**Międzyuczelniany Wydział Biotechnologii**
Uniwersytetu Gdańskiego i Gdańskiego Uniwersytetu Medycznego

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# Rola małych pęcherzyków zewnątrzkomórkowych wydzielanych przez keratynocyty w interakcji z układem odpornościowym w atopowym zapaleniu skóry

## The role of keratinocyte-derived small extracellular vesicles in the interaction with the immune system in atopic dermatitis

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A dissertation presented to
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Supervisor: Assoc. Prof. Danuta Gutowska-Owsiak
Laboratory of Experimental and Translational Immunology
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**My parents**

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**My friends**

You are my rock

# Contents

# Abbreviations

**ACSL** – long-chain-fatty-acid-CoA ligase

**AD** – atopic dermatitis

**ADAM10** – disintegrin and metalloproteinase domain-containing protein 10

**ADEH** – atopic dermatitis with a history of eczema herpeticum

**AE** – atopic eczema

**AhR** – aryl hydrocarbon receptor

**ALIX** – apoptosis-linked gene 2-interacting protein X

**AP** – apoptotic body

**APC** – antigen-presenting cell

**ARF6** – ADP ribosylation factor 6

**ARRDC1** – arrestin domain-containing protein 1

**ASC** – mesenchymal stem cell derived from adipose tissue

**BMH** – bleomycin hydrolase

**C1GALT1** – core 1 β,3-galactosyltransferase 1

**CASP14** – caspase-14

**CCL** – C-C motif chemokine ligand

**CD** – cluster of differentiation

**cDC** – conventional dendritic cell

**CDMS** – charge detection mass spectrometry

**CDSN** – corneodesmosin

**CE** – cornified envelope

**CLA** – cutaneous lymphocyte-associated antigen

**CLDN1** – claudin-1

**CLE** – cornified lipid envelope

**CLR** – C-type lectin receptor

**CTACK** – cutaneous T cell-attracting chemokine

**DAG** – diacylglycerol

**DC** – dendritic cell

**DC-SIGN** – dendritic cell-specific ICAM-3-grabbing non-integrin 1

**DDC** – dermal dendritic cell

**DISC** – death-inducing signaling complex

**DNA** – deoxyribonucleic acid

**DSG1** – desmoglein 1

**EBV** – Epstein-Barr virus

**ECM** – extracellular matrix

**EDC** – epidermal differentiation complex

**EDN** – eosinophil-derived neurotoxin

**EET** – eosinophil extracellular trap

**EH** – eczema herpeticum

**ELOVL** – enzymes of the elongation of very long fatty acid family

**ESCRT** – endosomal sorting complex required for transport

**EV** – extracellular vesicle

**FA** – fatty acid

**FasL** – Fas ligand

**FceRI** – high-affinity IgE receptor

**FcεRII** – low-affinity IgE receptor

**FLG** – filaggrin

**FUT4** – fucosyltransferase 4

**GM-CSF** – granulocyte-macrophage colony-stimulating factor

**GPI** – glycerophosphatidylinositol

**HaCaT** – spontaneously immortalized human keratinocyte cell line

**HEK293** – human embryonic kidney cell line

**HLA** – human leukocyte antigen

**Hla** – α-hemolysin

**HSV-1** – herpes simplex virus 1

**IDEC** – inflammatory dendritic epidermal cells

**IDO1** – indoleamine 2, 3-dioxygenase 1

**IF** – intermediate filament

**IFN** – interferon

**Ig** – immunoglobulin

**IL** – interleukin

**IL12B** – interleukin-12B

**IL12RB1** – interleukin-12 receptor subunit beta 1

**IL-13Rα1** – interleukin-13 receptor subunit α-1

**IL-31RA** – interleukin-31 receptor A

**IL-4Rα** – interleukin-4 receptor subunit α

**ILC** – innate lymphoid cell

**ILT** – immunoglobulin-like transcript

**ILV** – intraluminal vesicle

**iPSC** – induced pluripotent stem cell

**IRF** – interferon regulatory factor

**ISEV** – International Society of Extracellular Vesicles

**ITIM** – immune receptor tyrosine-based inhibition motif

**IVL** – involucrin

**K** – keratin

**K562** – human lymphoblastoid cell line

**K562-CD1a** – CD1a-overexpressing K562 cell line

**KC** – keratinocyte

**$KC_{sEV}$** – keratinocyte-derived small extracellular vesicle

**KHG** – keratohyalin granule

**KLK** – kallikrein-related peptidase

**LB** – lamellar body

**LC** – Langerhans cell

**LCE2B** – late cornified envelope protein 2B

**LC-PUFA** – long-chain polyunsaturated fatty acid

**LEKTI** – lympho-epithelial Kazal type-related inhibitor

**LMP1** – latent membrane protein 1

**LoF** – loss of function

**LOR** – loricrin

**LPS** – lipopolysaccharide

**LTA** – lipoteichoic acid

**Lyso-PC** – monoacylglycerophosphocholine

**m/lEVs** – medium/large extracellular vesicles

**MB** – midbody

**MBP** – major basic protein

**MB-R** – midbody remnant

**MCP-1** – monocyte chemoattractant protein-1

**MCP-4** – monocyte chemoattractant protein 4

**MDA-MB-231** – human epithelial breast cancer cell line

**MHC** – major histocompatibility complex

**MIP-3α** – macrophage inflammatory protein-3α

**miRNA** – micro-ribonucleic acid

**MMP** – matrix metalloproteinase

**MMR** – macrophage mannose receptor

**MnSOD** – manganese superoxide dismutase

**mRNA** – messenger ribonucleic acid

**MV** – microvesicles

**MVB** – multivesicular body

**MyD88** – myeloid differentiation factor 88

**N/TERT-1** – immortalized human keratinocyte cell line

**NET** – neutrophil extracellular traps

**NF-κB** – nuclear factor-κB

**NK** – natural killer

**NLR** – nucleotide-binding oligomerization domain-like receptors

**NMF** – natural moisturizing factor

**NOD** – nucleotide-binding oligomerization domain-containing protein

**N-SMase** – neutral sphingomyelinase

**OVA** – ovalbumin

**OVOL1** – ovo-like transcriptional repressor 1

**PAD** – peptidylarginine deiminase

**PC** – diacyl glycerophosphocholine

**PCA** – 2-pyrrolidone-5-carboxylic acid

**PCO** – ether-linked glycerophosphocholine

**pDC** – plasmacytoid dendritic cell

**PDGF** – platelet-derived growth factor

**PD-L1** – programmed death-ligand 1

**PEP1** – profilaggrin endopeptidase 1

**PIP3** – phosphatidylinositol 3-phosphate

**PKC** – protein kinase C

**PLA2** – phospholipase A2

**PLC-γ1** – phospholipase γ1

**PP2A** – protein phosphatase 2A

**PRR** – pattern recognition receptor

**PSM** – phenol-soluble modulins

**PTM** – post-translational modification

**qPCR** – quantitative polymerase chain reaction

**RAB31** – Ras-related protein Rab-31

**RAGE** – receptor for advanced glycation end products

**RANTES** – regulated upon activation, normal T cell expressed and presumably secreted

**SASPase** – skin aspartic acid protease

**SC-SFA** – short-chain saturated fatty acid

**SE** – staphylococcal enterotoxin

**sEV** – small extracellular vesicle

**SFA** – saturated fatty acid

**shC** – filaggrin-expressing (empty vector-transfected) HaCaT cells

**shFLG** – short hairpin RNA-mediated filaggrin knockdown HaCaT cells

**Siglec** – sialic acid-binding immunoglobulin-like lectin

**sMB-R** – secreted midbody remnant

**sMV** – small microvesicle

**SNP** – single nucleotide polymorphism

**SOCS3** – suppressor of cytokine signaling 3

**SPINK5** – serine protease inhibitor of Kazal type 5

**SPRR** – small proline-rich protein

**ST6GAL1** – ST6 β-galactoside α-2,6-sialyltransferase 1

**TARC** – thymus- and activation-regulated chemokine

**Tc** – cytotoxic T cell

**TCR** – T cell receptor

**TEM** – tetraspanin-enriched microdomain

**TEWL** – transepidermal water loss

**TG** – transglutaminase

**TGF** – transforming growth factor

**Th** – T helper cell

**TLR** – Toll-like receptor

**TMEM79** – transmembrane protein 79

**TNF** – tumour necrosis factor

**Tr1** – type 1 regulatory T cell

**Treg** – regulatory T cell

**TSG101** – tumour susceptibility gene 101

**TSLP** – thymic stromal lymphopoietin

**TSLPR** – thymic stromal lymphopoietin receptor

**TXP** – toxic granule protein

**TYR** – tyrosinase

**UCA** – urocanic acid

**UV** – ultraviolet

**UVB** – ultraviolet B

**VAMP3** – vesicle-associated membrane protein 3

**VPS4** – vacuolar protein sorting 4

# Streszczenie (abstract in Polish)

Keratynocyty są głównymi komórkami wchodzącymi w skład naskórka – najbardziej zewnętrznej warstwy skóry. **Atopowe zapalenie skóry (AZS) to przewlekła choroba zapalna skóry**, w której integralność naskórka jest naruszona w wyniku czynników genetycznych i stanu zapalnego. W efekcie patogeny, alergeny, promieniowanie UV i związki chemiczne, mające kontakt z naskórkiem, wnikają w głębsze warstwy skóry i aktywują odpowiedź układu odpornościowego oraz powodują uszkodzenia tkanki. W AZS dominuje odpowiedź immunologiczna typu 2, ale towarzyszą jej również czynniki prozapalne charakterystyczne dla odpowiedzi typu 17 i 22. **W AZS keratynocyty wpływają na aktywność komórek układu odpornościowego** i przyczyniają się do stanu zapalnego w skórze. **Skóra pacjentów z AZS jest podatna na infekcje patogenami**, takimi jak bakteria gronkowiec złocisty (*Staphylococcus aureus, S. aureus*) czy grzyb *Candida albicans* (*C. albicans*), które zaostrzają stan zapalny. Gen *FLG* koduje profilagrynę, której ekspresja zachodzi prawie wyłącznie w keratynocytach obecnych w naskórku. Profilagryna jest niezbędna do prawidłowego różnicowania keratynocytów i utrzymania integralności naskórka. Ponadto badania wskazują, iż **profilagryna bierze udział w regulacji odpowiedzi odpornościowych**. **Mutacje utraty funkcji (LoF) genu *FLG* są największym genetycznym czynnikiem ryzyka wystąpienia AZS.** Mutacje te zwiększają również ryzyko występowania u pacjentów z AZS różnych chorób o podłożu alergicznym już po ustąpieniu objawów AZS. Zjawisko to jest znane jako **„marsz alergiczny"** i dotyczy też tkanek, w których nie występuje ekspresja genu *FLG*.

**Egzosomy** to małe, otoczone dwuwarstwą lipidową pęcherzyki wydzielane przez wszystkie komórki jądrzaste. Pośredniczą one w komunikacji między komórkami i tkankami zarówno sąsiadującymi ze sobą, jak i tymi, które są znacznie od siebie oddalone. Egzosomy mogą być izolowane z pożywki pochodzącej z hodowli komórek lub płynów ustrojowych i znajdują się we frakcji **małych pęcherzyków zewnątrzkomórkowych (sEVs)**. Liczne badania wykazują, że sEVs produkowane przez różne komórki/tkanki wpływają na odpowiedź immunologiczną, ale **rola sEVs wydzielanych przez keratynocyty ($KC_{sEVs}$) w AZS nie została jeszcze poznana**.

