily K, member 1	1,06	2,09	D
GBP1	guanylate binding protein 1, interferon-inducible	1,06	2,09	
IL23A	interleukin 23, alpha subunit p19	1,05	2,08	D
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	1,05	2,07	D
C5orf20	chromosome 5 open reading frame 20	1,05	2,06	D
CCL8	chemokine (C-C motif) ligand 8	1,04	2,06	D
RELB	v-rel avian reticuloendotheliosis viral oncogene homolog B	1,04	2,06	
ACPP	acid phosphatase, prostate	1,03	2,05	E
TRAT1	T cell receptor associated transmembrane adaptor 1	1,03	2,04	D
PTX3	pentraxin 3, long	1,03	2,04	D
CD48	CD48 molecule	1,03	2,04	D
FPR3	formyl peptide receptor 3	1,02	2,03	D
TGM3	transglutaminase 3	1,02	2,03	E
CXCL11	chemokine (C-X-C motif) ligand 11	1,02	2,03	
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1	1,02	2,02	D

Table 1 Integration-Driven Discovery (IDD) Genes in the MADAD transcriptome, with indication of compartmental allocation as defined by Esaki et al. [63] *(Continued)*

CD6	CD6 molecule	1,02	2,02	D
SELPLG	selectin P ligand	1,01	2,02	D
ZC3H12D	zinc finger CCCH-type containing 12D	1,01	2,01	D
C15orf48	chromosome 15 open reading frame 48	1,00	2,00	E
Down				
PM20D1	peptidase M20 domain containing 1	−2,63	−6,19	
KRT79	keratin 79	−2,04	−4,13	
GAL	galanin/GMAP prepropeptide	−2,02	−4,06	
ELOVL3	ELOVL fatty acid elongase 3	−1,71	−3,26	
CYP4F8	cytochrome P450, family 4, subfamily F, polypeptide 8	−1,64	−3,11	
HAO2	hydroxyacid oxidase 2 (long chain)	−1,63	−3,10	
FADS2	fatty acid desaturase 2	−1,49	−2,80	
ANGPTL7	angiopoietin-like 7	−1,40	−2,63	D
CUX2	cut-like homeobox 2	−1,36	−2,56	
PON3	paraoxonase 3	−1,35	−2,55	E
SGK2	serum/glucocorticoid regulated kinase 2	−1,32	−2,50	
MSMB	microseminoprotein, beta-	−1,31	−2,47	E
FADS1	fatty acid desaturase 1	−1,30	−2,46	D
BPY2	basic charge, Y-linked, 2	−1,29	−2,45	
FAR2	fatty acyl CoA reductase 2	−1,29	−2,44	D
MUC7	mucin 7, secreted	−1,23	−2,35	
FA2H	fatty acid 2-hydroxylase	−1,20	−2,30	
ABHD12B	abhydrolase domain containing 12B	−1,18	−2,27	E
PNPLA3	patatin-like phospholipase domain containing 3	−1,18	−2,27	E
ACOX2	acyl-CoA oxidase 2, branched chain	−1,11	−2,16	D
PSORS1C2	psoriasis susceptibility 1 candidate 2	−1,10	−2,15	E
KRT19	keratin 19	−1,08	−2,11	
ATP6V1B1	ATPase, H+ transporting, lysosomal 56/58 kDa, V1 subunit B1	−1,07	−2,10	
SCGB2B2	secretoglobin, family 2B, member 2	−1,07	−2,09	
MOGAT1	monoacylglycerol O-acyltransferase 1	−1,07	−2,09	
NSUN7	NOP2/Sun domain family, member 7	−1,07	−2,09	E
COCH	cochlin	−1,05	−2,07	E
IL20RA	interleukin 20 receptor, alpha	−1,05	−2,07	E
SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	−1,03	−2,05	D
RHPN2	rhophilin, Rho GTPase binding protein 2	−1,03	−2,05	
AWAT1	acyl-CoA wax alcohol acyltransferase 1	−1,03	−2,04	
TMC4	transmembrane channel-like 4	−1,03	−2,04	
GPRC5A	G protein-coupled receptor, class C, group 5, member A	−1,02	−2,03	
TRHDE-AS1	TRHDE antisense RNA 1	−1,02	−2,03	
AQP5	aquaporin 5	−1,02	−2,03	
LINC00663	long intergenic non-protein coding RNA 663	−1,02	−2,02	
ZNF471	zinc finger protein 471	−1,01	−2,02	

Table 1 Integration-Driven Discovery (IDD) Genes in the MADAD transcriptome, with indication of compartmental allocation as defined by Esaki et al. [63] *(Continued)*

CYP2J2	cytochrome P450, family 2, subfamily J, polypeptide 2	−1,01	−2,02	E
MEGF10	multiple EGF-like-domains 10	−1,01	−2,01	
FAXDC2	fatty acid hydroxylase domain containing 2	−1,00	−2,01	D
SLC13A2	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2	−1,00	−2,01	

Suppression of lipid-related genes is coupled to increased Th2 activation

While we have previously shown inverse correlations between immune activation and terminal differentiation genes in AD lesions [11, 15], the relationship between immune activation and lipid metabolism genes has not been assessed in skin.

We thus investigated the relationship between expression of Th2-specific and epidermal lipid metabolism genes in the MADAD transcriptome. First we performed an unbiased database search for lipid related terms (see Methods) and chose genes with highest relevance scores. The correlation structure between lipid metabolism and immune genes in LS and NL skin is shown in Fig. 2b.

Unsupervised clustering identified two main sub-clusters in the Th2 gene-set and three main sub-clusters in the lipid metabolism gene-set. Cluster 3 consists only of SPTLC2, the only lipid metabolism gene with up-regulated expression in the MADAD transcriptome. This gene encodes serine palmitoyltransferase (SPT), a rate-limiting enzyme in sphingomyelin synthesis, whose elevated expression has been associated with increased barrier defects, including in AD [75–77].

Multivariate u-statistics correlate the dysregulation of immune and lipid metabolism genes [46]. Overall, an inverse correlation of −0.46 ($p = 0.003$) was obtained between Th2 and lipid subsets, supporting a proposed model of Th2 cytokine effects on lipid suppression [78]. Among Th2 genes, cluster 1 showed the highest negative correlation with lipid metabolism genes (−0.49; $p = 0.001$), and includes key AD genes (CCL22, IL-7R, and IL-4R) [63, 79–81]. Targeting IL-4R shows promise in early clinical trials as a possible therapeutic target for moderate-to-severe AD, and is now in phase 3 clinical trials for this disease (NCT02277743) [10, 18].

Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) offers insights into disease pathogenesis by studying weighted co-expression of genes within tissue samples [40]. This technique requires a large sample size [40, 82], which we were able to apply here for the first time in AD (see Methods).

Using WGCNA, 21 distinct sub-networks were identified, and each was correlated with age, IgE level, and disease severity (measured by Scoring of Atopic Dermatitis/ SCORAD) index (Fig. 3a-b, Additional file 12: Tables E7 and Additional file 13: Table E8). In LS skin, several networks showed significant positive correlations with SCORAD, including viral (M13) and innate immune response processes (M17), emphasizing cutaneous immune reactions to viral and/bacterial pathogens in LS AD skin. Proliferation and epidermal processes were also correlated with SCORAD (M10, M11), with a trend for negative correlations between SCORAD and structural epidermal modules (M4, M12). In NL skin, immune-related networks such as cytokine receptor signaling pathway and innate immune response modules (M4, M2) [63, 83] were also positively correlated with SCORAD, while extracellular matrix (ECM) organization (M10) and ECM-receptor interaction (M9) modules were negatively correlated (Fig. 3b). Interestingly, a trend for a positive correlation was observed between SCORAD and *Staphylococcus aureus* (*S.aureus*) infection in NL skin (M8), with possible clinical relevance, since AD patients are often colonized with *S.aureus*, even in NL skin [4, 84]. Similar but weaker correlations were seen with IgE, while minimal correlations where found with age (Fig. 3a-b).

A robust genomic AD classifier

Because clinical trials with specific and broad therapeutics are being tested in AD [5, 85], establishing a reliable gene set to discriminate between diseased and normal-appearing skin in AD is crucial. Here, we applied the classification algorithm Meta Threshold Gradient Directed Regularization (MTGDR) [41, 42], to determine the smallest set of genes that distinguishes LS from NL AD skin (see Methods). The final model identifies 19 genes (Fig. 3c and Table 2), including both epidermal and dermal genes, emphasizing the importance of both compartments to the disease model.

To assess the translational validity of these 19 discriminating genes, we investigated the effect of various therapies on gene expression using previously published studies with Dupilumab (300 mg, 4 weeks of treatment) [10], Cyclosporin A (CsA; at 2 and 12 weeks) [12], and narrow band UVB/NB-UVB (12 weeks of treatment) [11], shown in Table 2. Reversal of disease phenotype to the NL state was observed in 18 of the 19 discriminating genes. Overall, a higher reversal was seen with CsA, a broad immune suppressant [86, 87], particularly after 12 weeks of treatment, with an average recovery of 94.64 % (see Table 2).
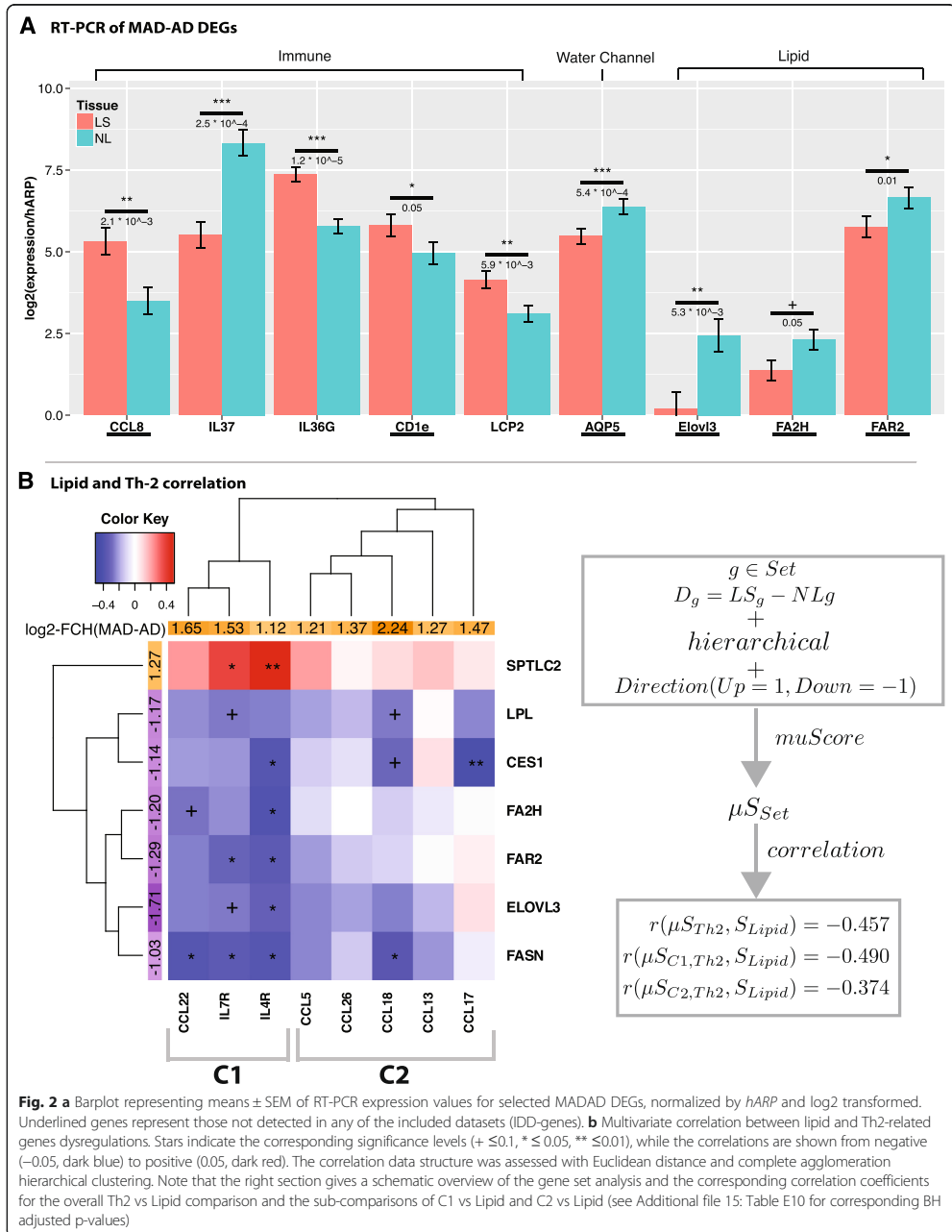
Fig. 2 a Barplot representing means ± SEM of RT-PCR expression values for selected MADAD DEGs, normalized by *hARP* and log2 transformed. Underlined genes represent those not detected in any of the included datasets (IDD-genes). **b** Multivariate correlation between lipid and Th2-related genes dysregulations. Stars indicate the corresponding significance levels (+ ≤0.1, * ≤ 0.05, ** ≤0.01), while the correlations are shown from negative (−0.05, dark blue) to positive (0.05, dark red). The correlation data structure was assessed with Euclidean distance and complete agglomeration hierarchical clustering. Note that the right section gives a schematic overview of the gene set analysis and the corresponding correlation coefficients for the overall Th2 vs Lipid comparison and the sub-comparisons of C1 vs Lipid and C2 vs Lipid (see Additional file 15: Table E10 for corresponding BH adjusted p-values)

Fig. 3 Module eigen-genes from weighted gene co-expression network analysis (WGCNA), correlated with Age, SCORAD and IgE, including annotation of top overrepresented BP GO-terms, KEGG Pathways or curated inflammatory skin diseases related gene-sets in (**a**) Lesional and (**b**) Non-lesional co-expression networks. Numbers in each cell represent the correlations with the respective trait and the associated p-values (see Additional file 16: Tables E11 and Additional file 17: Table E12 for corresponding BH adjusted p-values). **c** Radviz plots showing the separation of the lesional (LS) and non-lesional (NL) samples, as defined by the 19 genes signature identified by the MTGDR classifier (see Additional file 18: Table E13 for raw MTGDR results)

Among the genes down-regulated with treatment are inflammatory genes (S100A9, SELE) previously associated with AD [9, 59, 88]. Genes up-regulated with treatment (particularly with long-term CsA) include perilipin [89] and hydroxysteroid dehydrogenase [90], which are involved in lipid and steroid metabolism, respectively.

The LS and NL dysregulation in the MADAD correlates with the treatment change induced by CsA ($r = 0.99$; $p = 2.5*10^{-14}$), dupilumab ($r = 0.45$; $p = 0.189$), and UBV ($r = 0.90$, $p = 0.001$) treatments, leading to restoration of gene expression similar to that seen in NL skin (Additional file 14: Figure E4).

Discussion

A meta-analytic approach utilizes statistical processing and analysis to merge microarray studies from various populations and investigators, resulting in a single value that represents the estimated differential expression level of a gene between LS and NL skin. Combining multiple studies in a meta-analysis produces findings that more precisely reflect the differential expression of genes in a population, representing an accurate molecular characterization of a disease with increased power compared to individual analyses.

To address potential issues regarding meta-analysis application in gene expression studies, including laboratory effects, variations between probes and differential platforms, we planned this analysis beginning with data selection, through pre-processing and filtering, and finally to the meta-analysis model. This pipeline included the adjustment for study-related batch effects to minimize superficial inter-study discordance caused mainly by random noise and technical differences. Although in general this adjustment risk may confound true biological differences, we found no differences in disease severity, IgE and age across the four cohorts and thus feel confident that true biological differences are unlikely to be confounded. The resulting list of DEGs is presented here as a Meta-Analysis Derived Atopic Dermatitis (MADAD) transcriptome, utilizing 4 separate microarray studies for

Table 2 MTGDR robust classifier genes, compared to treatment response, and indication of compartmental allocation as defined by Esaki et al. [63]

SYMBOL	LAYER	logFCH (MAD-AD)	logFCH (Dupilumab, 300 mg, W4)	logFCH (Cyclosporin, W2)	logFCH (Cyclosporin, W12)	logFCH (UVB, W12)	Genename	Recovery (Dupilumab, 300 mg, W4)	Recovery (Cyclosporin, W2)	Recovery (Cyclosporin, W12)	Recovery (UVB, W12)
IGFL1	E	3.5	−2.4[a]	−2.6[a]	−3.6[a]	−2.3[a]	IGF-like family member 1	68.60	74.81	101.60	64.33
OASL	D	2.9	−2.7[a]	−2.7[a]	−3.3[a]	−1.8[a]	2'-5'-oligoadenylate synthetase-like	93.07	93.24	112.20	60.33
SELE	D	2.4	−2.9[a]	−1.6[a]	−1.6[a]	−2.3[a]	selectin E	121.06	66.99	66.24	94.94
AKR1B10	E	3.2	−1.3	−3.2[a]	−4.0[a]	−3.0[a]	aldo-keto reductase family 1, member B10 (aldose reductase)	42.39	100.29	127.48	95.22
MS4A14	D	1.4	−2.0[a]	−1.2[a]	−1.4[a]	0.0	membrane-spanning 4-domains, subfamily A, member 14	149.43	84.71	102.42	0.00
NUF2	E	1.4	−2.2[a]	−1.4[a]	−1.4[a]	−0.9	NUF2, NDC80 kinetochore complex component	155.33	96.79	96.65	59.46
S100A9	E	3.8	−1.1	−3.7[a]	−4.5[a]	−2.2[a]	S100 calcium binding protein A9	27.95	96.75	116.47	57.26
SERPINB3	E	3.8	−1.9	−3.2[a]	−3.8[a]	−2.4[a]	serpin peptidase inhibitor, clade B (ovalbumin), member 3	50.29	85.47	101.40	62.98
C15orf48	E	1.0	−0.9	−1.0	−1.3[a]	−2.1[a]	chromosome 15 open reading frame 48	93.70	103.85	131.53	211.69
MMP3	D	1.1	−4.0[a]	−2.0[a]	−0.9	−0.3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	374.65	181.39	81.06	25.91
MUC7		−1.2	−0.1	0.0	0.0	1.1	mucin 7, secreted	−9.82	0.00	0.00	87.60
HSPB7	D	−1.6	1.6	0.6	1.7[a]	0.2	heat shock 27 kDa protein family, member 7 (cardiovascular)	102.98	37.95	107.88	13.51
SCGB1D2	D	−1.2	0.3	0.5	1.2[a]	0.0	secretoglobin, family 1D, member 2	25.96	43.28	100.97	0.00
BTC	E	−2.1	−0.1	1.0	1.3[a]	1.3[a]	betacellulin	−6.53	48.15	65.56	65.17
CIDEC		−1.7	2.1	1.2[a]	1.8[a]	0.0	cell death-inducing DFFA-like effector c	126.22	72.11	106.23	−2.38
HSD11B1	E	−2.3	−1.8	0.1	2.1[a]	1.0[a]	hydroxysteroid (11-beta) dehydrogenase 1	−77.60	5.69	91.93	44.65
PLIN1		−1.9	1.9	1.7[a]	2.0[a]	0.3	perilipin 1	98.89	85.20	103.82	12.83
SCGB2A1		−1.8	−0.1	0.9	1.7[a]	1.5[a]	secretoglobin, family 2A, member 1	−7.33	50.36	92.70	80.79
WIF1	D	−2.2	0.7	1.2	2.1[a]	1.3[a]	WNT inhibitory factor 1	29.58	52.56	92.11	58.40
E = Epidermis, D = Dermis (Esaki et al. [63])							Average	76.78	72.61	94.64	57.51

[a]Indicate FDR ≤ 0.05

a total of 97 samples. It is our hope that the MADAD will prove helpful to investigators who may benefit from our robust characterization of the AD phenotype.