Celem badań, których wyniki zostały zaprezentowane w rozprawie, było poznanie czy **sEVs produkowane przez keratynocyty poddane czynnikom charakterystycznym**

**dla AZS wykazują funkcje związanie z regulacją układu odpornościowego.** W przeprowadzonych badaniach źródłem sEVs były ludzkie keratynocyty pierwotne, unieśmiertelnione linie ludzkich keratynocytów oraz ludzkie osocze.

Pierwsza część badań wykazała, iż poddanie keratynocytów działaniu prozapalnego środowiska charakterystycznego dla AZS i patogenu *C. albicans* zwiększa interakcje $KC_{sEVs}$ z komórkami dendrytycznymi. Wynikało to ze wzbogacenia powierzchni $KC_{sEVs}$ różnymi glikanami, również tymi zawierającymi kwasy sjalowe. Przeprowadzone zostały eksperymenty, w których blokowane były poszczególne receptory obecne na powierzchni komórek prezentujących antygen (APCs); wykazały one, że receptory Siglec-7 i Siglec-9, które wiążą kwas sjalowy i wyciszają odpowiedź immunologiczną biorą udział w interakcji $KC_{sEVs}$ z APCs. Ekspresja enzymów α2,6-sjalotransferaza-1 (ST6GAL1) i beta-1,3-galaktozylotranferaza rdzeniowa (C1GALT1) była zwiększona w keratynocytach stymulowanych cytokinami AZS, natomiast naskórek pacjentów z AZS charakteryzował się podwyższoną ekspresją ST6GAL1. Oba enzymy mogą więc przyczyniać się do zmienionego profilu glikozylacji powierzchni $KC_{sEVs}$, wywołanego stymulacją keratynocytów cytokinami AZS i *C. albicans.*

Eksperymenty wykonane w kolejnej części badań wykazały, że $KC_{sEVs}$ są źródłem lipidowych ligandów dla białka CD1a. Ligandy te musiały jednak zostać wcześniej uwolnione z błon $KC_{sEVs}$ przez enzym fosfolipazę A2. Po uwolnieniu, ligandy białka CD1a regulowały aktywność CD1a-zależnych limfocytów T. Wyciszenie genu *FLG* w keratynocytach (shFLG) skutkowało zmienioną zdolnością $KC_{sEVs}$ do regulacji tej aktywności. Objawiało się to wzmocnieniem odpowiedzi odpornościowej typu 2 i zahamowaniem odpowiedzi typu 1. Wspomniane różnice wynikały ze zmniejszonej ilości lipidowych ligandów białka CD1a stymulujących limfocyty T i jednocześnie zwiększonej zawartości tych, które je hamują w sEVs produkowanych przez komórki shFLG. Na zmiany w składzie lipidomu $KC_{sEVs}$ po wyciszeniu genu *FLG* wpływ miała zmniejszona w keratynocytach ekspresja enzymów odpowiadających za metabolizm lipidów. Jeden z nich, syntetaza acyloCoA 3 (ACSL3), odpowiada za włączanie długołańcuchowych wielonienasyconych kwasów tłuszczowych do fosfolipidów znajdujących się w błonach biologicznych. W skórze pacjentów z AZS zmniejszona była również ekspresja innych izoform ACSL. Skóra pacjentów charakteryzowała się też spadkiem ekspresji enzymów należących do rodziny elongaz kwasów tłuszczowych o bardzo długim łańcuchu (ELOVL), których funkcją jest wydłużanie łańcuchów kwasów tłuszczowych.

Jedną z obserwacji ostatniej części badań przeprowadzonych w ramach pracy doktorskiej była obecność produktów pochodnych profilagryny w $KC_{sEVs}$. sEVs pochodzące z osocza zarówno pacjentów z AZS jak i zdrowych osób również zawierały taki ładunek. Stymulacja keratynocytów bakterią. *S. aureus* spowodowała zwiększenie ilości produktów pochodnych profilagryny w sEVs wytwarzanych przez te komórki. Efekt ten był zależny od stymulacji receptora Toll-podobnego TLR2. TLR2 jest obecny w keratynocytach i rozpoznaje *S. aureus*. Bakteria ta spowodowała również rozregulowanie produkcji sEVs przez keratynocyty. Objawiało się to zwiększonym wydzielaniem sEVs o charakterze egzosomalnym, jak również małych mikropęcherzyków (sMVs).

Podsumowując, wyniki opisane w rozprawie doktorskiej wskazują istotną rolę $KC_{sEVs}$ w odpowiedzi odpornościowej w AZS. Pęcherzyki te mogą zostać wykorzystane przez patogeny w celach ochronnych; *C. albicans* zwiększa interakcje między $KC_{sEVs}$ i wyciszającymi odpowiedź immunologiczną receptorami Siglec, co może utrudniać eliminację tego patogenu. *S. aureus*, natomiast zwiększa ładowanie produktów cięcia profilagryny do $KC_{sEVs}$, dzięki czemu eliminuje je z keratynocytów i unika ich mikrobójczych właściwości. Co więcej, wzmożone usuwanie produktów pochodnych filagryny zmniejsza jej ilość w naskórku, przyczyniając się do pogorszenia stanu bariery skórnej. sEVs produkowane przez komórki shFLG zaostrzają charakterystyczną dla AZS odpowiedź odpornościową typu 2. Co za tym idzie, mogą one przyczyniać się do stymulowania tego rodzaju stanów zapalnych nie tylko w skórze, ale również w innych tkankach, do których dostarczane byłyby przez krwiobieg. Wszystkie opisane mechanizmy mogą nasilać stan zapalny w AZS poprzez przyczynianie się do naruszenia integralności bariery naskórka oraz zwiększenia ilości czynników prozapalnych w skórze. Można przypuszczać, że mechanizmy te mogłyby też wpływać na inne, dotknięte przez choroby „marszu alergicznego" tkanki i organy.

# Abstract

Keratinocytes are the main cellular component of the epidermis, the outermost layer of the skin. **Atopic dermatitis (AD) is a chronic inflammatory skin disease** in which integrity of the epidermis is compromised due to genetic factors and inflammation. As a result, pathogens, allergens, UV radiation and chemicals which come into contact with the epidermis penetrate into deeper layers of the skin triggering immune responses and causing tissue damage. Type 2 immune response predominates in AD but enhanced type 17 and type 22 responses also contribute to the inflammatory *milieu*. **Keratinocytes regulate the activity of immune cells in AD** and promote skin inflammation. **The skin of AD patients is prone to colonization by pathogens** such as *Staphylococcus aureus* (*S. aureus*) or *Candida albicans* (*C. albicans*) which exacerbate skin inflammation. *FLG* encodes profilaggrin which is expressed almost exclusively by epidermal keratinocytes. This protein is crucial for keratinocyte differentiation and maintenance of epidermal integrity. Additionally, mounting evidence suggests the involvement of **profilaggin in regulation of immune responses.** Loss-of-function (LoF) **mutations in the *FLG* gene are the greatest genetic risk factor for AD**. Such mutations also predispose AD patients to **'allergic march'** which is the appearance of additional allergic manifestations later in life; these can affect tissues devoid of *FLG* expression and occur even after the resolution of AD symptoms.

**Exosomes** are small lipid bilayer-enclosed vesicles produced by all nucleated cells; these vesicles mediate short- and long-distance communication between cells and tissues. Exosomes can be isolated from conditioned cell culture media or body fluids and are contained in the fraction of **small extracellular vesicles (sEVs)**. Extensive evidence shows the involvement of sEVs derived from different cells/tissues in immune responses but **the role of keratinocyte-derived sEVs ($KC_{sEVs}$) in AD is unknown**.

Research presented in this thesis aimed to elucidate whether **sEVs secreted by keratinocytes subjected to AD-relevant conditions carry out immune-related functions**; cultured human primary keratinocytes, human immortalized keratinocyte cell lines and human blood plasma were used as sources of sEVs.

First part of the work showed that exposure of keratinocytes to AD *milieu* and *C. albicans* increased interaction between $KC_{sEVs}$ and dendritic cells; this was due to an enrichment

of the $KC_{sEV}$ surface in certain glycans, including those containing sialic acids. Receptor blocking experiments revealed that sialic acid-binding immune inhibitory Siglec-7 and -9 receptors were involved in the interaction between $KC_{sEVs}$ and antigen presenting cells (APCs). ST6 β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) and core 1 β,3-galactosyltransferase 1 (C1GALT1) were upregulated by keratinocytes exposed to AD *milieu.* ST6GAL1 was also upregulated in the epidermis of AD patients. Hence, both enzymes may contribute to the remodeling of the $KC_{sEV}$ surface glycosylation pattern upon exposure of keratinocytes to AD cytokines and *C. albicans*.

The next part of research demonstrated that $KC_{sEVs}$ can be a source of lipid CD1a ligands which modulate CD1a-specific T cell responses; however, the activity of phospholipase A2 was required to liberate CD1a ligands from sEV membranes. *FLG* knockdown in keratinocytes (shFLG) resulted in the altered capacity of $KC_{sEVs}$ to modulate lipid antigen-driven CD1a-specific T cell responses; specifically, type 2 response was enhanced while type 1 response was reduced. This was a consequence of a decreased abundance of stimulatory and enrichment in inhibitory lipid CD1a ligands in the membranes of sEVs produced by shFLG keratinocytes. The differences in the sEV lipidome resulted from downregulation of enzymes involved in lipid metabolism in shFLG keratinocytes; one of these enzymes, long-chain-fatty-acid-CoA ligase 3 (ACSL3) is known incorporate long-chain polyunsaturated fatty acids into phospholipids of biological membranes. Additionally, downregulation of not only other isoforms of the ACSL enzyme but also the elongation of very long chain fatty acids enzyme (ELOVL) family was observed in AD skin. The role of ELOVL enzymes is to elongate fatty acid chains.

Finally, the last part of work documents the presence of profilaggrin-related cargo in sEVs produced by cultured keratinocytes. Additionally, such cargo was detected in sEVs derived from blood plasma of healthy individuals and AD patients. Moreover, exposure of keratinocytes to *S. aureus* enhanced the loading of profilaggrin-related products into $KC_{sEVs}$. The mechanism of this involved Toll-like receptor 2 (TLR2), which is known to recognize *S. aureus* in keratinocytes. *S. aureus* also dysregulated the production of sEVs by keratinocytes; a pronounced increase in the secretion sEVs of exosomal characteristics and small microvesicles (sMVs) by those cells was observed.