Similar to the MAD psoriasis transcriptome, our meta-analysis approach resulted in a more concise number of DEGs than that found in the individual studies [25]. However, this set of DEGs provides a more biologically relevant and powerful AD phenotype compared with previous studies [12], since important inflammatory and barrier pathways are more significantly enriched compared to individual studies, as is often observed with meta-analysis derived transcriptomes [91].

Our transcriptome is the first association of AD genomic fingerprinting with the atherosclerosis signaling pathway, which includes genes associated with vascular inflammation (SELE, IL-37, S100A8) [63, 77]. SELE has been independently associated with coronary heart disease and carotid artery atherosclerosis, and its expression in the vascular endothelium of the dermis of AD patients has also been observed [51, 88]. This supports the emerging view of AD as a systemic disease, which, like psoriasis, extends far beyond the skin [92, 93].

We have recently shown increased systemic immune activation in blood of moderate-to-severe AD patients among both skin homing and systemic T-cell subsets [94]. Furthermore, when comparing blood moderate-to-severe psoriasis and AD patients, we have shown that AD is associated with systemic activation and increased polar differentiation of effector and memory T-cell subsets, with higher and persistent activation within skin homing subsets. (Czarnowicki et al.-In press) AD patients also demonstrated higher levels of ICOS activation in circulating skin-homing subsets, (Czarnowicki- In press) consistent with the significant overrepresentation of ICOS signaling in our IPA analysis.

In large cohort studies, AD has also been recently associated with a variety of systemic diseases including inflammatory bowel disease [95, 96], Type 1 Diabetes Mellitus [97], and ADHD [98], providing further evidence for its systemic nature. Additionally, AD, like psoriasis, was recently shown to be associated with increased vascular inflammation using CT imaging [99–101]. The association of AD with systemic involvement emphasizes the great unmet need among severe AD patients for systemic therapeutic approaches, which are now in clinical trials (NCT00638989) [10, 18].

An interesting association between barrier defects and vascular inflammation in AD may be represented by the gene SPTLC2, which encodes a SPT, the rate-limiting enzyme in de novo synthesis of sphingomyelin and ceramides, and which has also been shown to influence atherosclerosis. Plasma sphingomyelin level was found to be an independent risk factor for coronary artery disease and is associated with subclinical atherosclerosis in

humans [102, 103]. In ApoE knockout mice, inhibition of SPT resulted in improved lipid profiles and prevented the development of atherosclerotic lesions [104]. Sphingomyelin is proposed to affect atherosclerosis by influencing lipid metabolism and regulating cell proliferation and apoptosis to modulate plaque growth and stability [105]. SPTLC2 increases with epidermal barrier abnormalities [75], as was observed in our MADAD transcriptome, and may be related to the previously unacknowledged systemic vascular inflammation in AD.

Lipid and differentiation abnormalities represent the hallmark of defective barrier function in AD [106, 107]. The Th2 cytokine effects on inhibition of terminal differentiation genes (e.g. filaggrin, loricrin) have been well documented by in vitro [1, 78] and in vivo studies [15, 108]. While the effects of Th2 cytokines on suppression of epidermal lipids have become recently available in model systems [78], our paper is the first to show negative correlations between expression of Th2 cytokines and epidermal lipids. It is established that AD LS skin shows alterations in lipid composition of the stratum corneum [109], with decreases in long-chain ceramides and free fatty acids in addition to disorganized lipid structure [107]. While Th1 cytokines, including TNFα and IFNγ, have been shown to induce ceramide synthesis, Th2 cytokines (including IL-4) inhibit the production of ceramides necessary for proper barrier function [76, 110]. Our findings support these in vitro models, and show significant negative correlations between increased Th2 cytokine production and decreased lipid expression.

We also identified for the first time as differentially expressed in AD several genes involved in lipid metabolism. These include FA2H, encoding protein essential to the de novo synthesis of specific ceramides that are critical in maintaining the permeability barrier of the epidermis [111, 112], and ELOVL3, encoding a protein involved in the elongation of long chain fatty acids and essential in prevention of transepidermal water loss [113, 114]. While not involved in fatty acid synthesis, aquaporin 5/AQP5 is a water channel that was found to be decreased in LS AD skin [115–117].

The network analysis of MADAD DEGs shows a significant correlation between AD disease activity/SCORAD and the *S. aureus* module in NL skin, emphasizing the role of colonization and infection in the onset of immune activation in background skin. *S. aureus* colonization/infection has been shown to occur at significantly higher rates in both LS and NL AD skin compared to healthy controls [118–120], and was shown to induce Th2 and Th22 immune activation [84, 121–123]. In LS skin, viral and innate immune process modules were significantly positively correlated with SCORAD, emphasizing the association between reactions to external pathogens and active inflammation in AD [121].

The treatment effect on the 19 discriminatory genes highlights the diverse mechanistic effects of these therapies with several observations worth mentioning. First, MMP3, a marker of general inflammation, displays an impressive recovery with the targeted therapy dupilumab compared to the more nonspecific immune suppressant treatments. This inflammatory gene improved over 300 % in only 4 weeks of treatment with dupilumab, while long-term treatment with CsA and UVB did not reach 100 % recovery. This may suggest the ability of dupilumab to suppress immune dysregulation in AD in a shorter time-frame than with less specific agents. Another noteworthy gene is Selectin E, encoding a protein involved in leukocyte extravasation, which also showed higher levels of recovery in dupilumab compared to CsA and UVB therapy. The difference in these markers may be related to the mechanism of action of each drug; UVB has direct effects on keratinocytes and thus mainly mediates signals originating there, while CsA is a nonspecific inhibitor of T-cells, B-cells and related pathways. Dupilumab more specifically modulates IL-4/IL-13 signaling, which has been implicated in the pathogenesis of AD, and this may account for the impressive recovery seen in genes central to the inflammatory response generated by AD.

Conclusions

This meta-analysis provides a stable and robust AD transcriptome worthy of future biomarker selection and evaluation of treatment response. The value of a standardized AD transcriptome will only increase with the bench-to-bedside translational approach, as a standardized measure of treatment response that cannot be confounded with placebo effects. This meta-analysis provides an integrative model of AD, emphasizing both immune and barrier abnormalities and also highlighting the systemic nature of the disease.

Additional files

Additional file 1: Supplementary materials and methods. (DOCX 111 kb)

Additional file 2: Table E9. Primer IDs for RT-PCR analysis. (XLSX 37 kb)

Additional file 3: Figure E1. A) Prisma. B) MADAD Datasets. C) Meta-Analysis Workflow. (PDF 608 kb)

Additional file 4: Table E1. All datasets intially considered before applying inclusion/exclusion criteria. (DOCX 65 kb)

Additional file 5: Table E2. Consensus of re-analyzed included datasets (by Probes). (XLSX 60 kb)

Additional file 6: Figure E2. QQ plot. Comparison of parameters. (PDF 1005 kb)

Additional file 7: Table E3. ALL MADAD DEGs (FCH ≥ 2, FDR ≤ 0.05). (XLSX 171 kb)

Additional file 8: Table E4. MADAD – Ingenuity Pathway Analysis. (XLSX 33 kb)

Additional file 9: Table E5. Khattri 2014 (GSE58558) – Ingenuity Pathway Analysis. (XLSX 26 kb)

Additional file 10: Figure E3. A) Inflammatory Skin Disease Gene Sets. B) MADAD IDD-DEGs IPA Canonical Pathways. (PDF 85 kb)

Additional file 11: Table E6. IPA Pathways and GO terms IDD genes. (XLSX 9 kb)

Additional file 12: Table E7. Overrepresented Hub30 Genes and module names. (LS) (XLSX 38 kb)

Additional file 13: Table E8. Overrepresented Hub30 Genes and module names. (NL) (XLSX 47 kb)

Additional file 14: Figure E4. logFCHs correlations of MADAD and treatments A) Dupilumab (300mg), B) Cyclosporin (W12), and C) UVB. (PDF 42 kb)

Additional file 15: Table E10. Adjusted P-values for the significance indicators in Figure 2B. (XLSX 8 kb)

Additional file 16: Table E11. BH adjusted p-values WGCNA LS Modules. (XLSX 9 kb)

Additional file 17: Table E12. BH adjusted p-values WGCNA NL Modules. (XLSX 9 kb)

Additional file 18: Table E13. MTGDR results. (XLSX 9 kb)

Abbreviations
AD: Atopic dermatitis; CsA: Cyclosporin A; DEGs: Differentially expressed genes (logFCH ≥ 1 and fdr ≤ 0.05); FDR: False Discovery Rate; FEM: Fixed-effects model (special case of REM); ICC: Integrated Correlation Coefficient; IDD: Integration-driven discovery; IgE: Immunoglobulin E; IPA: Ingenuity pathway analysis; LS: Lesional; MAD-AD: Meta-analysis derived AD; MTGDR: Meta Threshold Gradient Directed Regularization; NL: Non-lesional; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses; REM: Random-effects model; RT-PCR: Quantitative Reverse Transcription PCR; RPLP0: Ribosomal protein, large, P0 (formerly termed *human acidic ribosomal protein P0*/hARP); S. aureus: Staphylococcus aureus; SCORAD: Scoring of Atopic Dermatitis; SPT: Serine palmitoyltransferase; WGCNA: Weighted gene co-expression network analysis.

Authors' contributions
DAE carried out the main statistical and bioinformatics analysis, and prepared the manuscript. DM prepared the manuscript and carried out biological assessment and evaluations of the statistical results. JGK participated in the design of the study and manuscript preparation. CTW advised and participated in the bioinformatics analysis. TW and ST carried out the classification analysis. TL participated in the design of the study and manuscript preparation. EGY participated in the design of the study and directed manuscript preparation. MSF designed the study, directed manuscript preparation, and directed the statistical analysis. All authors have read and approved the final manuscript.

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2.2   Laser Capture Microdissection of Atopic Dermatitis

2.2.1   Prelude

The molecular signature of atopic dermatitis has been investigated by various groups using microarray expression analyses. However, no comparative layer specific expression analysis had been carried out on the disease.

In this work we applied laser capture microdissection to separate the epidermis from the dermis of lesional, non-lesional, and normal samples. The subsequent microarray expression analysis identified the differential expression of numerous novel barrier and immune related genes like IL34, IL22 or CLDNs 4 and 8, not detectable in full-thickness microarray analyses.

Overall this study adds layer specificity to the AD transcriptome, and reveals increased detection sensitivity.

Identification of novel immune and barrier genes in atopic dermatitis by means of laser capture microdissection

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Background: The molecular signature of atopic dermatitis (AD) lesions is associated with T_H2 and T_H22 activation and epidermal alterations. However, the epidermal and dermal AD transcriptomes and their respective contributions to abnormalities in respective immune and barrier phenotypes are unknown.
Objective: We sought to establish the genomic profile of the epidermal and dermal compartments of lesional and nonlesional AD skin compared with normal skin.
Methods: Laser capture microdissection was performed to separate the epidermis and dermis of lesional and nonlesional skin from patients with AD and normal skin from healthy volunteers, followed by gene expression (microarrays and real-time PCR) and immunostaining studies.
Results: Our study identified novel immune and barrier genes, including the IL-34 cytokine and claudins 4 and 8, and showed increased detection of key AD genes usually undetectable on arrays (ie, *IL22*, thymic stromal lymphopoietin *[TSLP]*, *CCL22*, and *CCL26*). Overall, the combined epidermal and dermal transcriptomes enlarged the AD transcriptome, adding 674 upregulated and 405 downregulated differentially expressed genes between lesional and nonlesional skin to the AD transcriptome. We were also able to localize individual transcripts as primarily epidermal (defensin, beta 4A *[DEFB4A]*) or dermal (*IL22*, cytotoxic T-lymphocyte antigen 4 *[CTLA4]*, and *CCR7*) and link their expressions to possible cellular sources.
Conclusions: This is the first report that establishes robust epidermal and dermal genomic signatures of lesional and nonlesional AD skin and normal skin compared with whole tissues. These data establish the utility of laser capture microdissection to separate different compartments and cellular subsets in patients with AD, allowing localization of key barrier or immune molecules and enabling detection of gene products usually not detected on arrays. (J Allergy Clin Immunol 2015;135:153-63.)

Key words: Atopic dermatitis, laser capture microdissection, IL-34, claudins 8 and 4, immune, barrier

Atopic dermatitis (AD) is the most common inflammatory skin disease.[1,2] Although its pathogenesis is not fully understood, both barrier and immune components have been suggested to play key roles in AD, as indicated by the "outside-in" and "inside-out" hypotheses.[3-10] Whereas barrier-related molecules are largely epidermal, inflammatory responses are derived from both the epidermal (ie, keratinocytes and Langerhans cells [LCs]) and dermal (ie, T cells and dendritic cells [DCs]) compartments.

Using genomic analyses on whole tissue/bulk samples, we have previously shown that the AD phenotype/transcriptome is associated with polar immune activation of T_H2/T_H22, as well as T_H1 and T_H17, pathways and corresponding epidermal alterations (epidermal hyperplasia and abnormal differentiation).[11-14] However, bulk sample genomic analysis (by using microarrays and real-time PCR [RT-PCR]) presents some limitations. First, it is difficult to determine whether altered gene expression is due to expansion (hypertrophy) of one tissue compartment versus altered gene expression at the cellular level. Second, it cannot localize a particular gene/transcript to an epidermal/dermal compartment. Finally, low-abundance genes are often present at less than the detection level of conventional microarrays because of dilution of mRNA within full-thickness samples with more dominant products.

Laser capture microdissection (LCM) is an established technique for procuring subpopulations of tissues/cells of interest

Abbreviations used

AD:	Atopic dermatitis
CE:	Cornified envelope
CLDN:	Claudin
CSF-1R:	Colony-stimulating factor 1 receptor
CTLA4:	Cytotoxic T-lymphocyte antigen 4
DC:	Dendritic cell
DEFB4A:	Defensin, beta 4A
DEG:	Differentially expressed gene
EDC:	Epidermal differentiation complex
FCH:	Fold change
FDR:	False discovery rate
FLG:	Filaggrin
GZMB:	Granzyme B
ICOS:	Inducible T-cell costimulator
LC:	Langerhans cell
LCM:	Laser capture microdissection
LOR:	Loricrin
MX1:	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
PI3:	Peptidase inhibitor 3, skin-derived
RT-PCR:	Real-time PCR
SPRR:	Small proline-rich protein
TJ:	Tight junction

under direct microscopic visualization to study alterations in disease states.[15] Our group has previously demonstrated that epidermal and dermal separation of lesional and nonlesional samples from patients with psoriasis and normal samples by using LCM complemented by microarrays largely increases the detection of low-abundance genes compared with whole-tissue analyses.[16,17] Despite the pathogenic relevance of separating the epidermal and dermal compartments, such studies are unavailable in patients with AD.

In this study we sought to determine the molecular phenotypes of the epidermal and dermal compartments of lesional and nonlesional AD skin (compared with skin from healthy subjects). Overall, our results (1) enlarged the AD transcriptome; (2) detected low-abundance genes (which are usually present at less than detection levels on whole-tissue microarrays [eg, *IL22* and thymic stromal lymphopoietin *[TSLP]*]); and (3) identified novel immune and barrier genes (ie, *IL34*, claudin 4 *[CLDN4]*, and *CLDN8*) and suggested possible cellular sources of immune markers (ie, CCR7).

METHODS
Skin samples

Paired nonlesional and lesional AD skin biopsy specimens were collected from 5 patients with moderate-to-severe chronic AD (3 male and 2 female patients; age, 27-59 years; mean age, 39.4 years; see Table E1 in this article's Online Repository at www.jacionline.org) under institutional review board–approved protocols. Normal skin samples from healthy volunteers (n = 10) that had been collected for a prior LCM publication[18] were also included. Paired epidermal, dermal, and full-thickness lesional and nonlesional samples were used for RT-PCR and microarray analysis (n = 5 in each group). Lesional and nonlesional expression values were compared with 10 epidermal, 6 dermal, and 6 bulk corresponding normal samples. For RT-PCR confirmation, 3 normal paired epidermal and dermal samples were used because of the limited available mRNA.

Slide preparation and LCM

LCM was performed according to the manufacturer's protocol for the CellCut system (Molecular Machines & Industries, Haslett, Mich; see the Methods section in this article's Online Repository at www.jacionline.org).

RNA extraction

Total RNA was extracted with the RNeasy Micro Kit (Qiagen, Valencia, Calif), according to the manufacturer's protocol, with on-column DNase digestion.

Sample preparation for gene chip analysis

Target amplification and labeling was performed according to the Affymetrix protocols for 2-cycle cDNA synthesis by using Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, Calif), as previously reported.[16]

Sample preparation for quantitative RT-PCR

Reverse transcription to cDNA from RNA of LCM samples was carried out by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, Calif), cDNA was amplified with TaqMan PreAmp Master Mix (Applied Biosystems), and the preamplified cDNA product was analyzed with TaqMan Gene Expression Master Mix, according to the manufacturer's instructions. The RT-PCRs for each assay were run in triplicate, and all data were normalized to human acidic ribosomal protein P0. The primers and probes used in this study are listed in Table E2 in this article's Online Repository at www.jacionline.org.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed on frozen skin sections, as previously described.[19] The antibodies used in this study are listed in Table E3 in this article's Online Repository at www.jacionline.org.