Taken together, the results presented in the thesis suggest an important role for $KC_{sEVs}$ in immune response in AD. The keratinocyte sEV system can be hijacked by pathogens as their evasion strategy; *C. albicans* promotes interaction of sEVs with immune inhibitory Siglec receptors on APCs which may impede pathogen clearance, while *S. aureus* seems to exploit the sEV-mediated removal of profilaggrin and its breakdown products to avoid their antimicrobial properties. Additionally, such enhanced removal profilaggrin-related products results in further decrease of its level in the epidermis, contributing to the barrier defect. Finally, $KC_{sEVs}$ produced on a filaggrin-insufficiency background exacerbate type 2 inflammation in the AD skin, and potentially also in the other tissues to which they could be delivered by circulation. All these identified mechanisms may intensify inflammation in AD skin by lowering barrier quality and contribution to the atopic *milieu,* and may potentially affect other tissues and organs, with relevance to the 'allergic march'.

# List of manuscripts included as part of the thesis

### Published:

**(P1)** **Kobiela A.***, Frąckowiak J. E.*, Biernacka A., Hovhannisyan L., Bogucka A. E., Panek K., Paul A. A., Łukomska J., Wang X., Giannoulatou E., Królicka A., Zieliński J., Deptuła M., Pikuła M., Gabrielsson S., Ogg G. S., Gutowska-Owsiak D. Exposure of keratinocytes to Candida albicans in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors. *Frontiers in Immunology* **13**, 884530 (2022). * joined first authors. IF = 8.786

### Under review:

**(P2)** **Kobiela A.**, Hewelt-Belka W., Frąckowiak J. E., Kordulewska, N., Hovhannisyan L., Bogucka A. E., Etherington R., Piróg A., Dapic I., Gabrielsson S., Brown S. J., Ogg G. S., Gutowska-Owsiak D. Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation. **Under review in *The Journal of Extracellular Vesicles* (2023)**. IF = 21.224

### In revision:

**(P3)** Hovhannisyan L.*, **Kobiela A.***, Bernardino de la Serna J., Bogucka A. E., Deptuła M., Paul A. A., Panek K., Czechowska E., Rychłowski M., Królicka A., Zieliński J., Gabrielsson S., Pikuła M., Trzeciak M., Ogg G. S., Gutowska-Owsiak D. Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion. **In revision in *The Journal of Extracellular Vesicles* (2022).** * joined first authors. IF = 21.224

Due to the amount of work put in during the revision stage I will become the sole first author of the revised manuscript. Hence, the new author order will be as follows:

**Kobiela A.,** Hovhannisyan, L., Bernardino de la Serna J., Bogucka A. E., Deptuła M., Paul A. A., Panek K., Czechowska E., Rychłowski M., Królicka A., Zieliński J., Gabrielsson S., Pikuła M., Trzeciak M., Ogg G. S., Gutowska-Owsiak D.

# 1. Introduction

## 1.1. The epidermis and its structure

The epidermis is a constantly renewing stratified epithelial tissue forming the outermost layer of the skin[1]. Around 90-95% of the epidermis is composed of a single cell type, keratinocytes which exist in various differentiation stages across 4 epidermal layers; starting with the deepest *stratum basale* through *stratum spinosum*, *stratum granulosum* to the impermeable *stratum corneum* on the epidermal surface (Figure 1.)[1].

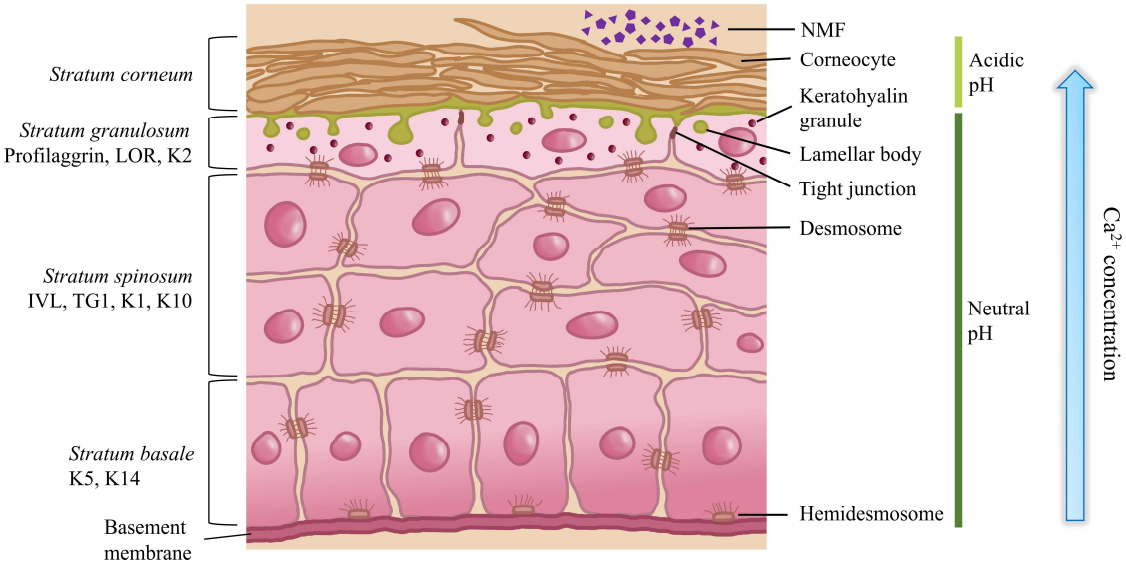


**Figure 1. Structure of the epidermis.** Keratinocytes are the main cellular component of the epidermis. *Stratum basale:* cells are attached to the basement membrane by hemidesmosomes, maintain a columnar shape and are highly proliferative; keratins 5 and 14 are expressed. *Stratum spinosum*: keratinocytes become flatter and are less proliferative; involucrin, transglutaminase 1, keratins 1 and 10 are expressed; cells are attached to each other by desmosomes. *Stratum granulosum:* keratohyalin granules containing profilaggrin and loricrin are formed; lamellar bodies contain lipids as well as lipid- and protein-processing enzymes; keratin 2 is expressed. *Stratum corneum*: keratin filaments are cross-linked, cytoskeleton collapses and organelles are destroyed; contents of lamellar bodies are released and they form a lipid matrix in which corneocytes are embedded. Profilaggrin processing products are a major component of the natural moisturizing factor that supports epidermal barrier function and skin hydration. Low pH of *stratum corneum* has anti-microbial properties and regulates enzymatic activity. Increasing $Ca^{2+}$ concentration in the epidermis drives keratinocyte differentiation by stimulating expression of differentiation-related genes and activity of enzymes. IVL, involucrin; K, Keratin; LOR, loricrin; NMF, natural moisturizing factor; TG1, transglutaminase 1.

In the *stratum basale* keratinocytes are highly proliferative and exhibit a columnar shape[1]. Basal keratinocytes are structurally interlinked with the elements building the underlying basement membrane through junctions known as hemidesmosomes[1]. The basement membrane is a layer of extracellular matrix (ECM) which directly and tightly links the epidermal *stratum basale* with the dermal layer located beneath[1]. As keratinocytes begin to differentiate, they gradually lose their ability to proliferate and detach from the basement membrane[1].

In the *stratum spinosum*, located directly above the *stratum basale* keratinocytes become more flattened and assume a polyhedral shape[1]. Keratinocytes of this layer are tightly connected with each other through desmosomes; these are connected to individual cells by bundles of fibrous keratinocyte-expressed proteins called keratins[1]. Keratins are crucial components of intermediate filaments (IFs), a key part of cell cycoskeleton[2]. Currently, over 50 different mammalian keratins have been described and some of them are characteristic for epidermal keratinocytes[3]. The keratin expression pattern changes in the differentiation process; while keratins 5 (K5) and 14 (K14) are expressed primarily by basal keratinocytes, in the *stratum spinosum* K1 and K10 are much more abundant[1]. K1 and K10 form very dense bundles and provide resistance of the upper epidermal layers to mechanical injury[3,4]. Keratinocytes in the *stratum spinosum* layer also start expressing involucrin (IVL) and transglutaminase (TG) 1 (TG1) which are both crucial for the formation of the impermeable *stratum corneum*[5].

The hallmark of keratinocytes in *stratum granulosum* is the formation of dense keratohyalin granules containing loricrin (LOR) and profilaggrin; both proteins are key for the formation of *stratum corneum*[5]. The *stratum granulosum* also contains lamellar bodies (LBs), secretory organelles which carry lipids, lipid-processing enzymes, proteases, protease inhibitors and antimicrobial peptides[5,6]. K2, characteristic for highly differentiated keratinocytes is expressed *stratum granulosum*[4].

Heavy cross-linking of keratin filaments that aggregate through the activity of filaggrin occurs at the interface of *stratum granulosum* and *stratum corneum*[5]. During this terminal differentiation process keratinocyte organelles, including the nucleus disintegrate[5]. The keratinocyte remnants are known as corneocytes and are interconnected by corneodesmosomes which are modified desmosomes[7]. Corneocytes of *stratum corneum* are enclosed by a network of cross-linked proteins known as the 'cornified envelope'

(CE)[8]. High calcium concentration stimulates the activity of TGs which are crucial enzymes for the formation of CE; TGs cross-link proteins such as IVL, LOR and small proline-rich proteins (SPRRs), giving rise to a rigid CE structure[2,5]. CE is, in turn surrounded by a different type of formation called the cornified lipid envelope (CLE); it is made from lipids that covalently bind to CE proteins[2]. TG1 has been shown to attach the ω-hydroxyceramide lipid to IVL and might therefore play a role in these lipid-protein interactions[9]. Together, the described programmed events give rise to a fully formed *stratum corneum,* which constitutes an impermeable barrier[5,7]. It consists of 15-20 layers of flat corneocytes surrounded by CE, CLE; this complex is further embedded in a lipid matrix formed by LB-derived lipids, processed by the enzymes present within these organelles[5,7]. Because of its structure *stratum corneum* is often referred to 'bricks and mortar', where corneocytes are the 'bricks' and the enclosing lipid matrix forms the 'mortar'[7]. Major lipid classes found in the *stratum corneum* are ceramides, cholesterol and free fatty acids but it is ceramides that constitute around 50% of the lipids in this epidermal layer by mass[10]. Correct formation of impermeable *stratum corneum* is indispensable for protection of the skin from environmental insults e.g., irritants, pathogens, allergens and UV radiation; importantly, this layer also prevents transepidermal water loss (TEWL)[7].

Calcium ions are the key driver of keratinocyte differentiation in the epidermis and *in vitro* cell culture[5,11,12]. The calcium ion concentration is at the lowest in the *stratum basale* and it progressively increases through the upper epidermal layers, orchestrating gradual differentiation of keratinocytes all the way to their terminal stage[5]. One of the crucial effects of the epidermal calcium gradient is the redistribution of the structural components involved in cell-to-cell adhesion; this allows for the formation of desmosomes, tight junctions or adherens junctions which are crucial for tight cellular connections which improve tissue impermeability[5]. Increased calcium levels also lead to the upregulation of K1, K10, IVL, TG1, LOR and profilaggrin[5,13,14]. Expression of many of the genes upregulated in differentiated keratinocytes, including those encoding K1 and IVL, contain DNA regulatory regions that are calcium-induced[15,16]. Phospholipase Cγ1 (PLC-γ1) is a critical component of calcium-dependent keratinocyte differentiation; downregulation of this enzyme or inhibition of its activity results in decreased expression of IVL and TG in human keratinocytes[17]. Similar effect has been observed in case of protein kinase C (PKC) which is activated downstream of PLC-γ1; specifically, inhibition of this enzyme

resulted in decreased abundance of IVL, TG and filaggrin in the spontaneously immortalized human keratinocyte cell line HaCaT[18].

Furthermore, additional proteins, such as components of complexes enabling cell-to-cell contacts also play a role in the calcium-dependent induction of keratinocyte differentiation[5]. Specifically, the E-cadherin-β--catenin complex, a critical component of adherens junctions, indirectly activates both PLC-γ1 and PKC[5]. It has been evidenced that under the increase in extracellular calcium stable E-cadherin--β-catenin complexes are formed in the plasma membrane[5]. β-catenin is a binding partner for kinases which phosphorylate phosphoinositides, components of the cell membrane[5]. Hence, upon recruitment of β-catenin to the cell membrane those enzymes are relocated to the vicinity of their substrates[5]. One of the products of their enzymatic activity directly stimulates PLC-γ1 which then generates diacylglycerol (DAG) and indirectly releases calcium ions from intracellular stores; both DAG and calcium ions activate PKC[5,19,20].

### 1.1.1. Filaggrin in epidermal homeostasis and differentiation

The filaggrin protein is indispensable for the maintenance of the barrier integrity and protection of the skin from environmental insults and dehydration[21]. The name itself was introduced in 1981 by Peter Steinert and Beverly Dale to reflect the ability of filaggrin to aggregate keratin intermediate filaments[22]. This protein is encoded by the filaggrin (*FLG*) gene and expressed in the form of a histidine-rich precursor called profilaggrin[21,23]. This large (>400 kDa) protein consists of 10-12 nearly identical filaggrin monomer repeats flanked by imperfect repeats on either side[21], as well as the N- and C-terminal domains[21]. Filaggrin repeats are separated by a short, conserved linker composed of 7 amino acids (FLYQVST in human profilaggrin)[21,24]. The varying number of filaggrin repeats reflects the *FLG* gene polymorphisms resulting from duplication of either the 8$^{th}$ filaggrin repeat or the 10$^{th}$ filaggrin repeat; alternatively, both can occur at the same time[21]. Therefore, the total number of filaggrin monomers copies carried by an individual can vary from 20 to 24 (on two gene copies)[21]. Complete cleavage of profilaggrin and trimming of the linkers yields filaggrin monomers 37 kDa in size[25]. The N-terminal domain of profilaggrin contains two S100 protein-like calcium binding motifs in its conserved A domain[25]. The less conserved B domain contains a nuclear localization signal which directs the N-terminal domain to the nucleus[25]. In has been shown that the N-terminal

domain knockdown results in the loss of filaggrin protein in a 3D skin model[26]. The exact function of the C-terminal domain of profilaggrin is currently unknown but it is believed to be necessary for processing of this protein into functional filaggrin monomers; it is evidenced by this process being impaired in the individuals carrying truncating *FLG* mutations that do not allow for the expression of the C-terminus[27].

Profilaggrin is heavily phosphorylated upon expression; this post-translational modification (PTM) is proposed to stop premature interactions between the protein and keratin filaments; only dephosphorylated filaggrin monomers are capable of keratin aggregation (Figure 2.)[25]. Profilaggrin expression is upregulated in *stratum granulosum* where it is stored in the form of tightly packed keratohyalin granules[25]. Profilaggrin undergoes dephosphorylation by phosphatases including acid phosphatases and a protein phosphatase 2A (PP2A)-type enzyme in the upper part of *stratum granulosum*[25]. Then, profilaggrin is cleaved into filaggrin monomers by proteases such as skin aspartic acid protease (SASPase), furin, profilaggrin endopeptidase-1 (PEP1), caspase-14, matriptase, prostasin or kallikrein-related peptidase (KLK) 5 (KLK5)[25,28,29]. Filaggrin monomers efficiently cross-link keratin fibers leading to the collapse of cytoskeleton, cell death and formation of flat corneocytes that form *stratum corneum*[30]. During profilaggrin processing both N- and C-terminal domains of the protein are cleaved off; the N-terminus then locates to the nucleus where it is broken down into A and B domains[25]. However, this is not the final stage of the profilaggrin processing cascade; arginine residues in filaggrin monomers are converted to citrulline by peptidylarginine deiminase (PAD) 1 (PAD1) and PAD3; such conversion is called deimination or citrullination[30]. Then, filaggrin is further broken down into amino acids by calpain 1, caspase-14 and bleomycin hydrolase (BMH)[21,30]. Among the generated free amino acids, histidine is converted into *trans*-urocanic acid (*trans*-UCA) by histidase and non-enzymatic conversion of glutamine to 2-pyrrolidone-5-carboxylic acid (PCA) also occurs[30]. Free amino acids, *trans*-UCA and PCA are key elements of the natural moisturizing factor (NMF) which helps maintain epidermal barrier function and supports skin hydration[21]. It has been shown that physiological concentrations of both UCA and PCA inhibit the growth of *Staphylococcus aureus (S. aureus)*[31,32]. Consistently, *FLG* gene mutation carriers in whom no functional profilaggrin protein is expressed, very often suffer from *S. aureus* skin infections[33]. When exposed to the UV radiation, *trans*-UCA undergoes isomerization into its *cis* form (*cis*-UCA) which has shown immunomodulatory function[34,35]. Free

amino acids released during filaggrin breakdown contribute to the maintenance of acidic pH (4.1-5.8) in the *stratum corneum* which protects the skin surface from pathogens[21,36]. The acidic pH further supports the function of *stratum corneum* since it provides an optimal environment for the activity of enzymes involved in ceramide synthesis[21]. Low pH also regulates the activity of KLK5 and KLK7 which are involved in homeostatic desquamation (shedding) of corneocytes[37]. Specifically, lympho-epithelial Kazal type-related inhibitor (LEKTI) inhibits both those proteases by forming complexes with them; however, these complexes dissociate in acidic pH allowing KLK5 and KLK7 to function[37]. On the other hand, both enzymes reach their optimal activity in slightly basic pH[37]. Therefore, low pH in the *stratum corneum* may prevent overactivity of these proteins which could lead to degradation of tightly cross-linked protein-lipid network in the tissue[37]. As a result, impermeability of the epidermal barrier could be compromised[37]; the importance of this system is highlighted in patients with the Netherton syndrome where this axis is dysregulated because of mutation in the serine protease inhibitor of Kazal type 5 *SPINK5*, encoding LEKTI[37].

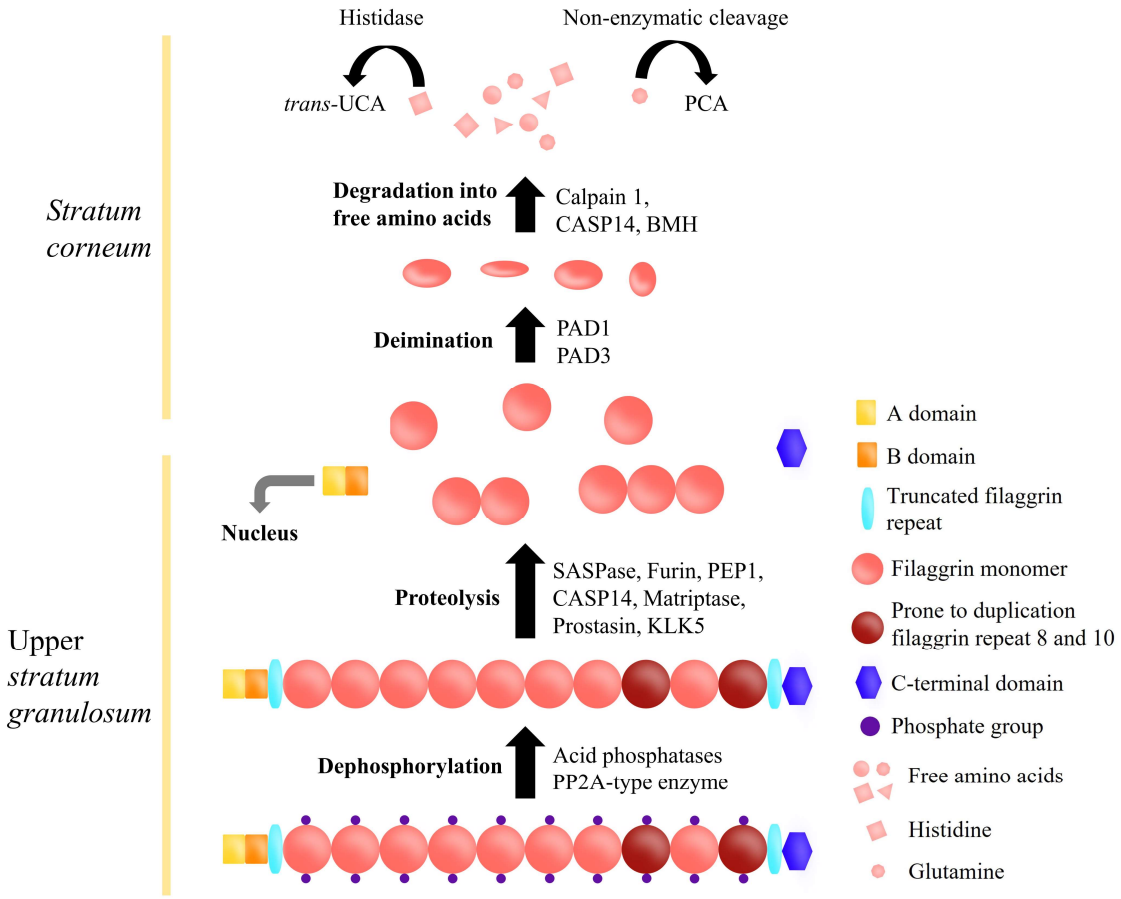


**Figure 2. Profilaggrin processing in the epidermis.** Profilaggrin is heavily phosphorylated upon synthesis. It is dephosphorylated in upper *stratum granulosum* by phosphatases and cleaved into filaggrin monomers by proteases. The N-terminal domain of profilaggrin containing A and B subdomains is translocated to the nucleus. Filaggrin monomers cross-link keratin fibers resulting in the collapse of the cytoskeleton; this is indispensable in the development of *stratum corneum*. Next, filaggrin undergoes deimination/citrullination and degradation to amino acids. Histidine is enzymatically converted to *trans*-urocanic acid (*trans*-UCA) while glutamine in cleaved to 2-pyrrolidone-5-carboxylic acid (PCA). Free amino acids, t-UCA and PCA contribute to skin moisturization. BMH, bleomycin hydrolase; CASP14, caspase-14; KLK5, kallikrein-related peptidase 5; PAD, peptidylarginine deiminase; PCA, 2-pyrrolidone-5-carboxylic acid; PEP1, profilaggrin endopeptidase 1; PP2A, protein phosphatase 2A; SASPase, skin aspartic acid protease; *trans*-UCA, *trans*-ucrocanic acid.

## 1.2. The immune cells of the skin

As skin constitutes a barrier surrounding the entire body it is vital that any pathogens which infiltrate the tissue are recognized as quickly as possible. However, it is equally important that innocuous substances e.g., allergens are recognized as non-threatening to avoid unwarranted activation of the immune system. This balancing act is fulfilled by a variety of skin-resident immune cells which are ready to respond to infections or tissue

injury or induce tolerance when the antigen encountered is harmless[38]. While keratinocytes are not classified as immune cells, they are immunologically active; their involvement in the immune response in a specific context will be discussed later.