Statistical analysis

Preprocessing and statistical analysis of microarray data were conducted with R (R-project.org) and Bioconductor software packages.[20] Full details of the pipeline and downstream analysis are described in the Methods section in this article's Online Repository. Succinctly, the Harshlight package[21] was used to quality control the images, and expression values were obtained by using the GCRMA algorithm.[22] Batch adjustments were carried out with the ComBat algorithm, and mixed-effect models in the limma package were used to model differential expression.[23-25] Genes with a false discovery rate (FDR) of less than 0.05 and a fold change of greater than 2 were considered significantly differentially expressed. Similar models were used to analyze $\log_2$-transformed values of normalized RT-PCR data.

RESULTS
LCM localizes genes selectively expressed in the dermis and epidermis

We performed LCM to collect epidermal and dermal (papillary, reticular, and inflammatory aggregate) cells in frozen sections of lesional, nonlesional, and normal skin samples, as shown in Fig 1, *A*. Microarray profiling of lesional, nonlesional, and normal epidermal and dermal tissues was performed with Affymetrix HGU133Plus2.0 microarrays to define the epidermal and dermal transcriptomes. A heat map of epidermis- and dermis-specific genes shows clear separation of differentially expressed genes (DEGs) localized to the epidermis and dermis across lesional, nonlesional, and normal samples (see Fig E1 in this article's
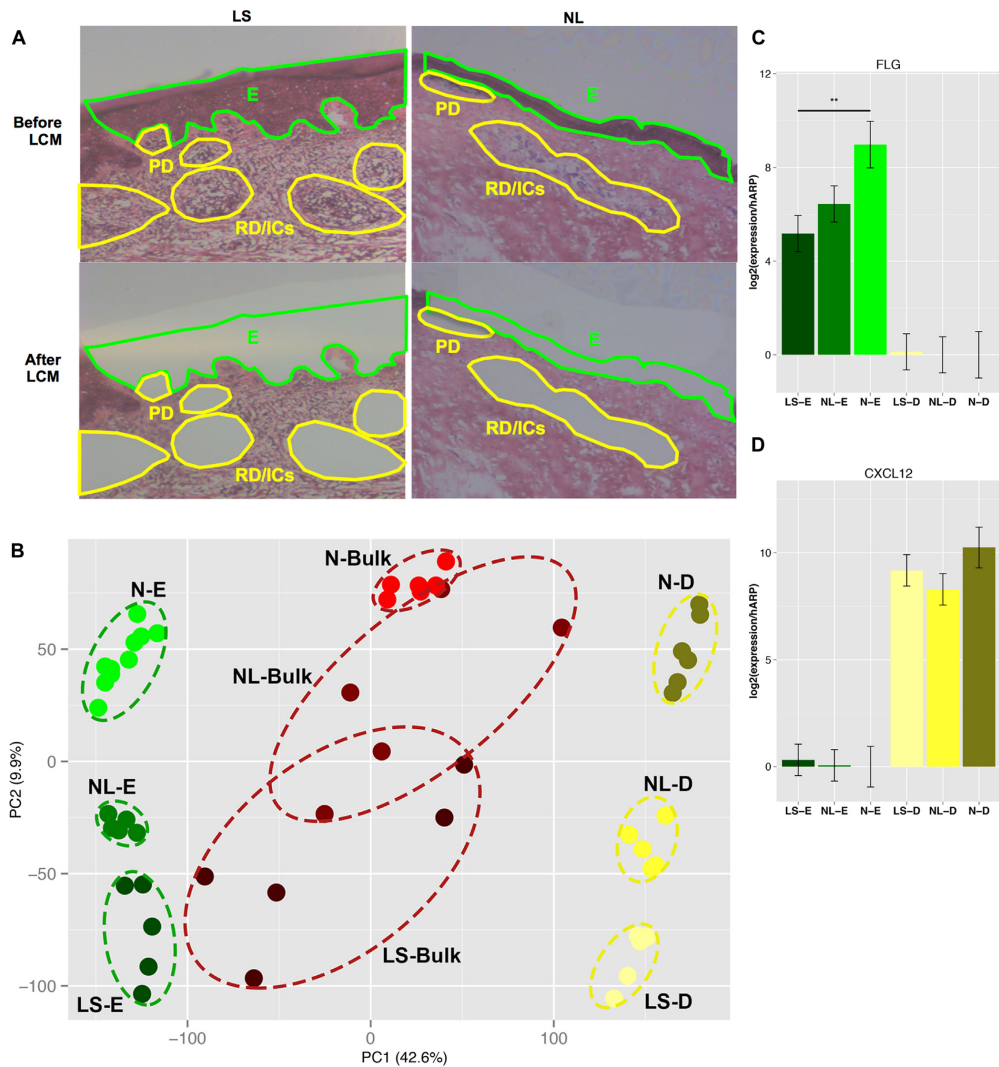
FIG 1. **A,** Representative hematoxylin and eosin staining of lesional *(LS)* and nonlesional *(NL)* AD skin. LCM was performed in the indicated zones *(green:* epidermis *[E]; yellow:* papillary dermis *[PD],* reticular dermis *[RD],* and inflammatory aggregates *[ICs]).* **B,** Principal component *[PC]* analysis plot showing clear separation of groups. **C** and **D,** mRNA expression of *FLG* (Fig 1, *C*) and *CXCL12* (Fig 1, *D*) in the epidermis *(E)* and dermis *(D)* of lesional *(LS)* and nonlesional *(NL)* AD and normal *(N)* skin. Values are presented as means ± SDs. **P < .01.

Online Repository at www.jacionline.org). A principal component analysis of expression values illustrates the lack of outliers and that samples cluster in accordance with tissue type (Fig 1, *B*). Markers primarily considered dermal *(CXCL12, CD93,* and collagens *[COL1A2* and *COL6A3]*) were highly represented in the dermis (see Tables E4, *A,* and E5, *A,* in this article's Online Repository at www.jacionline.org), and epidermal markers (filaggrin [FLG], loricrin [LOR], late cornified envelope [LCE], and small proline-rich proteins [SPRRs]) were localized to the epidermis, which is consistent with the clear
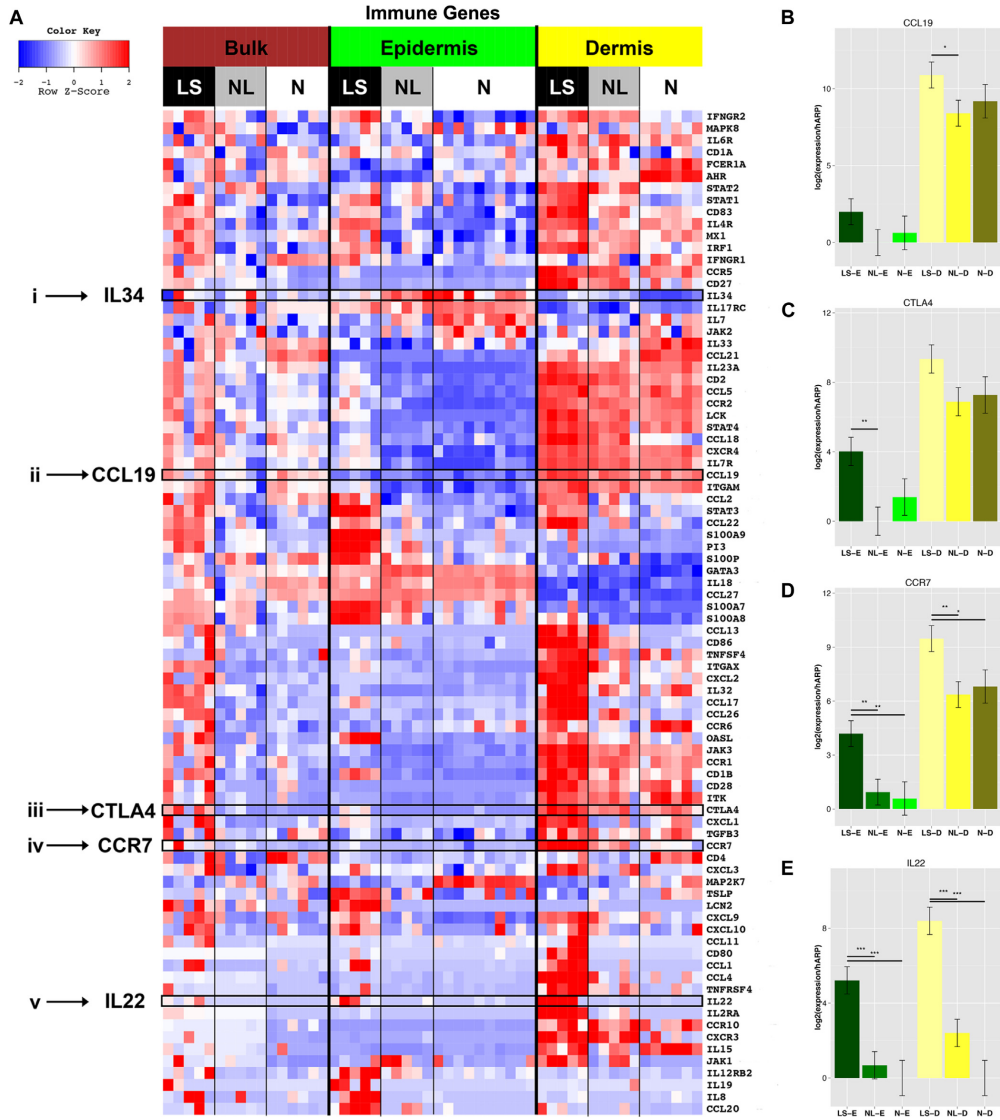
FIG 2. **A,** Heat map of immune-related genes organized by compartment. FCHs comparing lesional *(LS)*, nonlesional *(NL)*, and normal *(N)* skin in bulk samples *(B)* and the epidermis *(E)* dermis *(D)* are shown (n = 5 AD specimens and n = 6, 10, and 6 normal bulk, epidermal, dermal specimens, respectively). **B-E,** mRNA expression, as determined by using RT-PCR, of *CCL19* (Fig 2, *B*), *CTLA4* (Fig 2, *C*), *CCR7* (Fig 2, *D*), and *IL22* (Fig 2, *E*) in the epidermis *(E)* and dermis *(D)* of lesional *(LS)* and nonlesional *(NL)* AD skin and normal *(N)* skin. Values are presented as means ± SDs in Fig 2, *B* through *E*. *P < .05, **P < .01, and ***P < .001.
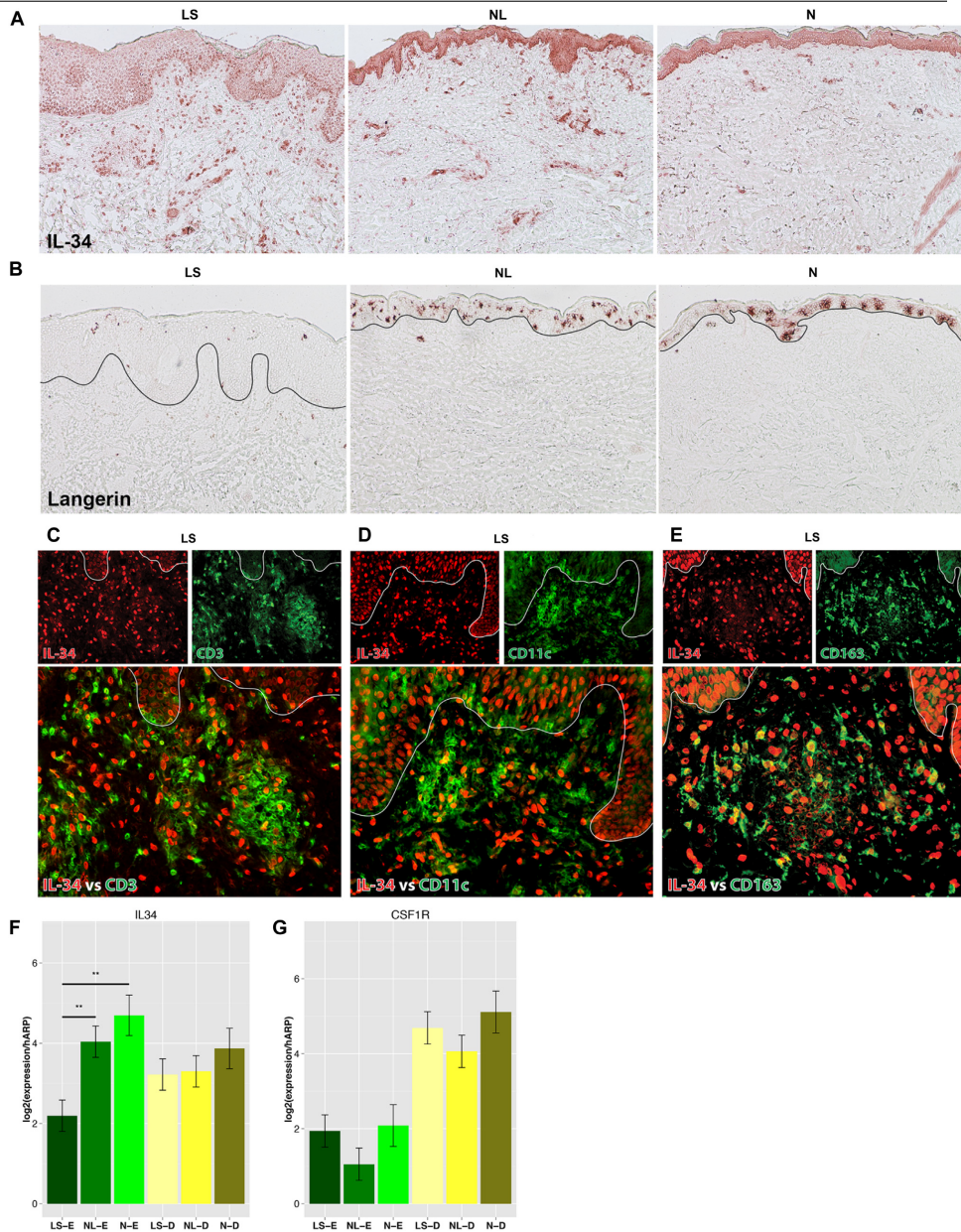
FIG 3. **A** and **B,** Representative staining of IL-34 (Fig 3, *A*) and langerin (Fig 3, *B*) in lesional *(LS)* and nonlesional *(NL)* AD and normal *(N)* skin. **C-E,** Representative double immunofluorescence for coexpression of IL-34 *(red)* versus CD3 *(green*; Fig 3, *C*), CD11c *(green*; Fig 3, *D*), and CD163 *(green*; Fig 3, *E*) in lesional *(LS)* skin. **F** and **G,** mRNA expression of *IL34* (Fig 3, *F*) and its receptor, *CSF1R* (Fig 3, *G*), in the epidermis *(E)* and dermis *(D)* of lesional *(LS)*, nonlesional *(NL)*, and normal *(N)* skin. Values are presented as means ± SDs. *******P* < .01.
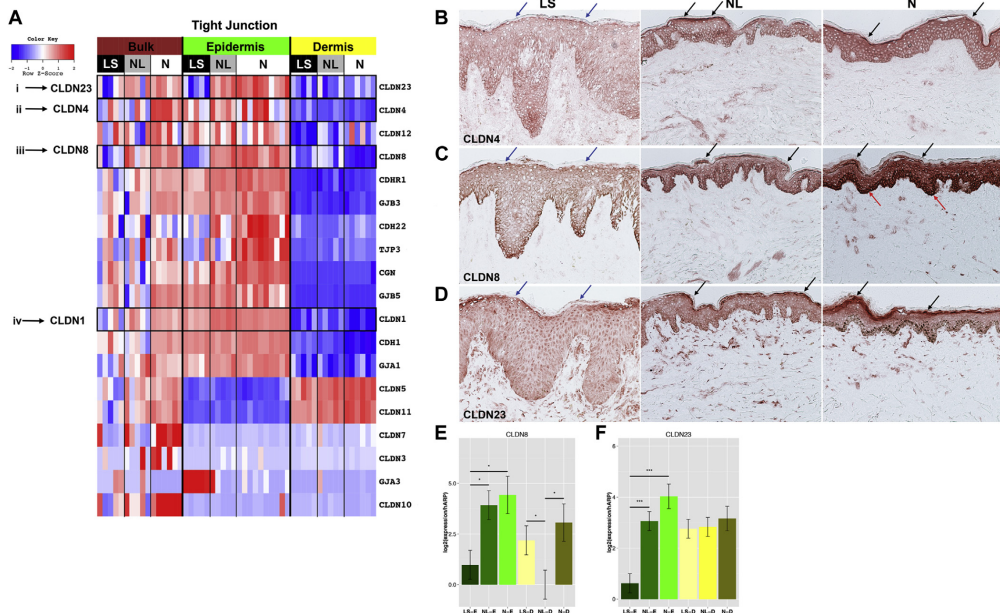
FIG 4. A, Heat map of barrier-related gene FCHs comparing lesional *(LS),* nonlesional *(NL),* and normal *(N)* skin in bulk, epidermal, and dermal specimens (n = 5 AD specimens and n = 6, 10, and 6 normal bulk, epidermis, and dermis specimens, respectively). **B-D,** Representative CLDN4 (Fig 4, *B*), CLDN8 (Fig 4, *C*), and CLDN23 (Fig 4, *D*) staining in lesional *(LS),* nonlesional *(NL),* and normal *(N)* skin. *Arrows,* Increased stratum corneum *(black),* decreased lesional stratum corneum *(blue),* and increased normal skin basal layer *(red)* staining. **E** and **F,** Expression of *CLDN8* (Fig 4, *E*) and *CLDN23* (Fig 4, *F*) in the epidermis *(E)* and dermis *(D)* of lesional *(LS),* nonlesional *(NL),* and normal *(N)* skin. Values are presented as means ± SDs. *$P < .05$ and ***$P < .001$.

epidermal-dermal separation (see Tables E4, *B,* and E5, *B*). The accuracy of the LCM separation was validated by using RT-PCR, with greater than 69.7-fold and greater than 846.2-fold enrichment of the mRNA expressions of *FLG* and *CXCL12* in the epidermis versus dermis, respectively, in normal tissues (Fig 1, *C* and *D*).

LCM enlarges the AD transcriptome and increases detection of low-abundance immune genes on microarrays

To characterize the AD phenotype within each compartment, we defined the lesional epidermal and dermal transcriptomes as the set of DEGs between lesional and nonlesional tissue within each compartment, respectively, by using the classical criteria of an FCH of greater than 2.0 and an FDR of less than 0.05. Combining the epidermal and dermal transcriptomes, many more compartment-specific DEGs were identified (860 upregulated and 495 downregulated, see Fig E2 in this article's Online Repository at www.jacionline.org), adding 674 upregulated and 405 downregulated DEGs to the recently defined AD transcriptome.[11,12,26] The bulk transcriptome consists of 710 upregulated and 487 downregulated DEGs, whereas the LCM epidermal transcriptome

contains 566 upregulated and 268 downregulated DEGs, and the LCM dermal transcriptome contains 330 upregulated and 244 downregulated DEGs. Little overlap was observed between the epidermal and dermal transcriptomes (only 36 upregulated and 17 downregulated DEGs were detected in both).

Overall, the top 25 upregulated and downregulated genes in each of the epidermal and dermal lesional transcriptomes included many genes that have been shown to contribute to the AD phenotype (see Tables E6 and E7 in this article's Online Repository at www.jacionline.org).[11,12] The top 25 upregulated genes in the lesional epidermal transcriptome consisted of proliferation-related (*KRT6A*, *KRT6B*, and *KRT16*), epidermal differentiation complex (EDC; S100 genes), inflammatory (matrix metalloproteinase 12 *[MMP12]*), and antimicrobial peptide–derived (defensin, beta 4A *[DEFB4A]* and peptidase inhibitor 3, skin-derived *[PI3]*/Elafin genes (see Table E6, *A*). The top 25 upregulated genes in the lesional dermal transcriptome included T-cell activation (granzyme B *[GZMB]* and IL-2 receptor α *[IL2RA]*), T_H2-related (*CCL17*, *CCL22*, and *CCL26*), T_H22/T_H17-related (*IL22*, *S100A8*, *CXCL1*, and *CXCL2*), and collagen (*COL6A6*, *COL6A5*, and *COL4A3*) genes (see Table E6, *B*). *IL22* expression was significantly increased in the lesional dermal transcriptome (12.46 FCHs and FDR < 0.001), whereas it was present
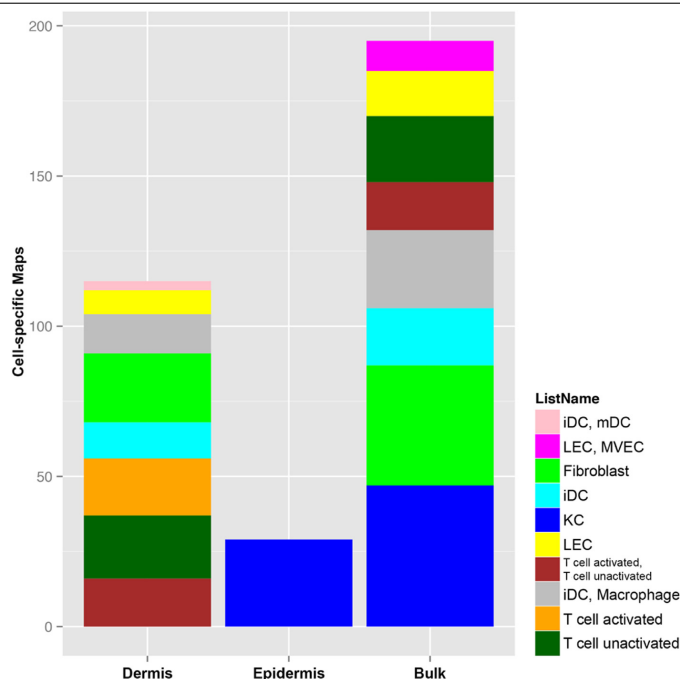
FIG 5. The cell-specific map of the LCM epidermal and dermal transcriptomes and bulk transcriptome. The cellular source of the epidermal transcriptome was mainly keratinocytes, whereas activated T cells were only significantly enriched in the LCM dermal transcriptome. *iDC,* Immature dendritic cell; *KC,* keratinocyte; *LEC,* lymphatic endothelial cell; *mDC,* mature dendritic cell; *MVEC,* microvascular endothelial cell.

at less than detection levels in previous reports,[11,12,26] indicating that LCM is a useful method to increase the sensitivity of detecting low-abundance genes.

Key inflammatory and barrier genes were uniquely detected in the LCM epidermal (see Table E8, *A*, in this article's Online Repository at www.jacionline.org) and dermal (see Table E8, *B*) transcriptomes. The unique lesional epidermal transcriptome included IL-17–related (*DEFB4A* and *CCL20*) and inflammatory (*CXCR4*, signal transducer and activator of transcription 3 *[STAT3]*, and *IL8*) genes (see Table E8, *A*). Key T$_H$2 (*CCL17*, *CCL22*, and *CCL26*) and T$_H$22 *(IL22)* markers and T-cell migration/activation markers (*CCR7* and inducible T-cell costimulator *[ICOS]*) were found within the unique lesional dermal transcriptome (see Table E8, *B*). Although the LCM approach detected an appreciably larger number of genes within bulk tissues, there was a subset of DEGs that were not identified in the corresponding epidermal and dermal transcriptomes (429 upregulated and 381 downregulated genes; Fig 2 and see Table E8, *C*). A deeper analysis of these data suggests that most of this discrepancy is created by increased or decreased gene abundance because of differing contributions of the epidermis and dermis in lesional versus nonlesional skin (see the Results section and Fig E3 in this article's Online Repository at www.jacionline.org).

We further explored the compartmental distribution of immune DEGs in lesional, nonlesional, and normal skin using our previously curated and reported immune gene subset,[19,26,27] as shown in a heat map (Fig 2, *A*, and see Table E9 in this article's Online Repository at www.jacionline.org). Among significantly upregulated genes in the lesional dermal transcriptome were T-cell (cytotoxic T-lymphocyte antigen 4 *[CTLA4]* and *CD28*), DC (integrin αX, complement component 3 receptor 4 subunit *[ITGAX]*/CD11c and *CD1A*), lymphoid-organizing chemokine (*CCR7* and its ligands *[CCL19* and *CCL21]*), and T$_H$2-related (*CCL11*, *CCL13*, *CCL17*, *CCL22*, *CCL26*, TNF receptor superfamily, member 4 *[TNFRSF4*/OX40], and *IL4R*) genes. Significant increases in T$_H$2-related *(IL7R)*, T$_H$17-related (*PI3*, lipocalin 2 *[LCN2]*, *CCL20*, and *STAT3*), T$_H$22/T$_H$17-related *(IL22, S100A8,* and *S100A9)*, and T$_H$1-related (2′-5′-oligoadenylate synthetase-like *[OASL]*, myxovirus [influenza virus] resistance 1, interferon-inducible protein p78 [mouse] *[MX1]*, *IL12RB2*, IFN-γ receptor 2 *[IFNGR2]*, and interferon regulatory factor 1 *[IRF1]*) products were found. Using RT-PCR, we validated the primarily dermal mRNA expression of selected markers, including *IL22*, *CTLA4*, *CCR7*, and *CCL19*. High *IL22* mRNA levels were characteristic of only lesional skin (Fig 2, *B-E*). A list of DEGs in all comparisons is presented in Table E8.

IL-34: A novel cytokine identified by means of LCM

IL-34 was significantly downregulated in lesional compared with both nonlesional and normal epidermis (Fig 2, *A*, and see Table E9). IL-34 is a recently identified cytokine in mice and normal human skin[28] and is suggested to regulate LC differentiation in steady states. It has been identified as an alternative ligand to the colony-stimulating factor 1 receptor (CSF-1R), which has been shown to be expressed in epidermal LCs, dermal monocytes, and DCs.[29] IL-34 has not been previously reported in human skin diseases. We performed immunohistochemical staining of IL-34, which showed stronger epidermal staining in normal and nonlesional skin compared with lesional skin and stronger dermal staining in lesional skin (Fig 3, *A*).

Because IL-34 was reported to induce LC differentiation only in steady states,[29] langerin (CD207) staining for LCs was performed. Many fewer LCs were detected in lesional compared with both nonlesional and normal skin (Fig 3, *B*). To identify the cellular distribution of IL-34 expression in the dermis, we performed double immunofluorescence for IL-34 (red) with CD3$^+$/T cells (green; see Fig E4, *A*, in this article's Online Repository at www.jacionline.org), CD11c$^+$/DCs (green; see Fig E4, *B*), and CD163$^+$/macrophages (green; see Fig E4, *C*) in normal, nonlesional, and lesional skin. Few double-positive IL-34$^+$/CD3$^+$ cells were found. Many IL-34$^+$ cells colocalized with CD11c$^+$ and CD163$^+$ cells (Fig 3, *C-E*). Thus IL-34 in the dermis is preferentially expressed by myeloid DCs and macrophages.

We validated the lower expression of IL-34 in lesional epidermis using RT-PCR, with significantly lower *IL34* mRNA expression observed in lesional versus both nonlesional and normal epidermis and with slightly lower dermal mRNA expression (Fig 3, *F*). We also measured *CSF1R* mRNA expression, which was significantly higher in the dermis, regardless of tissue (Fig 3, *G*).

LCM highlights tight junction defects in patients with AD

Because defective barrier function is a hallmark of AD,[1] we evaluated for EDC and cornified envelope (CE) markers. A heat map of EDC and CE genes (including *S100A* genes) is shown in Fig E5 in this article's Online Repository at www.jacionline.org. The majority of S100 genes (*S100A8/S100A9/S100A12/S100A13*), IL-17–induced *PI3*/Elafin, and SPRRs *(SPRR1A)* showed a primarily epidermal expression and higher expression in lesional versus both nonlesional and normal skin (FDR < 0.001 for most markers), whereas a few S100 genes (*S100A4/S100A6/S100A10*) showed predominantly dermal lesional expression. As previously reported in bulk tissues,[26] the differentiation genes (*LCE*, *LOR*, *FLG*, and periplakin *[PPL]*) showed decreased expression in lesional versus nonlesional and normal epidermis (see Fig E5 and Table E10 in this article's Online Repository at www.jacionline.org). Claudins, another crucial barrier component, are essential for tight junction (TJ) formation. Twenty-three claudins have been identified in human subjects; however, their contribution to AD is not fully defined.[30,31] To gain insight into which claudins are expressed in patients with AD, we evaluated a subset of TJ genes, the expression profiles of which are visualized in a heat map (Fig 4, *A*, and see Table E11 in this article's Online Repository at www.jacionline.org). Downregulation of claudins 1 and 23 was observed in lesional versus nonlesional and normal epidermis, as well as downregulation of claudins 4 and 8, which were previously unreported in patients with AD (FDR < 0.001).[32] We also detected dermal claudins (5 and 11), expression of which was significantly downregulated in the lesional versus normal skin comparison, which was previously not reported in patients with AD.

The differential expression of claudin genes in the epidermal and dermal compartments was validated by using immunohistochemistry and RT-PCR. CLDN4, CLDN8, and CLDN23 stained all epidermal layers, except the stratum corneum (Fig 4, *B-D*), with highly attenuated staining in lesional versus both nonlesional and normal epidermis. The reduced staining was particularly evident in the granular layer. CLDN8 showed increased intensity of basal layer staining in normal epidermis (Fig 4, *C*). The mRNA expression of *CLDN8* and *CLDN23* was also significantly reduced in lesional epidermis (*P* < .05; Fig 4, *E* and *F*).

Markers associated with activated T cells and other inflammatory cells are enriched by using LCM

Using individual cell-culture expression data (keratinocytes, fibroblasts, activated and unactivated T cells, DCs, and LCs),[33-35] we explored the distribution of DEGs identified in epidermal, dermal, and bulk AD transcriptomes[27] using a Gene Set Enrichment Analysis. Whereas only the keratinocyte gene subset was enriched in the epidermal transcriptome (Fig 5 and see Table E12, *A*, in this article's Online Repository at www.jacionline.org), the dermal transcriptome showed enrichment of key inflammatory subsets, including activated and unactivated T cells, various DC subsets (immature and mature DCs), macrophages, fibroblasts, and lymphatic endothelial cells (Fig 5 and see Table E12, *B*). The bulk transcriptome showed a significant enrichment for keratinocytes, fibroblasts, and inflammatory subsets, with no enrichment for activated T cells and mature DCs (Fig 5 and see Table E12, *C*).

The Gene Set Enrichment Analysis also linked key mediators (*IL22*, TNF superfamily, member 4 [*TNFSF4*/OX40L], and *CTLA4*) detected in the dermal transcriptome to the gene signature of activated T cells (see Table E12, *B*). Among the genes found to be associated with both activated and unactivated T cells in the dermal transcriptome were *GZMB*, *ICOS*, and *CD27*.

CCR7, which is associated with T-cell trafficking and cutaneous lymphoid aggregates,[36,37] was shown to be expressed by various cells types, including T cells and DCs. To investigate the predominant cellular expression of CCR7, we performed double immunofluorescence staining for CD3$^+$ T cells (green) or CD11c$^+$ myeloid DCs (green) with CCR7 (red) in lesional, nonlesional, and normal skin. CCR7 colocalized mainly with CD11c, showing increased expression in lesional versus nonlesional and normal dermis and some colocalization with CD3, primarily in lesional dermis (see Fig E6 in this article's Online Repository at www.jacionline.org). Thus CCR7 is preferentially expressed by myeloid DCs rather than T cells.

DISCUSSION

Evolving disease concepts associate the AD phenotype with barrier and immune abnormalities.[1,2] In this model altered proliferation and differentiation of keratinocytes result from

cytokines derived from distinct T-cell subsets.[2,12] It is important to understand the relative contributions of the epidermal and dermal compartments in creating the abnormal lesional phenotype to fully understand the pathogenic mechanisms driving AD.[26]

This is the first report that establishes robust epidermal and dermal genomic signatures of lesional and nonlesional AD and normal skin compared with corresponding whole-tissue fingerprinting. Using bulk skin, we have associated the AD phenotype with T_H2/T_H22 immune activation and abnormal epidermal differentiation.[11-14] However, in bulk genomic profiling we could not determine which compartments are responsible for individual gene expression or locate their cellular sources.[4,12,27] Furthermore, because of a dilution effect in bulk tissues, which are composed of functionally heterogeneous cells, many genes linked to AD pathogenesis are present at less than the detection level on microarrays.[11,12,26,27] Additionally, because a large subset of genes are expressed in either the epidermis or dermis only, some of the measured differential gene expression between lesional and nonlesional skin appears to be an artifact of the unequal contribution of epidermal expansion in lesional skin rather than because of true changes in gene expression at the cellular level. The previous disease model for AD, which has relied on bulk tissue genomic profiling, is limited by the inability to distinguish between these differences. A large proportion of DEGs found only in bulk tissue seem to derive from unequal contributions of lesional epidermis and dermis compared with nonlesional epidermis and dermis. Overall, we believe that the differences in cellular physiology created by AD are best detected in the LCM-generated DEGs.

Using the LCM method followed by genomic and cellular studies, we have identified a largely increased AD transcriptome, with an additional 674 upregulated and 405 downregulated genes compared with prior reports.[11,12,26,27] By separating the 2 compartments, we have also identified key immune and barrier markers that are usually undetectable on arrays (ie, IL-22 and TSLP) and obtained more robust genomic differences for most immune genes when comparing lesional, nonlesional, and normal tissues. Through this approach and previously acquired pathways and cell-specific genomic maps,[11-14,26,27] we have localized many immune and barrier genes to the epidermis or dermis (or both), leading to a deeper understanding of inflammatory circuits and the cellular subset involved in creating the AD phenotype. For example, dermal expression of CCL19 and CCL21 likely attracts $CCR7^+/CD11c^+$ DCs and might organize lymphoid tissue in the dermis. Our LCM and genomic approach also led to enrichment of gene products associated with activated T cells and inflammatory DCs that play a role in effector responses in patients with AD.[37]

Our data are the first to identify IL-34, the newly identified cytokine in mice models, and its receptor, CSF-1R, in a human skin disease. The mouse studies demonstrated a critical role for IL-34 in differentiation and proliferation of LCs in the epidermis during steady states, whereas repopulation of LCs in inflammatory states was independent of IL-34.[28] Although IL-34 is mainly produced by keratinocytes, its receptor, CSF-1R, originates from dermal macrophages and mononuclear cells.[29] Our data show decreased epidermal expression of IL-34 in lesional epidermis compared with that seen in both nonlesional AD and normal epidermis (Fig 3, B and C). This confirms the role of IL-34 in the maintenance phase, corresponding to prior mouse data.[28]

Thus the IL-34 cytokine might function as a negative regulator, and its induction in nonlesional skin might inhibit the propagation of the inflammatory cascade toward development of active skin lesions. Additionally, we found that IL-34 expression in lesional dermis colocalizes most commonly with myeloid DCs and macrophages. Future studies are needed to evaluate the possible functions of the IL-34/CSF-1R cytokine-receptor complex in background and diseased AD skin and whether strategies of increasing levels of this cytokine might be able to prevent development of skin lesions.

Separating the 2 compartments, we also identified 2 novel barrier genes, CLDN4 and CLDN8, for the first time in the AD transcriptome. Claudins are pivotal for TJ formation.[38] Prior mouse and human skin equivalent models demonstrated that CLDN4 colocalized with CLDN1 to the epidermal granular layer,[39,40] whereas CLDN8 was previously shown in human kidneys and intestines.[41,42] Nevertheless, our knowledge of claudins and their involvement in the barrier alterations seen in patients with AD remains incomplete.[29] De Benedetto et al[32] reported that CLDN1 and CLDN23 show significantly reduced expression in nonlesional AD skin compared with that seen in healthy skin. In our study CLDN8 and CLDN23 showed significantly reduced expression in lesional compared with nonlesional and normal epidermis by using microarrays (Fig 4, A, and see Table E11), with respective fainter epidermal staining of CLDN4, CLDN8, and CLDN23 in lesional versus nonlesional AD and normal epidermis (Fig 4, B and D). Claudin 11 was identified as a novel dermal claudin, which was previously shown in other tissues (brain, testis, and cochlea).[43-45] Future experiments are needed to investigate the function of the newly identified CLDN4 and CLDN8, but together with CLDN1 and CLDN23, these might contribute to the barrier defect associated with the AD phenotype.