### 1.2.1. Professional antigen-presenting cells

Professional antigen-presenting cells (APCs) are responsible for sampling of the tissue environment for pathogen-derived antigens. APCs recognize pathogen components with both membrane-bound and intracellular pattern recognition receptors (PRRs) they express; these include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the receptor for advanced glycation end products (RAGE), intracellular nucleotide-binding oligomerization domain-containing proteins (NODs) or nucleotide-binding oligomerization domain-like receptors (NLR)[38–40]. Upon encountering a pathogen, APCs can activate other tissue-resident immune cells in a contact-dependent manner or through production of cytokines; these cells also recruit additional immune cells from the periphery through the release of attracting chemokines[38]. Besides acting locally, APCs which have encountered antigens migrate to the lymph nodes where they present antigens to T lymphocytes. If the source of the antigen is harmful (e.g., pathogens), antigen-specific T lymphocytes are activated[38]. In case of self-antigens or antigens which are harmless, tolerance is induced and the immune system is not triggered[38]. Major histocompatibility complex (MHC) class I and class II molecules, which bind antigens are expressed on the surface of APCs[39]. After antigens are taken up by APCs, they are processed and cleaved into short peptides; these are then displayed ("presented") bound within the MHC proteins[39]. MHC-antigen complexes are recognized by T cell receptor (TCR) of antigen-specific T cells which become activated[39]. However, additional co-stimulation is necessary for an efficient T cell activation[39]. Such signal is provided by co-stimulatory molecules expressed on the surface of APCs; these include cluster of differentiation (CD) 40 (CD40), CD80 or CD86 which interact with their respective receptors on T cells[39]. On the other hand, APCs can inhibit the activation of antigen-specific T cells by expressing inhibitory proteins, such as programmed death-ligand 1 (PD-L1) or immunoglobulin-like transcript (ILT-3) and 4 (ILT-4)[41].

While the epidermis in steady state is, to a great extent, devoid of immune cells, it contains Langerhans cells (LCs) which are a specialized type of APCs that home exclusively to

this tissue[38,42]. Due to their epidermal location LCs are among the first "sentinel" cells to contact pathogens or allergens penetrating through the skin; therefore, they are crucial for quick initiation of the immune response and pathogen clearance. LCs can be distinguished by the presence of Birbeck granules containing langerin, a CLR which captures and internalizes antigens[42,43]. LCs are abundant in MHC class II and constitutively express very high levels of the lipid antigen-presenting molecule CD1a[38,44,45]. In fact, the skin is the predominant site of CD1a expression, which to a great extent can be attributed to the high CD1a levels in LCs[45]. Additionally, LCs also express the high-affinity immunoglobulin E (IgE) receptor (FceRI); this receptor allows for recognition and uptake IgE-bound antigens by LCs[46,47]. LCs activated in this manner can stimulate T cells to elicit a proinflammatory response[48]. When activated, LCs extend their processes into the dermis and stimulate other cells of the immune system[38]. LCs are also migratory and travel to lymph nodes where they present antigens to T cells[38].

Dermal dendritic cells (DDCs) present in the dermis are a major component of the skin APC population[38]. In humans these can be divided into conventional dendritic cell (cDC) type 1 (cDC1), type 2 (cDC2) and a separate population which expresses CD14, the marker which serves as a co-receptor for several TLRs[38,49]. These DDCs differ in the functional outcome produced following their interaction with T cells in the skin or lymph nodes[38].

Macrophages are phagocytic cells that are distributed in the peripheral tissues and can be derived from a range of progenitors[50]. These cells are very functionally plastic and their roles can vary depending on their origin as well as the environment of the tissue in which they reside[50]. However, the most distinct and best-described subsets are the proinflammatory M1 macrophages and anti-inflammatory M2 macrophages which polarize in response to different cytokines[50]. M1 macrophages are highly efficient in digesting microbes which are cleared from the environment and processed into antigens; these are then efficiently presented to T cells due to the high expression of MHC class II and co-stimulatory receptors CD40, CD80 and CD86 in M1 cells[50,51]. Moreover, M1 macrophages secrete cytokines promoting polarization of Th1 cells, produce anti-microbial agents and mediators which recruit other types of immune cells[51]. M2 macrophages, on the other hand, upregulate the macrophage mannose receptor (MMR, CD206), secrete immune inhibitory mediators, clear cellular debris and apoptotic bodies or facilitate tissue repair and wound healing[51]. Contrary to M1 cells they promote Th2 or

Treg phenotype in T cells[51]. In the skin macrophages localize to the dermis and express the CD64 marker which can be used to distinguish them from DDCs[38]. Dermal macrophages abundantly produce interleukin (IL) 10 (IL-10) in healthy skin suggesting their anti-inflammatory phenotype[38]. Their homeostatic function in the skin includes clearance of cellular debris and hair regeneration[38].

### 1.2.2. Mast cells

Mast cells are major contributors to allergic inflammation[38,52]. Those cells localize to the dermal layer of the skin to which they migrate as progenitors and mature in response to the keratinocyte-derived stem cell factor[38]. Cytokines secreted by T helper 2 (Th2) cells, which are typically activated in allergic inflammation induce expansion of mast cells[38]. The primary means of mast cell activation is through their membrane FcϵRI receptor[52]. IgE are often bound to FcϵRI receptors and can be cross-linked by antigens leading to mast cell activation and degranulation[52]. Due to their expression of a variety of PRRs mast cells can be also directly activated by pathogens[52,53]. Mast cell granules contain proteases, histamine, heparin, chondroitin sulfate and cytokines[52]. Histamine is a major mediator of allergic inflammation and one of the best-known inducers of itch[54]. This molecule decreases expression of filaggrin, inhibits keratinocyte differentiation and dysregulates expression of genes related to cell adhesion[55]. On the other hand, histamine upregulates BMH and protease inhibitors which are crucial for the function of *stratum corneum*[55]. Mast cells can also exacerbate allergic diseases by producing type 2 cytokines[56]. Moreover, these cells secrete lipid mediators of inflammation, such as prostaglandins and leukotrienes[52].

### 1.2.3. Eosinophils

Eosinophils infiltrate the dermis in many inflammatory skin diseases[57]. They can be directly activated by pathogens through PRRs and via receptors binding various inflammatory mediators[57]. Similarly to mast cells they contain granules but their composition differs; the granules store toxic granule proteins (TXPs) such as eosinophil peroxidase, eosinophil-derived neurotoxin (EDN), eosinophil cationic protein and major

basic protein (MBP)[57]. TXPs exhibit microbicidal properties, but EDN also recruits dendritic cells and can activate the TLR2 signaling pathway[57]. Eosinophils produce extracellular traps (EET) which contain DNA fibers and granule proteins which capture and kill extracellular bacteria, respectively[58]. In addition, eosinophils produce cytokines, leukotrienes and prostaglandin D2 characteristic for allergic inflammation[57]. Cytokine production by eosinophils also polarizes T cells towards the Th2 phenotype and stimulates B cells[57]. Apart from their role in allergic diseases eosinophils are recruited to tissues infected with helminths[59]. However, their involvement in the clearance of parasites in still elusive as data regarding this process vary between animal infection models and specific helminth species[59].

### 1.2.4. T lymphocytes

T lymphocytes are key cells of the adaptive immune response[39]. Following stimulation by APCs only T cells expressing TCR matching a cognate antigen presented within specific antigen presenting molecule are activated[60]. T cells can be divided into two types depending on their expression of co-receptors; CD4 or CD8 TCR co-receptors stabilize the interaction between the TCR and MHC class II or MHC class I, respectively[39]. The majority of T cells in healthy human skin is located in the dermis, usually in a close proximity to blood vessels and skin appendages[61]. Among CD4+ T cells a variety of Th subtypes can be distinguished including Th1, Th2, Th9, Th17 or Th22[39,62,63]. Differentiation into a particular Th subtype largely depends on the environmental cues. Every Th subset has its characteristic transcription factor, cytokine signature and is specialized to respond to specific pathogens[39]. Through the production of various mediators Th cells recruit and activate cells of the innate immune system, as well as B lymphocytes, cytotoxic T cells and non-immune cells which collectively play a role in pathogen clearance[64]. In contrast to the proinflammatory CD4+ T cells, there are subtypes of immunosuppressive CD4+ T cells[64]. Natural and induced regulatory T cells (nTreg and iTreg) as well as type 1 regulatory T cells (Tr1) can be distinguished within this subset[64]; their suppressive activity is necessary to prevent persistent inflammation which would lead to tissue damage[64]. Moreover, they also confer tolerance to harmless molecules such as allergens or self-antigens, hence protecting from allergic reactions and autoimmune diseases, respectively[64].

CD1a-autoreactive T cells which respond to lipid antigens presented by the CD1a protein are found among the CD4+ T cell population in the skin[65]. Even though these cells are major IL-22 producers, they have also been shown to secrete type 1 and type 2 cytokines[45,65]. Interestingly, CD1a-autoreactive T cells which recognize lipid self-antigens express skin-homing receptors and can be found even in non-diseased skin; this combined with constitutive high expression of CD1a in LCs suggests that CD1a-dependent T cell responses play an important role in skin homeostasis[45,66]. On the other hand, increased numbers of skin CD1a+ dendritic cells in inflammatory skin disorders is observed suggesting the involvement of CD1a-dependent recognition in pathology[66].

CD8+ T lymphocytes, also known as cytotoxic T lymphocytes (Tc) are activated by cells bearing antigen-MHC class I complexes[67]. The long-established role of cytotoxic T lymphocytes is direct killing of infected cells by inducing their apoptosis through two main mechanisms[68]. The first one involves the action of Tc-released perforin which creates pores in a target cell and granzyme B which activates caspases, i.e., serine proteases which are primary effectors of apoptosis[68]. Another mechanism of inducing apoptosis employed by Tcs involves receptor-mediated contact with the target cell; the Fas protein present on the target cell interacts with the Fas ligand (FasL) expressed on the Tc surface[68]. This interaction initiates the assembly of the death-inducing signaling complex (DISC) which activates caspases and leads to apoptosis[68]. Interestingly, despite cytotoxicity being the main function of CD8+ T cells, a heterogeneity similar to the CD4+ Th cells has been found within this subset[69]. Specifically, CD8+ cells populations secreting defined sets of cytokines have been identified; therefore, various Tc subtypes have been distinguished including Tc1, Tc2, Tc9, Tc17 or Tc22[69]. Apart from different cytokine profiles those subsets also vary in their cytotoxic capacity[69]. CD8+ regulatory T have also been identified; these can exert their immune inhibitory function by production of immunosuppressive IL-10 and transforming growth factor (TGF) β (TGFβ) or expression of inhibitory surface receptors[70]. Moreover, those cells were found to induce killing of pathogenic CD4+ cells or promote tolerogenic phenotype in monocytes and dendritic cells[70,71].