Our study also had some limitations. First, LCM is a labor-intensive method, usually allowing analyses of a small number of samples, as in our study (n = 5 lesional and nonlesional specimens each and n = 3 normal samples separated into epidermis and dermis categories), possibly resulting in weaker statistical power, particularly for bulk tissue comparisons. Second, although our LCM approach detected novel barrier genes, the downregulation of key differentiation markers (ie, FLG, LOR, and involucrin [IVL]) in the lesional and nonlesional (vs normal) epidermal transcriptomes was not as impressive as in prior bulk data,[13,26] which is contrary to the enrichment of FLG and LOR in a prior normal skin epidermis LCM study,[17,46] perhaps because of lower recovery of granular layer products using the epidermal-dermal separation approach.[47] Third, even though the combined LCM epidermal and dermal transcriptomes were larger than the bulk tissue transcriptome, many genes were only detected in the bulk transcriptome (see Fig E2 and Table E8, C). This might be explained by (1) the unequal contribution of lesional epidermis and dermis compared with their counterparts; (2) possible inclusion of subcutaneous tissue in bulk biopsy specimens; (3) platform/technical issues, as described in a previous LCM article separating the epidermis and dermis from only 3 psoriatic (lesional and nonlesional) and 3 normal tissues[16]; and (4) restriction of this analysis to patients with severe AD, in whom nonlesional skin has a more abnormal phenotype.[26] Thus many genes might not pass defined thresholds of FCH and significance when comparing lesional and nonlesional skin.

Our study establishes the utility of LCM in patients with AD to separate different skin compartments and cellular infiltrates. It provides complementary information to bulk analysis, allowing regional and/or cellular localization of key barrier or immune molecules, and enables detection of genes that are not usually detected on arrays because of the mixture of transcripts within the heterogeneous bulk tissue.[48,49] LCM is particularly beneficial for AD, in which a complex network of immune and barrier abnormalities results in the global phenotypes of active and "normal-appearing" skin, and dissecting the individual skin components and cells is crucial to unraveling their respective contributions to pathogenesis. Our combined LCM and genomic approach can be useful in future studies aimed at dissecting the relative roles of barrier versus immune activation of different AD phenotypes (ie, intrinsic and extrinsic AD)[11] and for dissecting therapeutic responses to various agents that are now in clinical trials for patients with AD.[50]

Clinical implications: Our approach can be useful to differentiate the ability of targeted treatments to reverse epidermal and immune alterations in AD skin.

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2.3 Evaluation of Atopic Dermatitis Mouse Models

2.3.1 Prelude

In preclinical evaluation of new treatments, proper disease models are of great importance. Apart from the disease phenotypes, the molecular signature of the models as compared to their respective controls is of great interest in a translational perspective.

In this work I compared the molecular signatures of two knockout, one spontaneous, and three induced murine models of AD, to my meta-analysis derived AD transcriptome.

In essence this study provides a comprehensive transcriptomic comparison of common murine models of AD, and reveals that no single model captures all aspects of AD on a molecular level. Therefor we recommend the choice of model to depend on the desired aspects to be covered.

1   **Major differences between human atopic dermatitis and murine models as**

2   **determined by global transcriptomic profiling**

3

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Abstract

Background: Atopic dermatitis (AD) is caused by a complex interplay between immune and barrier abnormalities. Murine models of AD are essential for preclinical assessments of new treatments. While many models have been used to simulate AD, their transcriptomic profiles are not fully understood, and a comparison of these models with the human AD transcriptomic fingerprint is lacking.

Objective: We sought to evaluate the transcriptomic profiles of six common murine models and determine how they relate to human AD skin.

Methods: Transcriptomic profiling was performed using microarrays and qRT-PCR on biopsies from NC/Nga, flaky-tail, *Flg*-mutated, ovalbumin-challenged, oxazolone-challenged, and IL-23-injected mice. Gene expression data of AD, psoriasis, and contact dermatitis were obtained from previous patient cohorts. Criteria of fold-change/FCH≥2 and false discovery rate/FDR≤0.05 were used for gene arrays.

Results: IL-23-injected, NC/Nga, and oxazolone-challenged mice show the largest homology with our human meta-analysis derived AD (MADAD) transcriptome (37%, 18%, 17%, respectively). Similar to human AD, robust Th1, Th2, and also Th17 activation are seen in IL-23-injected and NC/Nga mice, with similar, but weaker, inflammation in ovalbumin-challenged mice. Oxazolone-challenged mice show a Th1-centered reaction and flaky-tail mice demonstrate a strong Th17 polarization. *Flg*-mutated mice display FLG down-regulation without

4

85   significant inflammation.

86   **Conclusion:** No single murine model fully captures all aspects of the AD profile;

87   instead, each model reflects different immune or barrier disease aspects. Overall,

88   among the six murine models, IL-23-injected mice best simulate human AD; still,

89   the translational focus of the investigation should determine which model is most

90   applicable.

91

92   **Clinical Implications:** When testing new drugs for atopic dermatitis, murine

93   models might be used to study barrier or immune features, but human trials are

94   needed to determine effects on actual disease profile.

95

96   **Capsule Summary:** Differences between human atopic dermatitis/AD and

97   murine profiles are significant regardless of the model. While IL-23-injected mice

98   best replicate human AD, the choice of model should be guided by the

99   translational focus of the investigation.

100

101   **Keywords:** Atopic dermatitis; Th1; Th2; Th17; psoriasis; contact dermatitis;

102   mouse model; NC/Nga; filaggrin; oxazolone; ovalbumin; IL-2

103

104   **Abbreviations:**

105   AD: Atopic dermatitis

106   AQP7: Aquaporin 7

107   CCL: Chemokine (C-C motif) ligand

108   CD: Contact dermatitis

109   Claudin 8: CLDN8

110   CXCL: Chemokine (C-X-C motif) ligand

111   DC: Dendritic cell

112   DEGs: Differentially Expressed Gene

113   ELOVL3: Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3,

114   yeast)-like 3

115   FCH: Fold change

116   FLG: Filaggrin

117   HGNC: Human Genome Organization (HUGO) Gene Nomenclature Committee

118   IL: Interleukin

119   IFN: Interferon

120   KEGG: Kyoto Encyclopedia of Genes and Genomes

121   KRT16: Keratin 16

122   LCN2: Lipocalin 2

123   LOR: Loricrin

124   MADAD: Meta-analysis derived AD

125   PCA: Principal Component Analysis

126   PVCA: Principal variation component analysis

127   qRT-PCR: Quantitative reverse transcription PCR

128   SC: Stratum corneum

129   TEWL: Transepidermal water loss

130   Th: T helper

131   TLDA: TaqMan Low Density Array

132

Introduction

133

134   Atopic dermatitis (AD) is the most common inflammatory skin disease,[1] caused

135   by an interplay between immune and barrier abnormalities.[2,3] Unlike psoriasis,

136   another common inflammatory skin disease that is largely Th17/IL-23 polarized,[2]

137   AD is characterized by activation of multiple cytokine pathways.[4,5] In addition to

138   immune activation, reduced expression of barrier components (terminal

139   differentiation proteins, tight junctions and lipids) is also characteristic of AD.[6-9]

140   Transcriptomic profiling of AD tissues at baseline and after treatment with broad

141   and specific agents has recently increased our understanding of the disease.[10-12]

142   While these studies highlight a critical role for the Th2 axis in AD,[10,13,14] they also

143   suggest increased Th17/IL-23 and Th22 activation in AD, and particularly in some

144   subtypes (i.e. Asian and intrinsic AD).[4,5,15] Although human testing is needed to

145   elucidate the pathogenic contributions of various cytokines in AD, murine AD

146   models are essential for assessing new drugs at the preclinical level.[16,17]

147   An ideal murine model should replicate both the epidermal barrier

148   disruption and the cutaneous inflammation of the human disease. While many

149   models have been used to simulate human AD, their transcriptomic profiles and

150   relevance to the AD skin profile are not fully understood, since a direct

151   comparison between frequently used models and human AD is lacking. A number

152   of spontaneous genetically engineered, and inducible "AD-like" models have

153   been described. These include NC/Nga,[16] flaky-tail (*Tmem79^{ma/ma} Flg^{ft/ft}*),[18-20] *Flg*-

154   mutated (*Flg^{ft/ft}*),[21] oxazolone (Oxa)-challenged,[22,23] and ovalbumin (Ova)-

155   challenged mice.[24-27] Furthermore, IL-23-injected mouse model, which has been

156 traditionally considered to resemble psoriasis,[28,29] and exhibits the largest (25%)

157 transcriptomic homology with human psoriasis fingerprinting among existing

158 "psoriasis-like" models,[30] has also been found to display Th2 activation and

159 simulate "AD-like" skin inflammation.[31] While all murine "AD-like" models (with

160 the exception of *Flg*-mutated mice)[16,19-21,23,25,28] are visibly inflamed, it is difficult

161 to macroscopically differentiate "AD-like" dermatitis in mice from lesions

162 mimicking contact dermatitis (CD) or psoriasis. For example, since flaky-tail mice

163 exhibit Th17-dominated skin inflammation,[32,33] this murine model may more

164 closely simulate a "psoriasis-like" phenotype rather than an "AD-like"

165 fingerprinting.

166   To evaluate the transcriptomic profiles of common AD models and

167 determine how closely they resemble the intricate cytokine and epidermal

168 abnormalities in AD skin, we profiled five common "AD-like" murine models, as

169 well as the IL-23-injected model, previously considered to best mimic psoriasis.

170 To be able to contrast all polar cytokine responses in humans, the murine profiles

171 were compared with transcriptomic profiles of AD, and two other common

172 inflammatory skin diseases, psoriasis and CD, that share clinical and tissue

173 characteristics with AD.[9,34,35] We found that no single model fully captures all

174 aspects of AD, but surprisingly IL-23-injected mice show the most similar

175 transcriptomic profile to human AD.

176   **Methods**

177   **Murine models**

178   Transcriptomic profiling was performed using microarrays and qRT-PCR on

179   biopsies from six models, including NC/Nga, flaky-tail, *Flg*-mutated, Ova-

180   challenged, Oxa-challenged, and IL-23-injected mice. Controls for murine models

181   generally had similar genetic background, comprising the C57BL/6 (Flaky-tail,

182   *Flg*-mutated*,* Ova and IL-23), NC/Nga without mite infestation (NC/Nga) and

183   BALB/c (Oxa) laboratory strains. The samples of flaky-tail, *Flg*-mutated, Ova-

184   challenged, and NC/Nga mice were harvested at Kyoto University, Kyoto, Japan

185   (**Table 1**). The specimens from Oxa-challenged mice were collected at Leo

186   Pharma A/S, Ballerup, Denmark (**Table 1**). The IL-23-injected mice gene array

187   data were acquired from a previously published cohort (GSE50400),[30] and

188   available RNA samples from these mice were used for qRT-PCR. See

189   Supplementary Methods section in this article's Online Repository (OR) at

190   www.jacionline.org for more details. The Institutional Animal Care and Use

191   Committee of Kyoto University and Leo Pharma A/S approved all experiments.

192   Representative images of each model are shown in **Fig 1A**. Ear tissues

193   from flaky-tail, *Flg*-mutated, NC/Nga, and Oxa-challenged mice were used, while

194   dorsal skin was harvested from Ova-challenged and IL-23-injected mice. Flaky-

195   tail,[20] NC/Nga,[36] Ova-challenged,[25] and Oxa-challenged models[23] exhibit

196   increased serum IgE levels. *Flg*-mutated mice show normal IgEs,[18] and IgE data

197   are unavailable for IL-23-injected mice (**Table 1**).[30]

198

Human cohorts

199

200   The AD microarray data was obtained from our meta-analysis derived AD

201   (MADAD) transcriptome,[9] comprised of four AD lesional vs. non-lesional datasets

202   with a total of 97 samples (41 paired lesional/non-lesional samples).

203   This study also includes a previously published cohort of 25 AD patients (9

204   females and 16 males; age, 23-73 years [median, 45 years]) with SCORAD

205   scores from 33 to 77 (mean, 56.7; SD, 12.2) and serum eosinophils of 0.6% to

206   11.8% (median, 4.3%); among AD patients, 14/25 (56%) had extrinsic AD (serum

207   IgE levels, 254-3,000 kU/L; median, 1,292.8 kU/L).[5,11] Ten previously published

208   patients with moderate-to-severe psoriasis were included for comparisons

209   (Psoriasis Area Severity Index, 8.4-59.5; mean, 20.3; SD, 14.5).[5,37] Thirteen

210   previously published patients with allergic CD (n=10 with nickel allergy; n=3 with

211   fragrance allergy) were included.[34] Biopsies from positive patch tests to these

212   allergens were taken at 72 hours from patch test placement. All these subjects

213   were European Americans. Patients' characteristics are summarized in **Table E1**,

214   and the entire list is available in **Table E2**.

215

Quantitative RT-PCR and gene arrays

216

217   RNA was extracted with EZ-PCR Core Reagents (Life Technologies, Grand

218   Island, NY). RNA was reverse transcribed to cDNA using the High Capacity

219   cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). TaqMan

220   Low Density Array (TLDA) cards (384 well plates preloaded with TaqMan assays)

221   were used for qRT-PCR. For IFN-γ, IL-13, and IL-17A, which are frequently

11

222   undetectable with TLDA, single Taqman qPCR was performed. All primers are

223   listed in **Table E3**. For IL-23-injected mice, PCR analyses were performed using

224   stored samples from a previous report.[30] Expression values (threshold cycle/Ct)

225   were normalized to *Rplp0*.

226   Affymetrix Mouse Gene arrays 2.1 ST (Affymetrix, Santa Clara, CA) were

227   used for flaky-tail, *Flg*-mutated, Ova-challenged, and NC/Nga mice. Affymetrix

228   Mouse Gene arrays 1.0 ST were used for Oxa-challenged mice, while the

229   previously published IL-23-injected murine transcriptomic data were analyzed

230   with GeneChip Mouse Genome 430 2.0 Array (Affymetrix).

231   The MADAD transcriptome combined all effect sizes for each probe-set,

232   and identified a set of 595 differentially expressed genes (DEGs; 387 up- and

233   208 down-regulated) using classical fold change (FCH)≥2 and false discovery

234   rate (FDR)≤0.05 criteria.[9] For the qRT-PCR comparison, we used previously

235   published data obtained from above-mentioned human cohorts. Since non-

236   lesional AD skin is abnormal,[38] we also included a comparison of lesional AD to

237   normal skin.

238

239   **Bioinformatics and statistical analyses**

240   Murine microarray data were preprocessed in R by the *justRMA* function of the

241   *affy* package and normalized using the *normalize.quantiles.robust* or

242   *normalize.quantiles.use.target* (IL-23 and Oxa models) function of

243   *preprocessCore* package.[39,40]

244   Changes in expression profiles of each murine model and its controls

245   were performed using the linear model framework of *limma* package.[41] P-values

246   from moderated t-test were adjusted for multiple testing using Benjamini-

247   Hochberg procedure.

248   Cross-species-annotation from murine to human ensemble IDs was

249   performed using the *getLDS* function *biomart* package.[42-45]

250   Linear models were applied to analyze TLDA and qRT-PCR expression

251   data, based on *Rplp0* normalized and negative transformed Ct (-dCt) values.

252   Prior to applying these models to TLDA and qRT-PCR expression data, but after

253   normalization, we replaced non-detected -dCt values with 20% of the minimum

254   dCt for each gene across all samples, while keeping it in the possible range of -

255   40 to 40.

13

256   **Results**

257

258   **Transcriptomic profiling of "AD-like" murine models**

259   While transcriptomic fingerprinting has been thoroughly performed in AD

260   skin,[4,5,38,44-47] a similar analysis is lacking for "AD-like" murine models. To

261   establish the individual transcriptomic profiles of prominent "AD-like" models and

262   their similarity to human disease, we profiled skin from five common AD-like

263   models[20,21,23,25,36] as well as from the IL-23-injected model, considered to best

264   represent the IL-17/IL-23-centered inflammation in psoriasis,[30] using gene arrays

265   (**Table 1**). Criteria of FCHs≥2 and FDR≤0.05 were used to define DEGs between

266   diseased murine skin and respective controls. The lists of DEGs for each model

267   are listed in **Table E4**.

268   A Venn diagram, based on unique Human Genome Organisation (HUGO)

269   Gene Nomenclature Committee (HGNC) symbol orthologs and proportional to

270   DEGs, illustrates the overlap between murine models (**Fig. 1B**). Flaky-tail and

271   *Flg*-mutated models are excluded from the diagram since they display the lowest

272   number of DEGs (n=226 and n=47, respectively), despite being among the

273   largest sample sizes (n=5; **Table 1**). Overall, IL-23-injected mice show the

274   highest number of DEGs (n=2753), followed by Oxa-challenged (n=1004),

275   NC/Nga (n=742), and Ova-challenged (n=373) mice. Only 67 DEGs are shared

276   among NC/Nga, Ova-challenged, Oxa-challenged, and IL-23-injected mice, as

277   represented in the center of the Venn diagram (**Fig 1B**).

278

279   **Murine models show differential profiles compared to human AD**

280   To compare how well "AD-like", and the IL-23-injected models represent human

281   AD, each murine transcriptomic profile was compared with the meta-analysis

282   derived AD (MADAD),[9] a robust AD transcriptome across four independent

283   cohorts. The murine transcriptomes represent only 37%, 18%, 17%, and 11% of

284   the human AD profile (MADAD), for IL-23-injected, NC/Nga, Oxa-challenged, and

285   Ova-challenged mice, respectively (**Fig 1C**). Only 4% of DEGs in flaky-tail mice

286   and 1% in *Flg*-mutated mice overlap with MADAD. The poor homology between

287   human AD and the murine skin profiles is also illustrated as a heatmap, in **Fig E1,**

288   showing relative expressions of MADAD genes within all murine transcriptomes.

289   **Fig E2** illustrates expression data by sample. Surprisingly, the transcriptome of

290   IL-23-injected mice best represents the up- and down-regulated components of

291   the MADAD followed by NC/Nga, Oxa-challenged and Ova-challenged mice. *Flg*-

292   mutated and flaky-tail profiles are not well reflective of the MADAD increased or

293   decreased elements. The MADAD DEGs with corresponding FCHs and FDRs

294   are shown in **Table E5,** for each model.

295   Since the choice of cut-offs and hybridization kits can influence

296   intersections between DEGs of various transcriptomes,[48] the MADAD and

297   transcriptomes of six murine models were also compared using ranked gene lists

298   instead of cut-offs, as previously described.[29,30] The 5000 highest up- and down-

299   regulated genes in each mouse transcriptome were identified and ranked

300   according to FCHs, with lowest ranks assigned to the genes with highest

301   regulations. We then analyzed the overlap of MADAD with ranked genes in each

15

302   murine model. In **Fig. 1D**, the red and blue lines correspond to the overlap

303   between up- and down-regulated genes, respectively, of murine models and

304   MADAD at any given rank N, where N=1, …, 5000. The light blue line outlines

305   the level of overlap expected by chance (the 95% confidence region of

306   hypergeometric distribution under the null hypothesis), such that any line above it

307   indicates a significant level of overlap and farther distances from it show a higher

308   overlap.[49] For any given rank, IL-23-injected mice represent the highest and most

309   significant overlap with the MADAD, and in decreasing order for Oxa-challenged

310   and NC/Nga mice. The Ova-challenged model has significant overlap with genes

311   up-regulated in human AD, but fails to capture the down-regulated signature of

312   human AD. The transcriptomes of flaky-tail and *Flg*-mutated mice have poor

313   overlap with human AD, consistent with the standard cut-off based approaches

314   (**Fig 1 B-C**).