### 1.2.5. B lymphocytes

B lymphocytes produce antigen-specific antibodies (immunoglobulins, Ig) of several classes (isotypes), i.e., IgA, IgG, IgD, IgM and IgE. Mature but unstimulated B cells express only IgD and IgM[72]. However, stimulation by an antigen, immune mediators or interaction with T cells induces Ig class switching; as a result, B cells start to produce IgA, IgG or IgE depending on the type of stimulation[72]. B cells stimulated by IL-17 or cytokines of the type 2 response increase their production of IgE[73,74]. Additionally, B cells present antigens, co-stimulate T cells and produce a variety of immune mediators[75]. These cells are present in the dermis of healthy skin and two subtypes have been shown to localize to this tissue; these are conventional (B-2) and innate-like (B-1-like) B cells[76]. It is currently unknown how antibodies produced by those cells contribute to the skin homeostasis but in the intestine, they facilitate the regulation of the gut microbiome[76]. Whether these cells fulfil a similar role in the skin remains to be elucidated[76]. Skin-resident B cells in an ovine model have shown higher expression of CD80, CD86 and MHC class II molecules compared to the lymph node-localized B cells[77]. This suggests their high capability of efficient activation of antigen-specific T cells[77]. B cells are known to infiltrate the dermis in inflammatory skin diseases such as atopic dermatitis (AD), psoriasis or cutaneous leishmaniasis[38,75,78,79]. AD patients exhibit increased levels of immunoglobulin class E (IgE) in their serum which suggests a major involvement of those cells in the disease[80]. A subset of IL-10-producing immunosuppressive B cells has been identified among B-1-like B cells in the human skin but their role is not clear[76,81]. However, disruption of accumulation of IL-10-producing B cells in the skin aggravated cutaneous inflammation in mice[82]. Moreover, the abundance of IL-10-producing circulating B cells in decreased in patients suffering from AD and psoriasis[83,84].

### 1.2.6. Innate lymphoid cells

Innate lymphoid cells (ILCs), similarly to T lymphocytes differentiate from common lymphoid progenitor cells[85,86]. Their subsets mirror Th cells in terms of cytokine secretion profile and transcription factors that govern their polarization[85,86]. The three ILC subtypes identified are ILC1 resembling Th1 cells, ILC2 which are counterparts of Th2 lymphocytes and ILC3 that share similarities with Th17 cells[85]. Unlike T cells, ILCs lack

antigen-specific receptors and are primarily activated by cytokines secreted by neighboring cells[85]. Additionally, ILC2 can recognize pathogens via PRRs[86]. ILCs are scarce in the circulation but enriched in mucosal and barrier sites; therefore, they are primarily tissue-resident and carry out their immune functions mainly in the periphery[86]. ILC2 is the ILC subtype the most enriched in the skin and present in both the epidermis and dermis[86]. Increased numbers of ILC2s have been observed in human AD lesional skin and these cells upregulated receptors for cytokines relevant to the disease[87]. Furthermore, ILC2s from lesional skin of AD patients exhibit increased expression of cytokines promoting type 2 response[87]. This ILC subtype is also known to express CD1a and present endogenous lipid antigens[88]. Expression of CD1a in ILC2s can be upregulated by inflammatory mediators[88]. These observations suggest an important role for ILC2s in skin disease.

## 1.3. Extracellular vesicles

Extracellular vesicles (EVs) are lipid bilayer-enclosed structures produced by all cellular organisms[89]. Due to their role in intercellular communication EVs have been extensively studied in both physiology and pathology[89]. EV populations can be divided into apoptotic bodies (APs), ectosomes and exosomes, based on differences in their biogenesis which also affect their size, cargo content and functionality (Figure 3. and Table 1.). Additionally, a novel type of EV, secreted midbody remnants (sMB-Rs) have been described but their function is poorly described[90,91]. Ectosomes are generated at and released from the cell membrane into the extracellular space[89]. These vesicles constitute a very heterogenous group and are named after types and functionality of the producers to reflect their cell-specific origin (e.g. oncosomes produced by tumour cells or migrasomes released by migratory cells)[89]. However, the best characterized members of the ectosome family are microvesicles (MVs) which like APs and exosomes are produced by all nucleated cells[89,92,93].

Several EV subtypes have been distinguished based on their pathway of generation, size or cargo composition[90]. However, current EV isolation techniques and lack of subset-specific markers impede isolation and identification of separate, highly pure EV populations for the experimental purposes[94]. Hence, one of the recommendations regarding EV nomenclature set up by the International Society of Extracellular Vesicles

(ISEV) is the use of broader nomenclature referring to the size of vesicle subtypes; the terms "small EVs" (sEVs) and medium/large EVs (m/lEVs) were advised[94]. Accordingly, those terms were applied in the description and discussion of the original research included as the core part of this thesis as well as studies cited throughout as background knowledge.

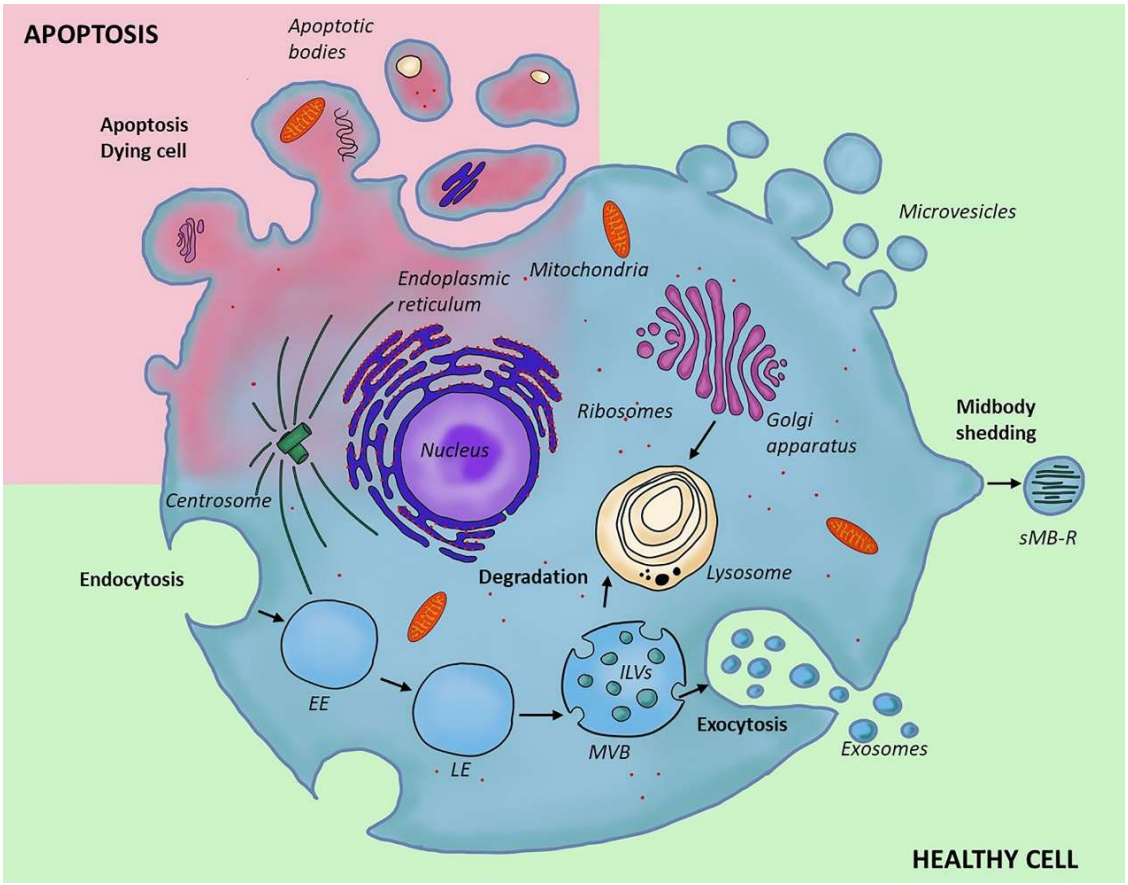


**Figure 3. Generation of extracellular vesicles.** The figure is from Hovhannisyan *et al.*[90] Apoptotic bodies are the largest extracellular vesicles; they are formed by blebbing of the membrane of cells undergoing programmed cell death, apoptosis. Those vesicles contain organelles which are expelled during cell death. Microvesicles are shed from the cell surface and their cargo largely resembles the content of the cytoplasm. Secreted midbody remnants also originate from the plasma membrane and are formed during the late stage of cell division. They contain the midbody which participates in the separation of dividing cells. The biogenesis of exosomes occurs via the endosomal system; it begins with the inward budding of late endosomal membrane which leads to the formation of intraluminal vesicles; these accumulate inside the late endosome which matures into a multivesicular body. The multivesicular body can then either fuse with a lysosome, leading to degradation of its content or merge with the cellular membrane which results in the release of exosomes into the outside of the cell. EE, early endosome; LE, late endosome; MVB, multivesicular body; ILV, intraluminal vesicle; sMB-R, secreted midbody remnant.

| | Medium/large extracellular vesicles (m/lEVs) 150 – 5000 nm | | | Small extracellular vesicles (sEVs) 50 – 150 nm | |
|---|---|---|---|---|---|
| | **Apoptotic bodies (APs)** | **Larger microvesicles (Larger MVs)** | **Secreted midbody remnants (sMB-Rs)** | **Smaller microvesicles (Smaller MVs)** | **Exosomes (Exos)** |
| **Cargo** | Fragmented DNA, RNA, proteins, lipids, histones, apoptotic organelles | DNA, RNA, proteins, receptors, lipids | Midbody, cellular proteins, MAPK pathway proteins, Rho GTPase effectors, cytokinetic proteins, prominin-1 | DNA, RNA, proteins, receptors, lipids | DNA, RNA, lipids, proteins, tetraspanins, integrins, receptors |
| **Membrane** | Protein-rich | Protein-rich | Currently unknown | Protein-rich | Lipid-rich |
| **Origin** | Membrane of apoptotic cells | Cell membrane | Intercellular bridge of dividing cells | Cell membrane | Endosomal compartment |
| **Release mechanism** | Cell membrane blebbing during apoptosis | Cell membrane budding | Cytokinetic abscission | Cell membrane budding | Multivesicular body fusion with cell membrane |
| **Cargo selectivity** | No | No | Not known | No | Yes |
| **Sedimentation** | 2,000 g 10 min | 10,000 g 30 min | 10,000 g 30 min | 100,000 g 1-16 h | 100,000 g 1-16h |

**Table 1. Characteristics of extracellular vesicle populations.** m/lEV, medium/large extracellular vesicle; sEV, small extracellular vesicle; AP, apoptotic body; MV, microvesicle; sMB-R, secreted midbody remnant; MAPK, mitogen-activated protein kinase.

APs are large (1-5 µm) vesicles formed as a result of an apoptotic cell disassembly during the late stage of apoptosis and contain many types of cellular components such as organelles, degraded proteins, micronuclei, chromatin and DNA fragments[95]. APs are important in the clearance of apoptotic cell components which may be toxic to the surrounding tissues by attracting phagocytes that engulf and clear those vesicles[95]. Interestingly, APs have been shown to also mediate horizontal DNA transfer which can lead to integration of the genetic material into the genome of the recipient cell[95,96].

Microvesicles (MVs) are smaller compared to APs (100-1000 nm) and are shed from the cell surface through a highly regulated process of cell membrane pinching and scission of the nascent MV[92]. Due to their biogenesis mechanism surface markers present within their lipid membrane reflect the composition of the membrane of the cell of origin[90]. Several proteins implicated in the process of formation and release of MVs such as ADP ribosylation factor 6 (ARF6) and arrestin domain-containing protein 1 (ARRDC1) or vesicle-associated membrane protein 3 (VAMP3) sites[92,97,98]. Apart from the proteins, a variety of lipids and nucleic acids have also been identified within the cargo carried by MVs[90,92].

Exosomes, the smallest membrane-surrounded vesicles (50-150 nm) originate through the endosomal pathway[89]. First, the late endosomal membrane buds inwards which

generates an intraluminal vesicle (ILV)[89,90,93]. As the number of ILVs increases endosomes mature into multivesicular bodies (MVBs)[89,93]. MVBs then fuse with the cell membrane leading to the release of ILVs, which are now called 'exosomes', from the cell[90]. In addition to their small size exosomes are abundant in lipids which improves their flexibility; therefore, these vesicles produced by tissues can enter the bloodstream, reach targets distant from their origin and even penetrate the blood-brain barrier[99,100]. Hence, exosomes participate in both local and long-distance signaling[99,101]. One of the main exosome biogenesis pathways involves the endosomal sorting complex required for transport (ESCRT) machinery; alternatively, these vesicles can be generated through ESCRT-independent mechanisms[90].

ESCRT pathway proteins include ESCRT-0, -I, -II and -III subcomplexes which are supported by accessory proteins[102,103]. The formation of exosomes is initiated by the binding of ESCRT-0 to the outer endosomal membrane; this is possible through high affinity of this subcomplex for phosphatidylinositol 3-phosphate (PIP3), a phospholipid species abundant in the endosomal membranes[102,103]. Moreover, ESCRT-0 binds ubiquitin and therefore interacts with ubiquitinated cargo, also prevalent in the endosomal membrane[103]. ESCRT-0 then recruits ESCRT-I and -II which results in the formation of protein-rich microdomains on the cytoplasmic surface of the endosomal membrane; the consequence of this is the invagination of the membrane[102]. ESCRT-II then recruits and activates ESCRT-III which assembles at the neck of the budding vesicle and cleaves it, resulting in the release of the vesicle into the endosomal lumen[103]. Following this event, the ESCRT-III complex is disassembled and the vacuolar protein sorting 4 (VPS4) which is an ATPase provides energy required for this process[102,104]. Due to their involvement in exosome biogenesis, some ESCRT or accessory proteins, such as the apoptosis-linked gene 2-interacting protein X (ALIX) or tumour susceptibility gene 101 (TSG101) are used as markers of those vesicles[103].

While ESCRT-dependent mechanism of exosome generation is now broadly appreciated, other pathways leading to the exosome formation have also been described[105,106]. For example, Ras-related protein Rab-31 (RAB31) has been shown to interact with flotillin proteins in the endosomal membrane microdomains and induce inward budding of the membrane *in vitro*[105]. Exosome biogenesis is also dependent on the types and the arrangement of the lipids in the endosomal membrane[106]; *in vitro* studies have shown that accumulation of ceramides in the endosomal lipid bilayer promotes invagination of the

membrane[106,107]. Such enrichment in ceramides is driven by neutral sphingomyelinases (N-SMases) and their inhibition reduces sEV secretion[106,108]. Another mechanism of exosome formation involves the family of tetraspanin proteins and exosomes are particularly enriched in those, including CD9, CD63, CD81 and CD82 which are commonly used as exosomal markers[109,110]. Tetraspanins, which are transmembrane proteins are present in cellular and endosomal membranes of nearly all cell types; they are involved in the spatial organization of their own members, transmembrane or cytosolic proteins as well as lipids into distinct microdomains known as tetraspanin-enriched microdomains (TEMs)[109]. Due to their ability to alter the curvature of biological membranes, tetraspanins have been suggested to participate in exosome formation[109,111]. For example, an *in vitro* study showed that dendritic cells from CD9 knockout mice exhibited decreased production of sEVs compared to wild type while CD63 knockout in the human embryonic kidney cell line HEK293 resulted in a decrease of exosome-size particles produced by those cells[112,113]. Moreover, sEVs secreted by a human epithelial breast cancer cell line MDA-MB-231 in which CD81 was knocked out showed disrupted membrane integrity, suggesting that this protein is involved in biogenesis of those vesicles[114].

The composition of the exosomal cargo does not necessarily reflect the content of the parental cell suggesting that these vesicles employ selective cargo sorting mechanisms[115]; these involve some of the components of the exosome biogenesis pathways including the ESCRT complex[102,115]. Since ESCRT-0 recognizes ubiquitinated cargo, proteins bearing this post-translational modification can be sorted into the vesicles during ILV formation[115]; indeed, enrichment in the ubiquitinated proteins has been found among sEV cargo[116,117]. At the same time a lot of non-ubiquitinated proteins are also carried within sEVs, suggesting that other pathways of exosomal cargo sorting are also employed[118]. Many other PTMs have been implicated in the regulation of trafficking of proteins into exosomes; these include SUMOylation, ISGylation, acetylation, glycosylation, citrullination and more. However, some PTMs such as acetylation or ISGylation directed the modified proteins for degradation rather than exosomal release[115,119,120]. Another proposed mechanism of the exosomal cargo loading is by its association with lipid rafts; i.e., regions of the lipid membrane particularly rich in cholesterol, sphingolipids and glycerophosphatidylinositol (GPI)-anchored protein, identified also in the exosomal bilayer[121]. Some proteins known to associate with lipid rafts such as the ganglioside GM1,

MHC class II or flotillin-1 have been identified in those rafts within the sEV membrane suggesting that localization of cargo in those sites may facilitate their trafficking into sEVs[121]. Additionally, an *in vitro* study in retinal pigment epithelial cells revealed that the αB-crystallin protein known to localize to lipid rafts was present in sEVs and disruption of lipid raft formation inhibited release of this protein within sEVs[122]. Tetraspanins have also been implicated in the process of the exosomal cargo sorting through their interaction with such cargo[123]. For example, CD63 has been linked with localization of the melanocyte protein PMEL to ILVs or trafficking of the latent membrane protein 1 (LMP1) from Epstein-Barr virus (EBV) to sEVs[124,125]. Another tetraspanin, CD9 has been suggested to increase the abundance of the metalloproteinase CD10 in sEVs[126]. Both CD9 and CD82 have shown to enhance the amount β-catenin in secreted sEVs[112].

Following the release into the extracellular space sEVs can interact with and propagate signals to the recipient cell by binding of cell membrane-embedded proteins[115,127]. This mode of action is used by sEVs produced by DCs which can present antigens to T lymphocytes by MHC-peptide complexes present within the vesicle membrane[127]. In addition, sEV cargo may be released into the cell by fusion of exosome with a cell membrane or whole intact vesicles might be internalized by the cell through a variety of processes[115,128,129].

As far as the cargo is concerned, internalization of exosomes which are abundant in proteins, lipids and nucleic acids affects processes occurring inside the recipient cell[129]. A particular interest has been expressed in the functionality of exosomal microRNA (miRNA) species[130], which can regulate gene expression in recipient cells[130].

Secreted midbody remnants (sMB-Rs), sized between 200 nm and 600 nm are generated at the final stage of the cell division[90,91], when the daughter cells remain connected via a narrow intercellular bridge. This bridge contains the midbody (MB) which is abundant in microtubules; this transient organelle is involved in cytokinetic abscission resulting in separation of the dividing cells[91]. One of the daughter cells retains the MB in the form of a midbody remnant (MB-R)[91]. However, it has been shown that MB-Rs can be secreted into extracellular space (sMB-Rs) and taken up by non-sister cells[91]. Since MB-Rs are involved in cell signaling, sMB-Rs may also exhibit such properties[91]. Currently, there are very little on the function of those vesicles; however, the study by Rai *et al.* showed

that human colorectal cancer cell line-derived sMB-Rs induce invasive phenotype in quiescent cells of a mouse fibroblast cell line[91].

## 1.4. Atopic dermatitis

Atopic dermatitis (AD), also known as atopic eczema (AE), is among the most prevalent chronic inflammatory skin diseases presenting with skin lesions and pruritus (itch) affecting both children and adults (Figure 4.)[131]. Various sources estimate the incidence of AD in children and adolescents to be at 2-30% while 2-10% of the adult population are reported to be affected by the disease[132–135]. There is high country-to-country variability in the percentage of the population suffering from AD as more AD cases have been reported in the developed countries and among urban populations indicating that lifestyle and environmental factors may influence the occurrence of this disorder[132–136]. Around 50% of patients start experiencing AD symptoms within the first year of life while 85% develop the disease by 5 years of age[137]. A meta-analysis of pediatric AD patients from 15 highly developed countries found that in 20% of cases the disease persisted beyond the age of 8 years[138]. However, single studies indicate that even 30-40% of AD patients may experience persistent symptoms[139–141]. Even though the duration of patient follow-up in those studies vary, overall, they support the idea of AD as a chronic disease[138]. Although AD symptoms mostly develop in the childhood, adult onsets of the disorder have been reported; such presentation has been found to differ pathophysiologically and clinically[142]. Due to its chronic nature and debilitating symptoms AD negatively impacts individual quality of life and poses a great socioeconomic burden[131]. Additionally, AD has been positively correlated with psychological disorders such as anxiety or depression and the propensity of such co-morbidities has been found to increase with disease severity and duration[131,143,144]. The psychological and emotional burden is also experienced by the closest family members, mostly caregivers of children suffering from AD; disruption of everyday life and lack of sleep, financial strains imposed by the treatment cost and special care those children require affect the mental health of members of their closest circle[145], not to mention the emotional load. The complexity of AD stems from multiple factors contributing to its pathophysiology which include the impairment of epidermal integrity, dysregulation of the immune response, gene mutations, epigenetic mechanisms, skin infections and multiple environmental factors[131,142]. Such complexity poses a question of

which of the disease components initiates the cascade of events leading to the onset of AD[146]. Two main hypotheses are currently proposed with one suggesting that the breach of epidermal integrity occurs first and is responsible for the activation of aberrant immune response ("outside-in" hypothesis), while the other proposes that it is the activation of immune system that leads to epidermal disruption ("inside-out" hypothesis)[146]. Moreover, AD patients are known to develop other allergic manifestations such as rhinitis, asthma or food allergy later in life even if AD symptoms had resolved; such progression of allergic manifestations into spatially-distant tissues and organs is known as the 'allergic march' or 'atopic march'[134].