315   To associate different mice profiles with relevant biological functions in

316   human AD, an enrichment analysis was also conducted, using KEGG

317   pathways.[50,51] Selected pathways significantly enriched in human AD and in the

318   murine models are shown in **Fig 2A** and **Table E6,** with the black vertical line

319   representing FDR=0.05. The genes represented in each pathway are listed in

320   **Table E7**. The most significantly enriched pathways in the MADAD includes

321   PPAR signaling, cytokine-cytokine receptor interaction, JAK-STAT signaling, T

322   cell receptor signaling, chemokine signaling, cell adhesion molecules, and

323   adipocyte signaling pathways. IL-23-injected mice best simulate the inflammation

324   and lipid pathways in human AD, with enrichments in some pathways also seen

16

325   with NC/Nga and Oxa-challenged mice. Ova-challenged mice only share the

326   cytokine-cytokine receptor interaction, PPAR signaling and biosynthesis of

327   unsaturated fatty acids pathways with MADAD. A single lipid pathway

328   (biosynthesis of unsaturated fatty acids) is shared between *Flg*-mutated and

329   flaky-tail mice and the MADAD, although this pathway is not significant for the

330   MADAD. Retinol metabolism, hedgehog signaling and melanogenesis pathways

331   are enriched in flaky-tail mice, but are not enriched in MADAD.

332   **Fig 2B** illustrates the top 30 up- and down-regulated MADAD DEGs and

333   their representation in each model (**Table E5**). Again, IL-23-injected mice best

334   represent not only the up- (e.g. CCL18, S100A8, KRT16) but also the down-

335   regulated DEGs (e.g. CLDN8) in the MADAD, while NC/Nga, Ova-challenged,

336   and Oxa-challenged mice mostly capture the up-regulated but not the down-

337   regulated genes in the MADAD. The flaky-tail model only poorly represents key

338   AD genes, and *Flg*-mutated mice entirely lack the top up- and down-regulated

339   human AD genes. The IL-23 model captures some lipid (ELOVL3), tight junction

340   (CLDN8) and water channel (AQP7) deficiencies, previously reported to

341   characterize the barrier defects in AD.[9,44,52,53]

342

343   **Transcriptomic homology between mice and inflammatory skin diseases**

344   Since the signatures of the six murine models showed limited resemblance to

345   human AD, we expanded our comparison to also include transcriptomic profiles

346   of two other well-characterized polar inflammatory skin diseases, psoriasis and

347   contact dermatitis (CD).[5,11,34,37] We then performed unsupervised clustering with

17

348   percentages reflecting robustness of each branch in the dendrogram (**Fig 3A**).

349   The resultant dendrogram establishes a tight cluster of inflammatory skin

350   diseases, with clear separation from murine models. *Flg*-mutated and flaky-tail

351   mice cluster together and farther apart from other models. To elucidate the

352   relative distances between individual models and human disease and to identify

353   which murine model is closest to the human diseases, we performed a principal

354   component analysis (PCA) (**Fig. 3B).** The PCA plot shows that murine models

355   are separated from inflammatory skin diseases. IL-23-injected mice are closest to

356   the AD profile (MADAD) on the PC1 axis, followed by CD (to fragrance) and

357   surprisingly only then by psoriasis (**Fig. 3B**). Indeed, a principal variation

358   component analysis (PVCA) also indicates that the organism explains 41.5% of

359   the variations, with disease or mouse model accounting only for 12% (data not

360   shown).

361

362   **qRT-PCR highlights significant differences in polar immune activation**

363   **among murine models**

364   To validate and extend the microarray findings to polar cytokines, qRT-PCR was

365   performed on a wide array of hallmark human AD inflammatory and barrier genes

366   that are often below detection levels in arrays and share murine orthologs. The

367   mRNA expressions were also compared to those obtained in our AD and

368   psoriasis cohorts, and, for selected, available genes also to CD (to nickel and

369   fragrance) populations (**Fig. 4)**.[34] Overall, similar patterns are seen in qRT-PCR

370   and gene arrays. IL-23-injected, NC/Nga, Oxa-challenged and Ova-challenged

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371   mice show large increases in innate, Th2, and Th1 cytokine pathways (IL-1β, IL-

372   13, IFN-γ), as well as increased KRT16 (marking epidermal hyperplasia),[54] and

373   the Oxa-challenged model has the highest activation of the Th2 cytokine, IL-13.

374   Only IL-23-injected and NC/Nga mice reflect all diverse cytokine pathways,

375   including IL-17A activation. The down-regulation of terminal differentiation genes

376   (filaggrin/FLG, loricrin/LOR) that characterizes AD is best captured by *Flg*-

377   mutated and flaky-tail models, and to some extent by IL-23-injected, Ova, and

378   Oxa models but is missing in NC/Nga mice.

379   **Discussion**

380   Over the past decades, many murine "AD-like" models,[20,21,23,25,36] including

381   humanized AD,[55] topical vitamin D analogue application,[56,57] or

382   allergen/staphylococcal enterotoxin B models,[56-58] have been developed as tools

383   for understanding the AD pathogenesis and as preclinical models. The most

384   complete view of the human AD profile is based on transcriptomic profiling of skin

385   lesions.[4,5,9] Key AD features include prominent activation of inflammatory

386   pathways and large defects in epidermal differentiation, tight junctions and lipid

387   profiles.[38,44-46,59,60] Unlike psoriasis that is largely Th17/IL-23-polarized, AD shows

388   more diverse cytokine expression, with Th2, but also Th22 pathway activation,[2]

389   with possible roles also suggested for IL-23/Th17 axis, particularly in specific AD

390   populations, such as Asian AD.[5]

391   Global transcriptomic profiling of individual murine models is essential for

392   understanding their distinct cytokine and barrier alterations and how well they

393   resemble human AD fingerprinting, as compared with profiles of other common

394   polar inflammatory skin diseases.[29,30] This study is the first to evaluate the

395   fingerprinting of commonly used "AD-like" (and a representative "psoriasis-like")

396   murine models and their relevance to human AD, but also to psoriasis and CD.

397   A proper model of human AD skin should appropriately represent its two

398   main features, the relevant cytokine networks and the epidermal pathology.

399   However, our data suggest that no single murine model is able to accurately

400   simulate both aspects of AD. Our data separates the models into two clusters.

401   One group includes flaky-tail and *Flg*-mutated mice, which are least reminiscent

402   of human AD, and particularly of its immune skewing. Flaky-tail mice show a

403   Th17-dominated polarization and *Flg*-mutated mice demonstrate no apparent

404   clinical or tissue inflammation, which is quite different from the human AD profile.

405   The other group, including IL-23-injected, NC/Nga, Oxa-challenged, and Ova-

406   challenged mice, show a broad range of polar immune activation. With the

407   exception of the IL-23-injected model, which simulates some barrier features of

408   human AD, such as reductions in tight junctions (CLDN8), lipids and LOR, other

409   models in this group mostly do not reflect the epidermal abnormalities of AD.

410   Surprisingly, among all evaluated models, IL-23-injected mice show the

411   largest overlap with human AD skin, which is unexpected since this model has

412   been traditionally used for psoriasis.[61] This model demonstrates remarkable

413   activations of innate immunity, Th1, Th2, and Th17/IL-23 axes, as well as some

414   epidermal alterations. Still, IL-23-injected mice have increased neutrophils and

415   only sparse eosinophils and mast cells,[30,31] that can be found in human AD.[62,63]

416   Furthermore FLG down-regulation, which represents a hallmark of AD, is absent

417   in this model. This model thus replicates the Th2 activation of AD, as well as the

418   Th17/Th22 signature of psoriasis and some AD subtypes. While IgE levels have

419   not been evaluated in our IL-23 model,[30,31] IgE responses take at least several

420   weeks to develop.[25] Perhaps, unlike other models, which have more chronic T-

421   cell activation, and are able to induce IgE responses over time, IL-23 model is a

422   short-term skin inflammation model (profile is created within 7d).

423   While the NC/Nga model does not reflect the various barrier defects of

424   human AD, including decreases in differentiation, tight junctions, and lipids (i.e

21

425   FLG, LOR, CLDN8, ELOVL3),[9,38,44-46] it clearly shows the diverse immune

426   polarizations in human AD, including innate, Th1, Th2, and Th17 pathways. Thus,

427   use of NC/Nga mice might be useful to investigate the complex cytokine

428   interactions, but not the barrier aberrations of AD. Ova-challenged mice also

429   replicate activation of inflammatory pathways in AD, although to a lesser extent

430   than IL-23-injected and NC/Nga models.

431   Oxa-challenged mice demonstrate high Th1 and marginal Th2 inductions,

432   with only limited barrier defects (LOR). IL-17A induction is absent in these mice,

433   but a few downstream mediators (S100A8, lipocalin 2/LCN2) are up-regulated.

434   Flaky-tail mice show selective Th17 activation, as previously reported.[32,33]

435   While the Th17 axis is a central driver of psoriasis, some role of this pathway in

436   AD, including induction of the Th2 axis, has recently been suggested.[5,64,65] Thus,

437   while flaky-tail mice replicate some Th17 activation and reductions in FLG and

438   LOR seen in AD, data obtained from these mice should be applied cautiously to

439   human AD as this model lacks Th2 activation, which is the hallmark of the AD

440   immune responses.

441   *Flg*-mutated mice are the only murine model showing significant down-

442   regulation of FLG, similar to reductions observed in human AD.[66-68] However, *Flg*-

443   mutated mice lack both clinical and transcriptomic skin inflammation,[21] and

444   additional barrier abnormalities that are seen in AD, suggesting its limited use as

445   a model to replicate the inflammation and global barrier defects in AD. A

446   summary of clinical and laboratory characteristics of all analyzed models is

447   provided in **Table 2**.

22

448   Human inflammatory skin diseases share a number of features, but

449   maintain distinct differences in tissue structure and immune activation. While

450   down-regulation of differentiation genes clearly differentiates AD from the other

451   two inflammatory skin diseases, other immune and epidermal features are

452   shared among the 3 conditions. These characteristics include increased

453   epidermal hyperplasia and activation of Th1, Th17, and Th22 pathways, although,

454   as expected, Th2 polarization is minimal in psoriasis compared to AD.[5]

455   Our study has several limitations: 1. Only mRNA, but not protein levels

456   were measured, and assessments were limited to a single time-point; 2. We

457   mainly used B6 mice, although BALB/c might be more appropriate in certain

458   cases; 3. We did not perform functional *in-vivo* tests (i.e., transepidermal water

459   loss/TEWL, corneal water content, and pruritus measures).[16,23] The biopsies

460   were taken from different locations and at different ages in mice, possibly

461   affecting gene expression.[69] Last, comparisons with different AD endotypes were

462   not possible here, and should be addressed by future larger cohort studies of

463   various ages, races, etc.

464   Overall, no single murine model fully captures all immune and barrier

465   aspects of human AD skin, and phenotypical differences between AD and murine

466   models are significant regardless of which model is chosen. Nevertheless, of the

467   models studied, IL-23-injected mice appear to best replicate the human AD

468   profile. IL-23-injected and NC/Nga mice demonstrate a wide array of

469   inflammatory axes, including Th2 activation, and are best suited to assess this

470   AD-centric axis, and how it interacts with other activated cytokines in AD. Oxa-

471 challenged and Ova-challenged mice, despite showing remarkable inflammation,

472 are not necessarily AD-specific. IL-23-injected, Oxa-, and Ova-challenged

473 models have the advantage that inducing agents can be used on different mouse

474 backgrounds, including existing knockouts of specific genes, to dissect

475 cutaneous inflammation. Flaky-tail mice fail to capture any key features of human

476 AD. *Flg*-mutated mice are best only to represent the *FLG* down-regulation in AD,

477 but not other AD characteristics. Thus, the choice of which murine model to use

478 depends on the translational focus of the investigator, but caution should be

479 exercised when translating murine data to human inflammatory skin diseases.

480 **Tables**
481
482 Table 1. Mouse sample data

Sample	n	Age	Sex	Biopsy location	Serum IgE elevation	Description	#DEGs (unique HGNC symbols)
Flaky tail	5	26 weeks	female	Ear	+	*Flg* and *ma* mutation	226
Flg-mutated	5	26 weeks	female	Ear	-	*Flg* mutation	47
C57BL/6	5	26 weeks	female	Ear	-	Controls for flaky tail and *Flg*-mutated mice	
NC/Nga (+mite)	5	10 weeks	male	Ear	+	Mite exposure	742
NC/Nga (-mite)	5	10 weeks	male	Ear	-	No mite exposure (controls for NC/Nga +mite)	
Ova-challenged	3	15 weeks	female	Dorsal skin	+	C57BL/6 with ovalbumin application	373
Control	3	15 weeks	female	Dorsal skin	-	Controls for Ova-challenged mice	
Oxa-challenged	3	~10 weeks	female	Ear	+	BALB/c with oxazolone application	1004
Acetone-challenged	3	~10 weeks	female	Ear	-	Controls for Oxa-challenged mice	
IL-23-injected	5	6 weeks	female	Dorsal skin	n/a	C57BL/6 with IL-23 injections	2753
PBS-injected	5	6 weeks	female	Dorsal skin	n/a	Controls for IL-23-injected mice	

483

484 Table 2. The summary of clinical and histological features of mouse models

	Clinical					Histology			
	Visible inflammation	TEWL increase	SC water content reduction	Pruritic activity	Serum IgE increase	Epidermal hyperplasia	Parakeratosis	T cell	DC
Flaky tail[16,20]	+	+	+	+	+	+	+	+	+
Flg-mutated[21]	-	-	-	-	-	-	-	-	-
NC/Nga[16]	+	+	+	+	+	+	+	+	+
Ova[25]	+	+	+	n/a	+	+	+	+	+
Oxa[23]	+	+	+	+	+	+	+	+	+
IL-23-injected[28,31]	+	n/a	n/a	n/a	n/a	+	+	+	+

485

25

486   **Figure legends**

487

488   **Figure 1**: **A.** Representative pictures of murine models. **B.** Venn diagram of

489   overlapping DEGs for 4 AD models. Flaky-tail and *Flg*-mutated models are

490   excluded due to their small number of DEGs. **C.** Proportion of MADAD DEGs

491   represented in each model.[9] IL-23-injected mice appear separated because

492   these data are from our previous study (GSE50400).[30] **D.** Overlap between top

493   up- (red) and down-regulated (dark blue) genes in each model and MADAD was

494   estimated for rank N=1…5000.[70] The light blue line outlines the overlap expected

495   by chance, such that any line above it indicates significant overlap and distances

496   farther from it show higher overlap.[49]

497

498   **Figure 2:** **A.** KEGG analysis in the respective MADAD and murine model

499   transcriptomes. The black horizontal line indicates FDR=0.05. **B.** Heatmap of the

500   top 30 up- and down-regulated MADAD DEGs with orthologs in murine models.[9]

501   Corresponding $\log_2$(FCH) are shown. *P<0.05; **P<0.01; ***P<0.001. Coloring

502   (blue: down-regulated; red: up-regulated) appears for absolute $\log_2$(FCH)≥1, with

503   adjusted p-values<0.05.

504

505   **Figure 3**: Genome-wide neighboring analysis of the 8400 genes represented in

506   human-murine orthologs. **A.** Dendrogram applying unsupervised clustering using

507   Pearson's correlation distance and McQuitty agglomeration. Significant clusters

508   have approximately unbiased (AU) p-values ≥ 95%[71] **B.** PCA using $\log_2$(FCH) for

26

509   the 8400 genes.

510

511   **Figure 4**: Heatmap of qRT-PCR-derived mRNA expression differences. CD data

512   are shown separated because data from only 13 genes was available.[34]

513   Corresponding $\log_2$(FCH) are shown. +P<0.1; *P<0.05; **P<0.01; ***P<0.001.

514   Dendrograms represent the unsupervised clustering with Euclidean distance and

515   McQuitty   agglomeration.   LS=lesional,   NL=non-lesional,   PSO=psoriasis,

516   AD=atopic   dermatitis,   CD=contact   dermatitis

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517

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751

Figure 1   Click here to download Figure No.: Mouse AD Figure 1 0426.pdf

Figure 2

Figure 3

A   **Click here to download Figure No.: Mouse AD Figure 3 0426.pdf**

Figure 4   Click here to download Figure No.: Mouse AD Figure 4 0426.pdf

Color Key — Value: -4, -2, 0, 2, 4

	LOR	FLG	PPL	TNFSF4	IL20	IL17A	IL19	S100A8	CCL22	CCL17	CCL20	IL12B	IL13	STAT1	IL23A	MX1	IFNG	CXCL10	LCN2	CXCL1	IL1B	KRT16
FLG mutant	-0.7+	-2.4***	-0.1	1.5	-2.7	-1.2	-0.7	-1.8	0.1	-0.4	-0.4	0.8	0.6	0.6	0.2	0.1	1.2	1.4	0.2	-0.2	-1.1	-2.1+
Flaky-tail	-1.0***	-0.6	0.1	-0.7	4.5*	8.6***	2.2*	4.9***	-0.1	-1.0*	0.9*	-0.5	-0.6+	0.6+	1.0	-2.7*	-0.6	0.0	2.8***	2.6**	1.1	1.0
Ova	-1.0**	1.9*	-0.9*	-0.1	10.5**	1.9	-2.9*	2.3+	-0.5	0.5	0.8+	0.9	1.2*	0.2	1.9*	1.6	1.4	1.3	2.9***	2.0*	1.2	3.5*
Oxa	-0.9*	1.0*	-1.6*	0.7	-0.7	0.0	8.9*	6.5***	-0.4+	0.2	0.7	1.9	4.4+	2.8*	3.2	5.0**	6.5***	10.8***	1.5*	2.0	5.5**	3.6
NC/Nga	0.6*	3.3***	-0.1	0.7	0.9	5.7***	5.1*	6.9***	1.0***	1.4**	-0.2	1.2	1.1***	0.9**	2.1**	0.1	1.8*	1.8*	3.3***	2.9***	5.6**	6.8***
IL-23	-2.7***	1.4*	-0.5	2.9**	2.5+	2.9***	6.0***	9.6***	0.6	0.3	0.2	0.3	1.8**	1.8**	1.1*	1.8*	3.1***	3.7***	5.9***	4.5***	5.8***	6.6
PSO (LS vs. NL)	-0.7+	-0.5	0.0	-0.1	4.5***	8.5***	7.4***	6.3***	1.9***	1.4*	3.4***	4.0***	0.7	1.2**	1.5**	1.8**	2.7***	2.3*	4.3***	3.1***	1.9***	4.5***
AD (LS vs. NL)	-1.3*	-0.6	0.0	0.2	3.6***	2.5+	4.1***	4.7***	1.5***	2.5***	1.9**	1.9**	3.0***	0.8*	1.2***	1.0**	1.1	2.3*	1.8***	2.7***	2.0*	4.6***
AD (LS vs. Normal)	-3.2**	-1.4*	0.4	1.2*	3.9***	5.6**	6.1***	4.3***	2.8***	4.5***	2.9***	5.0***	5.4***	1.5***	1.4	1.8*	2.8*	3.4*	-0.3	3.9***	2.4*	4.3***
CD (Nickel vs. NL)	6.7***	N/A	N/A	3.2	N/A	4.0***	N/A	N/A	N/A	0.6	1.5*	2.6***	N/A	2.7***	2.9***	2.2*	N/A	4.1***	N/A	1.1	1.9*	3.4**
CD (Fragrance vs. NL)	4.2	N/A	N/A	2.2	N/A	4.0*	N/A	N/A	N/A	0.3	1.2	1.8	N/A	4.3***	3.3*	1.0	N/A	5.6**	N/A	1.5	1.0	3.9

1   **Supplementary Methods**

2

3   **Murine models**

4   The murine sample information is summarized in **Table 1**.

5

6   1. Flaky-tail and *Flg*-mutated mice[1,2]

7   Flaky-tail mice carry a spontaneous *Flg* homozygous frameshift mutation

8   (c.5303delA) and a nonsense mutation (c.840C>G) in the *Tmem79* gene causing

9   a matted (*ma*) hair phenotype (*Tmem79^{ma/ma} Flg^{ft/ft}*).[3,4] *Flg*-mutated mice only

10   have the *Flg* homozygous frameshift mutation (c.5303delA).[1,2] C57BL/6 (B6)

11   mice serve as controls for both models. While flaky-tail mice spontaneously

12   develop skin inflammation, *Flg*-mutated mice do not, since flaky-tail mice lack the

13   *Tmem79* mutation, which is responsible for the eczematous lesions on a B6

14   background.[4] Ear samples were taken at the age of 20 weeks from flaky tail mice

15   which were confirmed to have elevated total IgE, *Flg*-mutated mice, and B6 mice

16   (n=5 per group).

17

18   2. NC/Nga mice[5]

19   NC/Nga mice with mite infestation develop dermatitis-like lesions, whereas

20   their controls, NC/Nga mice without mite infestation, do not.[5] Ear samples were

21   harvested from 10-week-old male NC/Nga mice with and without mite infestation

22   (n=5 per group).

23

24   3. Ova-challenged mice[6]

25   Ova-challenged B6 mice were treated with 10mg/mL of Ova and

26   compared to untreated mice 24hrs after the last application of Ova.[6] Hairs on the

27   dorsal trunk skin of B6 mice were shaved with an electric razor (THRIVE Co Ltd,

28   Osaka, Japan). A single dorsal skin site on each mouse was tape stripped at

29   least five times with adhesive cellophane tape (Nichiban, Tokyo, Japan). After

30   stripping, 10 mg/ml of Ova (Hyglos GmbH, Regensburg, Germany) in phosphate

31   buffered saline (PBS) was applied to the dorsal skin site and covered with

32   transparent film (Suprasorb F; Lohmann & Rauscher, Miami, FL) for 3 days.

33   Next, the film was removed and the skin remained open for 4 days. Then, new

34   Ova was applied on the same skin area as the first application. We repeated this

35   process three times. Elevated Ova-specific serum IgE and IgG1 levels were

36   found. Dorsal trunk skin samples were harvested from 15-week-old female mice

37   at 24 hours after the last application (n=3 per group). Mice without treatment

38   were used as controls.

39

40   4. Oxazolone (Oxa)-induced mice[7]

41   We sensitized both dorsal and ventral faces of the right auricle with 0.8%

42   Oxa in 10 μl acetone, and 7d later elicited with low 0.4% Oxa on the ear on days

43   7, 10, 12, 14, 17, 21, 23, 25, and 27. Skin samples were harvested 2hrs after last

44   application. Mice with acetone application served as controls.

45

46   5. IL-23-injected mouse mice[8]

2

47   The data from a previous cohort was used for gene array (GSE50400) and

48   previously collected RNA samples were used for qRT-PCR.[8] In 6-week-old

49   female B6 mice, the induction of dorsal skin lesions was achieved by daily

50   intradermal injections of recombinant murine IL-23 in 100µl PBS (3 µg,

51   eBioscience, San Diego, CA) from day 1 to 4. All mice were euthanized on day 7

52   and skin samples at the IL-23 injection site were collected for evaluation (n=5 per

53   group).

54   Except the Oxa-challenged model, all mouse models were processed in

55   the same lab, by the same personnel, and with the same protocol.

56

57   **Expression Analysis Preprocessing**

58   The Affymetrix Mouse Gene 2.1 ST chip definition files (cdf) were not available

59   on bioconductor.org and were therefore manually created from the available pd

60   file (pd.mogene.2.1.st) using the functions *writeCdf.AffyGenePDInfo* and

61   *make.cdf.package* from the *aroma.affymetrix* and the makecdfenv packages

62   respectively.

63   The IL-23 and Oxa model expression sets were normalized to the same

64   target expression set, comprised of the Flaky-tail, *Flg*-mutated, NC/Nga and Ova-

65   challenged models, making use of target normalization.

66

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Supplementary Legends

Figure E1: Heatmap of the human MADAD genes with orthologs in murine models. The leftmost column represents the direction of dysregulation in MADAD (blue: down-regulated; red: up-regulated) whereas for each murine model, the group-wise Z-score represents the relative expression of MADAD genes in each model.

Figure E2: The murine samples' normalized expression values presented by: **A**) A boxplot of all mouse samples colored by dataset, and **B**) A heatmap of all overlapping mouse genes.

Table E1: Summary of clinical characteristics and demographics of patients (mean ± SE if not specified).

Table E2: Characteristics of studied populations.

Table E3: Primers used for qRT-PCR.

Table E4: DEGs in each murine AD model (models vs. controls).

Table E5: The log2(FCH) values are presented with their corresponding Benjamini adjusted p-value of each murine model contrast, for the MADAD DEGs

(cut-off absolute FCH ≥ 2 and FDR ≤ 0.05). The status columns indicate the direction of dysregulation in each murine model (0 = not significant, 1 = significant up-regulation, -1 = significant down-regulation).

Table E6: -log(FDR) values for KEGG pathways in MADAD and murine models (significant pathways > 1.3 corresponding to FDR<0.05)

Table E7: Genes represented in each KEGG pathway

Figure E1

Figure E2

A)

Legend:
Group
- FLG,Flakytail,Nc/Nga, and OVA
- OXA
- IL23

B)

Color Key

−1.5 −0.5 0.5 1.5
Group wise z−scale

Mouse Expressions scaled by group

Legend:
- FLG/Flkaytail
- OVA
- OXA
- IL23
- Nc/Noa
- Control
- Diseased

Tissue
Model
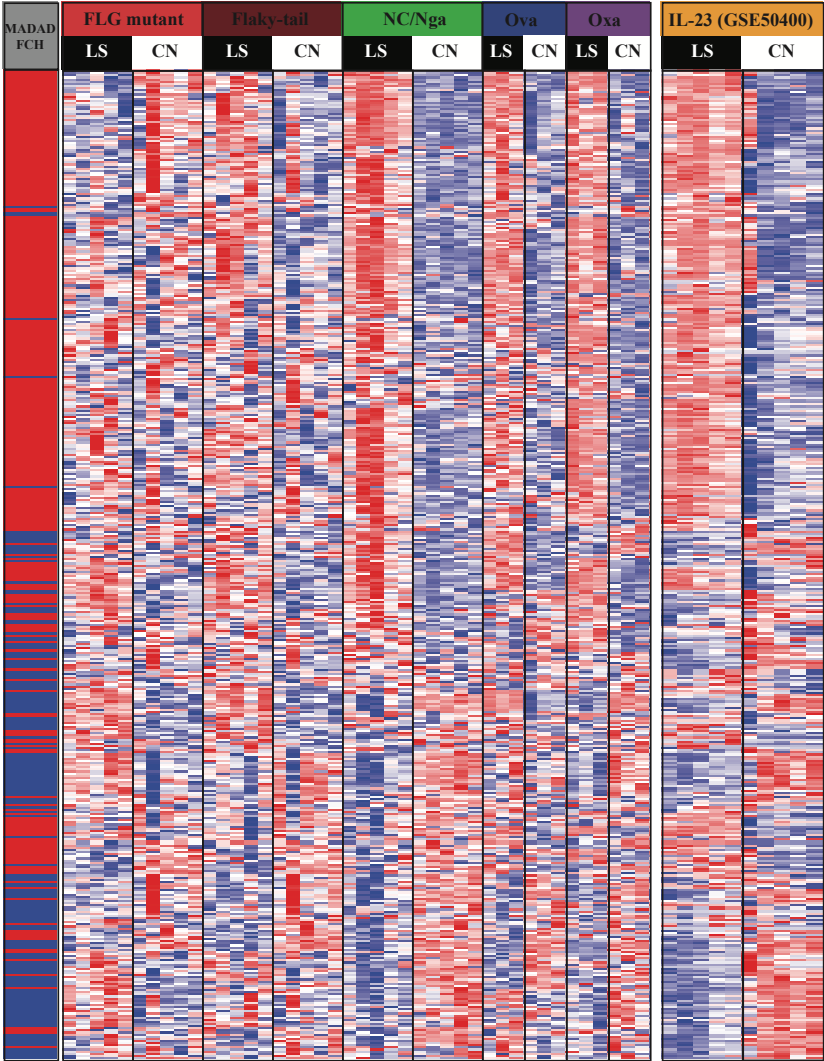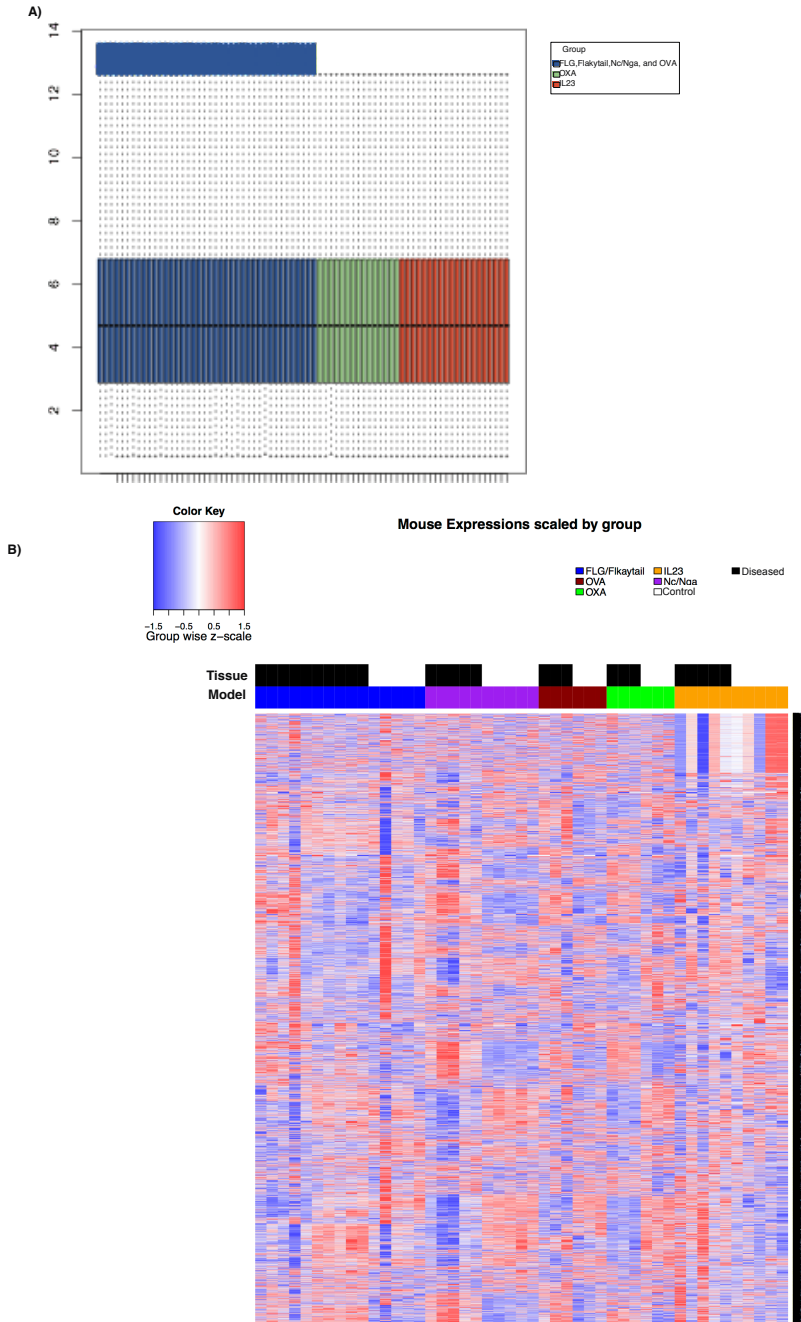
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Table E1. Summary of clinical characteristics and demographics of patients (mean ± SE if not specified)

	AD (n = 25)	Psoriasis (n = 10)	CD (nickel) (n = 10)	CD (frangrance) (n = 3)	Control (n = 8)
Age (y), mean (SE)	45.7 (2.3)	51.3 (4.9)	46 (3.2)	37.3 (9.0)	50.9 (2.0)
Sex (male/female; n)	16/9	6/4	0/10	2/1	7/1
SCORAD index, mean (SE)	56.7 (3.4)				
IgE kU/L, log10, (SE)	2.4 (0.17)				
%Eosinophils (SE)	4.9 (1.2)				
Extrinsic AD, no. (%)	14/25 (56)				

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Table E2. Characteristics of studied populations

Number	Disease or control	Race	Gender	Age	SCORAD	PASI	IgE (U/ml)	Extrinsic/ intrinsic	%eosinophil
1	AD	EA	M	45	56	NA	1281.5	Extrinsic	2.6
2	AD	EA	F	47	77	NA	3000	Extrinsic	5.4
3	AD	EA	F	49	56	NA	1304	Extrinsic	8
4	AD	EA	M	48	51	NA	41	Intrinsic	3.6
5	AD	EA	F	59	45	NA	14.1	Intrinsic	1.4
6	AD	EA	F	50	44	NA	1269	Extrinsic	6.3
7	AD	EA	M	61	52	NA	71	Intrinsic	1.2
8	AD	EA	M	33	65	NA	56.6	Intrinsic	10.3
9	AD	EA	F	67	53	NA	6	Intrinsic	0.6
10	AD	EA	F	40	72	NA	92.2	Intrinsic	2.9
11	AD	EA	F	59	56	NA	3.6	Intrinsic	3.5
12	AD	EA	M	69	61	NA	72.9	Intrinsic	2.36
13	AD	EA	M	73	55	NA	471	Extrinsic	7.5
14	AD	EA	F	36	65	NA	492	Extrinsic	11.8
15	AD	EA	M	42	53	NA	1821	Extrinsic	9.2
16	AD	EA	M	34	76	NA	100	Intrinsic	4.2
17	AD	EA	F	39	37	NA	2000	Extrinsic	6
18	AD	EA	M	48	51	NA	39	Intrinsic	4.3
19	AD	EA	M	36	36	NA	1021	Extrinsic	4.4
20	AD	EA	M	26	33	NA	254	Extrinsic	7.6
21	AD	EA	M	30	57	NA	2000	Extrinsic	2.1
22	AD	EA	M	50	56	NA	907	Extrinsic	3.7
23	AD	EA	M	23	76	NA	93	Intrinsic	6.7
24	AD	EA	M	38	64	NA	2000	Extrinsic	1.3
25	AD	EA	M	41	70	NA	1562	Extrinsic	5.9
26	Psoriasis	EA	M	59	NA	20.1	NA	NA	NA
27	Psoriasis	EA	F	49	NA	8.4	NA	NA	NA
28	Psoriasis	EA	F	30	NA	8.5	NA	NA	NA
29	Psoriasis	EA	M	43	NA	31.6	NA	NA	NA
30	Psoriasis	EA	M	59	NA	13.5	NA	NA	NA
31	Psoriasis	EA	F	42	NA	13.9	NA	NA	NA
32	Psoriasis	EA	M	64	NA	59.5	NA	NA	NA
33	Psoriasis	EA	F	62	NA	14.5	NA	NA	NA
34	Psoriasis	EA	M	44	NA	21.6	NA	NA	NA
35	Psoriasis	EA	M	61	NA	11.7	NA	NA	NA
36	CD (nickel)	EA	F	45	NA	NA	NA	NA	NA
37	CD (nickel)	EA	F	33	NA	NA	NA	NA	NA
38	CD (nickel)	EA	F	52	NA	NA	NA	NA	NA
39	CD (nickel)	EA	F	48	NA	NA	NA	NA	NA
40	CD (nickel)	EA	F	51	NA	NA	NA	NA	NA
41	CD (nickel)	EA	F	55	NA	NA	NA	NA	NA
42	CD (nickel)	EA	F	37	NA	NA	NA	NA	NA
43	CD (nickel)	EA	F	54	NA	NA	NA	NA	NA
44	CD (nickel)	EA	F	28	NA	NA	NA	NA	NA
45	CD (nickel)	EA	F	57	NA	NA	NA	NA	NA
46	CD (fragrance)	EA	M	42	NA	NA	NA	NA	NA
47	CD (fragrance)	EA	F	50	NA	NA	NA	NA	NA
48	CD (fragrance)	EA	M	20	NA	NA	NA	NA	NA
49	Control	EA	F	46	NA	NA	NA	NA	NA
50	Control	EA	M	56	NA	NA	NA	NA	NA
51	Control	EA	M	55	NA	NA	NA	NA	NA
52	Control	EA	M	57	NA	NA	NA	NA	NA
53	Control	EA	M	49	NA	NA	NA	NA	NA
54	Control	EA	M	40	NA	NA	NA	NA	NA
55	Control	EA	M	53	NA	NA	NA	NA	NA
56	Control	EA	M	51	NA	NA	NA	NA	NA

AD = atopic dermatitis
EA = European American
SCORAD = scoring atopic dermatitis
PASI = psoriasis area and severity index
IgE levels of greater than 200 kU/L defining extrinsic AD and values of less than 200 kU/L defining intrinsic AD

Table E3: Primers used for qRT-PCR

Gene symbol	Assay ID
18S	Hs99999901_s1
Ccl17	Mm01244826_g1
Ccl20	Mm01268754_m1
Ccl22	Mm00436439_m1
Cxcl1	Mm04207460_m1
Cxcl10	Mm00445235_m1
Flg	Mm01716522_m1
Ifng	Mm01168134_m1
Il12b	Mm00434174_m1
Il13	Mm00434204_m1
Il17a	Mm00439618_m1
Il19	Mm01288324_m1
Il1b	Mm00434228_m1
Il20	Mm00445341_m1
Il23a	Mm01160011_g1
Krt16	Mm01306670_g1
Lcn2	Mm01324470_m1
Lor	Mm01962650_s1
Mx1	Mm00487796_m1
Ppl	Mm00447206_m1
Rplp0	Mm00725448_s1
S100a8	Mm00496696_g1
Stat1	Mm00439531_m1
Tnfsf4	Mm00437214_m1

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Table E4: DEGs in each murine AD model (models vs. controls)

Flaky-tail Log FCH	Flaky-tail BHadj P value	Flaky-tail HGNC Symbol	Flaky-tail Symbol	FLG Log FCH	FLG BHadj P value	FLG HGNC Symbol
4.68	0.012	NA	Gm3893	1.51	0.000	NA
4.40	0.019	NA	Gm3893	1.51	0.015	SCD
4.31	0.018	NA	Gm3893	1.29	0.001	VSNL1
4.27	0.000	S100A8	S100a8	1.22	0.025	TMEM47
4.00	0.023	NA	4933409K0	1.22	0.025	LGALS12
3.89	0.000	CSTA	Stfa3	1.20	0.011	DHDH
3.82	0.025	NA	Gm3893	1.15	0.000	PLAC9
3.44	0.031	CCL27	Gm21075	1.15	0.049	NA
3.41	0.000	KRT84	Krt84	1.14	0.002	TSHR
3.37	0.000	S100A9	S100a9	1.13	0.000	PLAC9
3.34	0.030	NA	Gm3893	1.13	0.000	PLAC9
3.33	0.024	NA	Gm3893	1.11	0.000	TRHDE
3.23	0.040	KRT28	Krt28	1.10	0.004	LRRN4CL
3.21	0.000	SERPINB3	Serpinb3a	1.10	0.016	MYF6
3.21	0.000	SERPINB4	Serpinb3a	1.08	0.018	PAPSS2
2.82	0.019	KRT73	Krt73	1.06	0.017	RETN
2.81	0.000	ENTPD4	Gm21464	1.02	0.043	CEACAM1
2.74	0.045	NA	Gm21158	1.02	0.043	CEACAM6
2.72	0.031	FAM205A	Gm7819	1.02	0.043	CEACAM7
2.64	0.048	NA	Gm3893	1.02	0.043	CEACAM8
2.64	0.048	NA	Gm3893	1.02	0.043	
2.60	0.001	CSTA	Gm5416	1.02	0.043	CEACAM5
2.54	0.000	CSTA	Stfa1	1.01	0.035	ACOT1
2.52	0.029	FAM205A	LOC100862	1.01	0.035	ACOT2
2.51	0.036	DSG4	Dsg4	1.01	0.023	PPP1R27
2.34	0.047	LYG2	Lyg2	-1.01	0.012	KLK13
2.31	0.000	AADACL3	Aadacl3	-1.03	0.005	C10orf10
2.29	0.033	NA	Gm7819	-1.07	0.016	NPBWR1
2.27	0.002	SPTSSB	Sptssb	-1.11	0.002	NA
2.25	0.000	AADACL4	Gm436	-1.12	0.002	NA
2.23	0.000	IL17F	Il17f	-1.12	0.002	SLC5A1
2.22	0.001	NA	Uox	-1.40	0.037	KRT16
2.14	0.000	FABP7	Fabp7	-1.40	0.033	SERPINA3

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Gene	Flaky-tail Log FCH	Flaky-tail BHadj P value	Flaky-tail Status	FLG Log FCH	FLG BHadj P value	FLG status	NC/Nga Log FCH	NC/Nga BHadj P value	NC/Nga Status	Ova Log FCH	Ova BHadj P value	Ova Status	Oxa Log FCH
MC2R	0.00	0.995	0	0.14	0.827	0	-0.23	0.553	0	0.54	0.066	0	-0.29
ATXN7L1	-0.39	0.220	0	-0.14	0.752	0	-0.41	0.056	0	-0.22	0.349	0	0.28
CLDN8	0.03	0.971	0	0.35	0.557	0	-0.95	0.001	0	-0.82	0.004	0	-0.32
C1QB	-0.06	0.933	0	-0.22	0.674	0	1.62	0.002	1	1.26	0.150	0	0.48
SMPDL3B	0.73	0.015	0	-0.14	0.750	0	0.42	0.117	0	0.44	0.162	0	0.69
KIF20A	0.51	0.107	0	0.13	0.779	0	1.23	0.001	1	0.72	0.052	0	0.28
TTC39B	-0.11	0.771	0	0.14	0.702	0	-0.07	0.724	0	-0.02	0.946	0	0.30
LY6G6D	1.69	0.103	0	0.54	0.712	0	-0.05	0.952	0	-2.24	0.053	0	2.04
STAP1	0.78	0.005	0	0.52	0.100	0	-0.17	0.440	0	0.28	0.610	0	0.12
ALOX5AP	-0.09	0.812	0	-0.10	0.793	0	1.16	0.014	1	0.55	0.173	0	0.25
CHRNB4	-0.55	0.087	0	-0.01	0.991	0	-0.54	0.012	0	-1.78	0.021	-1	-0.35
RBMX	-0.14	0.720	0	0.06	0.902	0	-0.22	0.168	0	0.11	0.711	0	0.12
MX1	-0.09	0.888	0	-0.05	0.938	0	0.00	0.994	0	0.25	0.742	0	2.91
NKAP	-0.06	0.890	0	0.16	0.680	0	-0.30	0.128	0	0.42	0.251	0	-0.34
CBLN1	0.19	0.633	0	0.04	0.944	0	1.39	0.052	0	-0.15	0.702	0	0.73
CXorf21	-0.53	0.286	0	0.13	0.858	0	0.47	0.518	0	0.64	0.050	0	0.40
IL18BP	-0.45	0.073	0	-0.09	0.815	0	0.01	0.969	0	-0.09	0.800	0	0.51
ADAMTS15	0.05	0.921	0	-0.16	0.711	0	1.12	0.003	1	0.79	0.115	0	0.47
SLC6A20	-0.49	0.055	0	-0.66	0.027	0	0.39	0.090	0	0.94	0.002	0	0.44
PSMG4	-0.15	0.674	0	-0.21	0.514	0	-0.17	0.317	0	0.16	0.780	0	-0.29
DPPA5	0.04	0.917	0	-0.04	0.926	0	-0.09	0.735	0	-0.03	0.947	0	0.02
PAQR7	-2.55	0.000	-1	0.47	0.352	0	-1.64	0.002	-1	-1.59	0.002	-1	-2.53
TMEM125	0.22	0.583	0	-0.39	0.304	0	0.38	0.103	0	0.12	0.785	0	0.35
RNF213	-0.05	0.918	0	0.32	0.299	0	0.65	0.025	0	0.39	0.116	0	2.01
HPX	-0.09	0.824	0	-0.17	0.650	0	0.11	0.681	0	-0.10	0.836	0	-0.13
DKKL1	-0.93	0.002	0	-0.25	0.497	0	-0.53	0.210	0	-0.55	0.026	0	-1.04
HLA-B	0.02	0.966	0	0.30	0.415	0	0.57	0.197	0	0.38	0.457	0	1.43
TSC22D3	-0.61	0.003	0	-0.51	0.028	0	-0.94	0.023	0	-0.63	0.096	0	-1.22
TCAF2	-0.62	0.013	0	-0.29	0.324	0	-0.50	0.020	0	1.18	0.002	1	1.44
NDUFA4L2	0.49	0.257	0	0.20	0.725	0	0.93	0.025	0	-0.22	0.577	0	1.19
CILP	-0.16	0.786	0	-0.13	0.844	0	-0.10	0.790	0	1.21	0.234	0	-0.14
XPNPEP2	-0.41	0.383	0	-0.21	0.727	0	0.16	0.576	0	1.45	0.006	1	-0.88
HRNR	-0.15	0.585	0	-0.33	0.172	0	0.21	0.224	0	1.53	0.000	1	0.22

Table E5: MADAD DEGs in each mouse model

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Table E6: KEGG pathways

KEGG PATHWAY	MADAD	Flaky-tail	FLG mut	NcNga	Ova	Oxa	IL-23
PPAR SIGNALING	6.205	1.020	0.000	0.092	1.950	0.000	2.685
CYTOKINE CYTOKINE RECEPTOR INTERACTION	4.550	1.131	0.000	8.271	1.950	18.453	19.899
JAK/STAT SIGNALING	3.858	0.596	0.000	2.947	0.416	9.129	10.866
T CELL RECEPTOR SIGNALING	3.829	0.000	0.000	0.020	0.000	1.734	6.713
CHEMOKINE SIGNALING	2.921	0.000	0.000	5.195	0.942	4.127	15.619
CELL ADHESION MOLECULES (CAMS)	2.329	0.000	0.000	4.175	0.000	3.025	7.956
ADIPOCYTOKINE SIGNALING	1.817	0.000	0.000	0.113	0.000	0.348	4.281
NATURAL KILLER CELL MEDIATED CYTOTOXICITY	1.217	0.000	0.000	1.066	0.000	2.289	7.959
BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	1.217	2.206	3.937	0.000	2.432	0.000	0.347
COMPLEMENT AND COAGULATION CASCADES	1.104	0.000	0.000	2.010	0.106	0.005	0.700
TOLL LIKE RECEPTOR SIGNALING	0.883	0.000	0.000	0.044	0.126	2.617	3.750
LEUKOCYTE TRANSENDOTHELIAL MIGRATION	0.734	0.000	0.000	4.713	0.000	0.371	5.774
FC EPSILON RI SIGNALING	0.556	0.397	0.000	3.006	0.460	0.948	3.803
RETINOL METABOLISM	0.541	4.521	0.000	0.167	6.670	4.919	2.774
B CELL RECEPTOR SIGNALING	0.369	0.000	0.000	1.436	0.000	0.053	4.257
HEDGEHOG SIGNALING	0.000	1.669	0.000	0.000	0.000	0.000	2.227
MELANOGENESIS	0.000	1.020	0.000	0.000	0.000	0.000	2.786

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Table E7: Genes represented in each KEGG pathway

Pathway	Gene 1	Gene 2	Gene 3
PPAR Signaling Pathway	SLC27A5	SLC27A4	SORBS1
Cytokine Cytokine Receptor Interaction	CCL26	TNFSF13	HGF
JAK STAT Signaling Pathway	STAT3	STAT4	STAT1
T Cell Receptor Signaling Pathway	JUN	ZAP70	MALT1
Chemokine Signaling Pathway	CCL26	STAT3	STAT1
Cell Adhesion Molecules (CAMs)	CDH5	JAM3	CDH3
Adipocytokine Signaling Pathway	STAT3	CHUK	PTPN11
Natural Killer Cell Mediated Cytotoxicity	ZAP70	TNFSF10	ITGAL
Biosynthesis of Unsaturated Fatty Acids	ACOT4	TECR	BAAT
Complement and Coagulation Cascades	F2	F2R	VWF
Toll Like Receptor Signaling Pathway	JUN	CD80	CD86
Leukocyte Transendothelial Migration	CDH5	JAM3	MLLT4
FC Epsilon RI Signaling Pathway	SOS2	RAF1	PRKCB
Retinol Metabolism	RPE65	CYP3A5	UGT2B28
B Cell Receptor Signaling Pathway	JUN	SOS2	CHUK
Hedgehog Signaling Pathway	CSNK1A1L	HHIP	PTCH2
Melanogenesis	CALM2	CALM1	TYR

Epilogue

3.1 Summary and Discussion

In this work I characterized the transcriptional basis of the inflammatory skin disease atopic dermatitis (AD) from three perspectives, a microarray meta-analysis of publicly available expression data, an in-depth analysis of the two main skin layers epidermis and dermis by laser capture micro-dissection (LCM), and an evaluation of available and popular murine models.

As a whole, my work gives a good all-round view on various transcriptional aspects of AD, highlighting the important involvement of immunological and barrier related genes, recommending considerate choice of murine model, and indicating the importance of layer specific investigations for increased transcriptional detection specificity.

The **meta-analysis derived AD (MADAD) transcriptome (paper 1)** presented as the first study in this work, provides the research community with a robust AD transcriptome, defined by a random effects model, that combined the lesional versus non-lesional effect sizes of four published AD transcriptomics studies. This MADAD transcriptome or set of gene-wise effect sizes could (and to some degree already does) serve as reference for future research, especially with respect to translational studies evaluating treatment effects on a molecular level.

The design of this study was primarily based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA, http://www.prisma-statement.org), where the inclusion criteria where clearly defined prior to searching the public repositories. Only four studies met all these criteria, coincidentally all initially stemming from one laboratory, though performed by different researchers. As previous studies had highlighted improved overall performance of microarray meta-analyses by probe-set level comparisons, rather than gene level, I decided to keep the strict inclusion criteria and not to loosen them to gene level comparisons. The detection of multi-

ple differentially expressed genes (DEGs) by this meta-analysis not detected by the individual included studies, and subsequent confirmation by the more sensitive qRT-PCR method, suggests that my approach was able to increase the power to detect also subtle changes in expression levels between LS and NL skin samples.

The **laser capture microdissection (paper 2)** of AD skin biopsies with subsequent compartment specific expression analysis, revealed 674 up- and 405 down-regulated genes in LS versus NL samples (epidermis and dermis combined), that had not been described as DEG in the corresponding full-thickness (or bulk tissue) microarray expression analyses. Among these DEGs various barrier and immune related genes appeared. Importantly, my analysis highlighted the possible importance of barrier function related genes like CLDN23 and CLDN8 that appeared to be expressed significantly lower in LS versus NL samples. Also, the cytokine IL-34 showed lower expression in LS samples, suggesting a role as a negative regulator of inflammation. Despite these interesting and important findings, this study was limited by the relatively small samples size (n=5 for both LS and NL samples) and the use of a non-stratified set of patient samples.

The transcriptional **evaluation of atopic dermatitis mouse models (paper 3)** was carried out on six common murine models for inflammatory skin diseases, in comparison to the human MADAD transcriptome defined in the first paper presented in this work. The six murine models included the three main model categories for inflammatory skin diseases, namely 1) induced models (oxazolone, ovalbumin, and IL-23), 2) knock-out models (FLG-mutant, and flakytail), and 3) spontaneous (NC/Nga). Overall, ranked list comparison of the 5000 most up and down regulated genes between each model and the MADAD transcriptome, indicated the largest overlap between the MADAD transcriptome and the IL-23, oxazolone-challenged and NC/Nga models. Despite these relatively solid overall overlaps, none of the models fully captures the molecular basis of human AD. Thus, for pre-clinical research it is essential to chose the murine model that best captures the aspects to be investigated, e.g. the NC/Nga might be well suited for investigating immune aspects of the disease, but will be limited with respect to covering barrier characteristics of AD.
Initially, we planned to compare only four models (FLG-mutant, flakytail, ovalbumin-challenged, and NC/Nga), but had the ability to include data from two other sources (oxazolone-challenged and IL-23 injected). This certainly provided a wider angle to

our study, but also adds a number of limitations. Importantly, the inclusion of the oxazolone-challenged and IL-23 injected mouse models, introduced two extra batches, as the expression data of both were produced on different microarray chip types, at different times, and, as is the case for the oxazolone model, in different laboratories. I sought to limit these possible batch effects by applying target normalization towards the initial dataset (first batch), and re-analyzing the oxazolone-challenged and IL-23 injected mouse model data in the standard pipeline used for the first batch. Furthermore I limited direct comparisons of the models, but sought to compare each model to the reference AD transcriptome.

3.2   Conclusion and Perspectives

Overall, this work provides a solid transcriptional reference for atopic dermatitis research, especially in the context of translational medicine, covering a broad range of important aspects of the disease, ranging from a robust disease transcriptome, over layer specific transcription profiling, to comparative disease model evaluation.

Future work, building on the findings presented her, could or should address some of the shortcomings. An obvious next step would be LCM on a much larger cohort, ideally stratified to intrinsic and extrinsic patients. Even-though it has to be noted that this technology is labour and time intensive, which certainly limits its applicability in larger cohort studies. Eventually, the application of LCM on larger cohorts is an evaluation of the insights gained versus the increased amount of work-hours and resources invested in the project.

Evaluating an increased cohort of model organisms, controlling the ages, sites of biopsies and the microarray technologies, will certainly also add to the understanding of both the disease mechanisms and the applicability of the different models. In the context of this, a comparative analysis of the mouse data to other animal models (e.g. Canis lupus familiaris/dog) could broaden the mechanistic perspective.

From a technological perspective, it would be of great interest to increase and improve my work by highly relevant methods like proteomics and microbiomics. Here, proteomics could add focus to the presented transcriptional aspects of AD, by indicating differential protein levels between lesional and non-lesional samples. Correlating the transcriptional aspects of the disease to the corresponding skin (or even gut)

microbiome might add interesting insights into the interplay between the skin, the disease, and the microbiota populating the respective sites on the skin.

Furthermore, my findings can be applied to evaluate minimally invasive skin micro-biopsy technologies, which would, if proving reliable and applicable, enable time-series analysis of AD. This could be applied not only to investigate disease progression, but more importantly, also in clinical trials for rapid and repeated monitoring of treatment response.

Also, the maturation of RNA-seq methods, their gradually drop in price, and the establishment of solid bioinformatics analysis algorithms has made researchers shift from microarray to RNA-seq expression analysis in many fields. It is very likely that this tendency will be increasingly observed in the field of inflammatory skin diseases, where microarray technology is still widely applied. This might lead to interesting new findings, but also leads to challenges in terms of comparability and reproducibly of microarray and RNA-seq findings, especially with respect to the transcriptional evaluation of clinical trials.

Finally, it would be of great benefit to the atopic dermatitis research community, if the results of my comprehensive work became accessible in an easy to use database, where researchers could rapidly look-up and compare the expression levels of genes of interest. Such a database is under development, and I hope to make it available online in the near future.

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