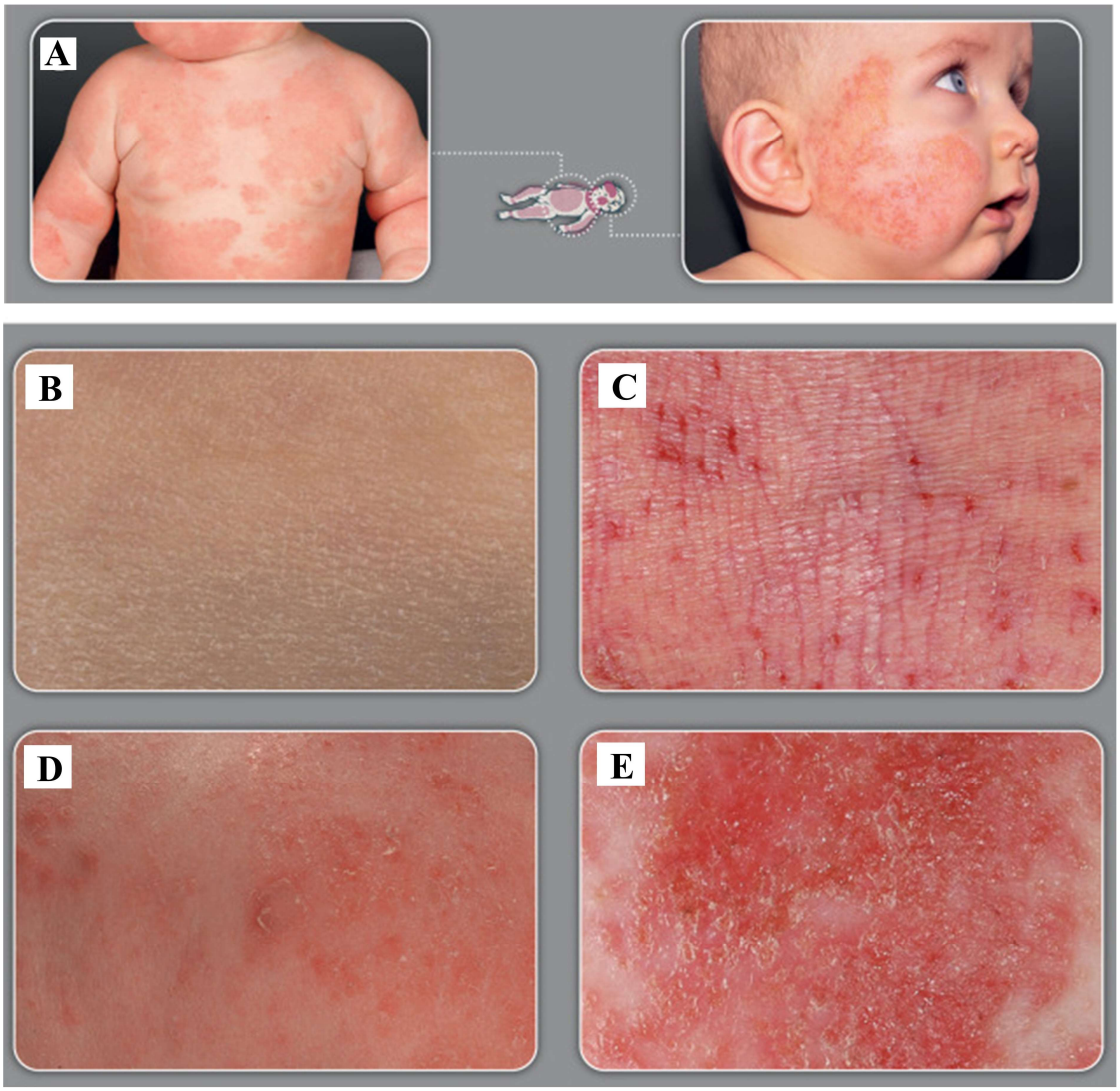


**Figure 4. Skin lesions in atopic dermatitis.** Figure adapted from Langan *et al.*[131]. Lesions in infants can affect vast areas of the skin (A). Non-lesional (B), acute (C), subacute (D) and chronic (E) skin lesions in atopic dermatitis. The appearance of lesions often differs between acute and chronic disease and skin thickening (lichenification) often occurs in the latter.

### 1.4.1. Immune response in atopic dermatitis

#### 1.4.1.1. Keratinocytes

Keratinocytes have a crucial role in driving immune responses in AD by producing various mediators[147]. They activate in response to physical epidermal damage but also through PRR stimulation triggered by pathogens and allergens which penetrate into deeper layers of the epidermis due to its compromised integrity[147]. In terms of the PRRs, keratinocytes are known to express extracellular TLR1-6 and intracellular TLR3 and 9 as well as most NLRs and CLRs[147,148].

Upon activation by the proinflammatory environment, keratinocytes produce an array of chemokines that attract cells of the immune system to the epidermis[149]; elevated monocyte chemoattractant protein-1/C-C motif chemokine ligand 2 (MCP-1/CCL2) is known to recruit monocytes, dendritic cell precursors, Th2 and Th1-skewed T cells, while the regulated upon activation, normal T cell expressed and presumably secreted (RANTES/CCL5) additionally attracts eosinophils[149,150]. MCP-1 is also involved in stimulation of IL-4 production by T cells and promotes the Th2 phenotype[149]. Other chemotactic agents relevant to AD include eotaxin targeting eosinophils as well as T cells, CCL17 and CCL18 recruiting T cells and macrophage inflammatory protein-3α (MIP-3α/CCL20) attracting T cells, monocytes and antigen-presenting cells including dendritic cells and LCs[150–154]. I-309/CCL1 which attracts T cells and LCs precursors, while monocyte chemoattractant protein 4 (MCP-4/CCL13) is a chemoattractant for T cells, eosinophils, monocytes and dendritic cell precursors; eotaxin-3/CCL26 recruits eosinophils and T cells and CTACK/CCL27 attracts T cells[150].

In a response to stimuli such as tissue damage, microbes and allergens or signaling molecules keratinocytes produce a plethora of immune mediators, including IL- 1β, IL-3, IL-6, IL-17C, IL-18, IL-19, IL-23, IL-25, IL-33, thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor alpha (TNFα), TGFα and TGFβ or platelet-derived growth factor (PDGF)[155–158]. Of those, keratinocytes directly influence T cell response bias by secretion of the type 2 response-promoting mediators, i.e., TSLP, IL-25 and IL-33 (also known as “alarmins”) which are hallmark keratinocyte-derived cytokines upregulated in AD[159,160]. IL-1β, IL-6 and TGFβ synergistically trigger type 17 response which has been found as characteristic for early AD onset in children; the subsequent release of IL-17A can stimulate the type 2

response[161–163]. Among transcription factors contributing to the allergic inflammation in AD is p63; its overexpression induces pathological skin phenotype, very similar to that in lesional skin of AD patients[164]. p63 has been shown to drive expression of IL-31 and IL-33 which both promote the activated phenotype in T cells and significantly contribute to the pruritus experienced by AD patients[164–166].

TSLP is a cytokine characteristic for AD and its expression is increased in AD lesions but not in non-lesional skin, suggesting a confined distribution of this mediator[167]. Cytokines known to be upregulated in AD *milieu* such as TNFα, IL-1β, IL-4 and IL-13 work in combination to further stimulate TSLP production from keratinocytes, thus exacerbating the inflammation[168]. TSLP is a potent stimulator of T cell polarization, activating dendritic cells to prime naïve T cells for development into the Th2 phenotype[134,147,169]. Studies in murine models revealed that induction of TSLP expression in the skin resulted in the development of lesions resembling those present in patients with AD; type 2 cytokines were also increased in the skin; mast cells, eosinophils and lymphocytes were identified in dermal infiltrates and elevated IgE levels were observed in the circulation[170]. TSLP was also found to be crucial in eliciting inflammation in antigen-induced dermatitis[170]. This mediator has also been shown to promote pruritus by stimulation of sensory neurons[134,171].

Keratinocytes are the major producers of IL-25 in AD; increased levels of this cytokine are found in both lesional and non-lesional skin of AD patients[172]. Induction of the type 2 inflammation by IL-25 can occur by direct stimulation of either naïve T cells or ILC2 cells[158,173]. On the other hand, IL-25 has been shown to promote Th17- rather than Th2-biased inflammation in contact hypersensitivity[174]; however, it is still uncertain whether IL-25 play a role in Th17 inflammation occurring in the skin of early onset pediatric AD patients[161].

IL-33 is constitutively expressed by keratinocytes and stored in the nucleus as a full-length precursor[175]. Upon cell damage, this precursor is released and cleaved into shorter, much more biologically active forms; such cleavage can be carried out by proteases derived from allergens or those produced by neutrophils and mast cells[175,176]. Similarly to the other two alarmins, IL-33 promotes a type 2-skewed response; a study in which the cytokine was overexpressed in murine keratinocytes *in vivo* resulted in increased levels of type 2 cytokines in skin lesions accompanied by elevated circulating IgE[177,178].

Moreover, IL-33 directly enhances production of IL-13 and IL-5 by Th2 cells, activates eosinophils, stimulates basophils to secrete IL-4 and IL-13 as well as induces ILC2 proliferation and type 2 cytokine secretion by ILC2[87,178,179]. IL-33 also contributes to pruritus by direct stimulation of sensory neurons but also by potentiating the release of IL-31, the main cytokine involved in the induction of chronic itch in AD, by Th2 lymphocytes[165,166,180].

Keratinocytes are known to constitutively express MHC class I while MHC class II expression can be induced by inflammatory conditions, primarily by IFNy stimulation; moreover, studies in mice have shown that skin colonization by AD-relevant pathogens such as *S. aureus* and *C. albicans* as well as the IL-22 cytokine also stimulate MHC class II expression in keratinocytes[181–185]. Importantly, these cells are capable of antigen processing which is a prerequisite for efficient presentation of protein-derived antigens[182,184]. Several studies have shown the capability of keratinocytes to act as non-professional APCs they activate both CD4+ and CD8+ T cells in an antigen-specific manner[184,186,187]. However, exposure of keratinocytes to inflammatory conditions or their components such as IFNy is often required for T cells to be efficiently activated[184,187]. T cells can also become activated non-specifically upon cross-linking of their TCRs and IFNy-induced keratinocyte MHC class II molecules by *S. aureus* enterotoxin B, acting as a superantigen[188].

#### 1.4.1.2. T cells

Lesional and non-lesional AD skin is infiltrated by immune cells, incl. antigen-presenting LC and DC populations, ILC2s and CD4+ T cells skewed towards the Th2 phenotype; increased levels of IL-4, IL-5 and IL-13 are reported as a consequence in the affected skin[74,87,134]. Lesional skin of AD patients exhibits differential expression patterns in genes related to Th2 response (IL-4, IL-10, IL-13) and IL-22, a hallmark cytokine of the Th22 response[189–191].

#### 1.4.1.2.1. Type 2-dominated inflammation in AD skin

Cytokines linked to the type 2 response dominate complex allergic *milieu* and play multiple roles which collectively compound AD pathology[192] (Figure 5.). Increased numbers of Th2/Tc2, Th22/Tc22 and decreased frequency of Th1/Tc1 T cells has been reported among cutaneous lymphocyte-associated antigen (CLA)+ T cell population in the blood of adult AD patients presenting with moderate-to-severe symptoms[193]. Type 2 cytokines are known to stimulate eosinophils and mast cells[74]. Moreover, IL-4 and IL-13 activate basophils and mast cells which release immune mediators[74]. IL-4 and IL-13 cytokines are involved in a positive feedback loop that is crucial for maintenance of the Th2-skewed phenotype of T cells and their production of Th2 cytokines[74].

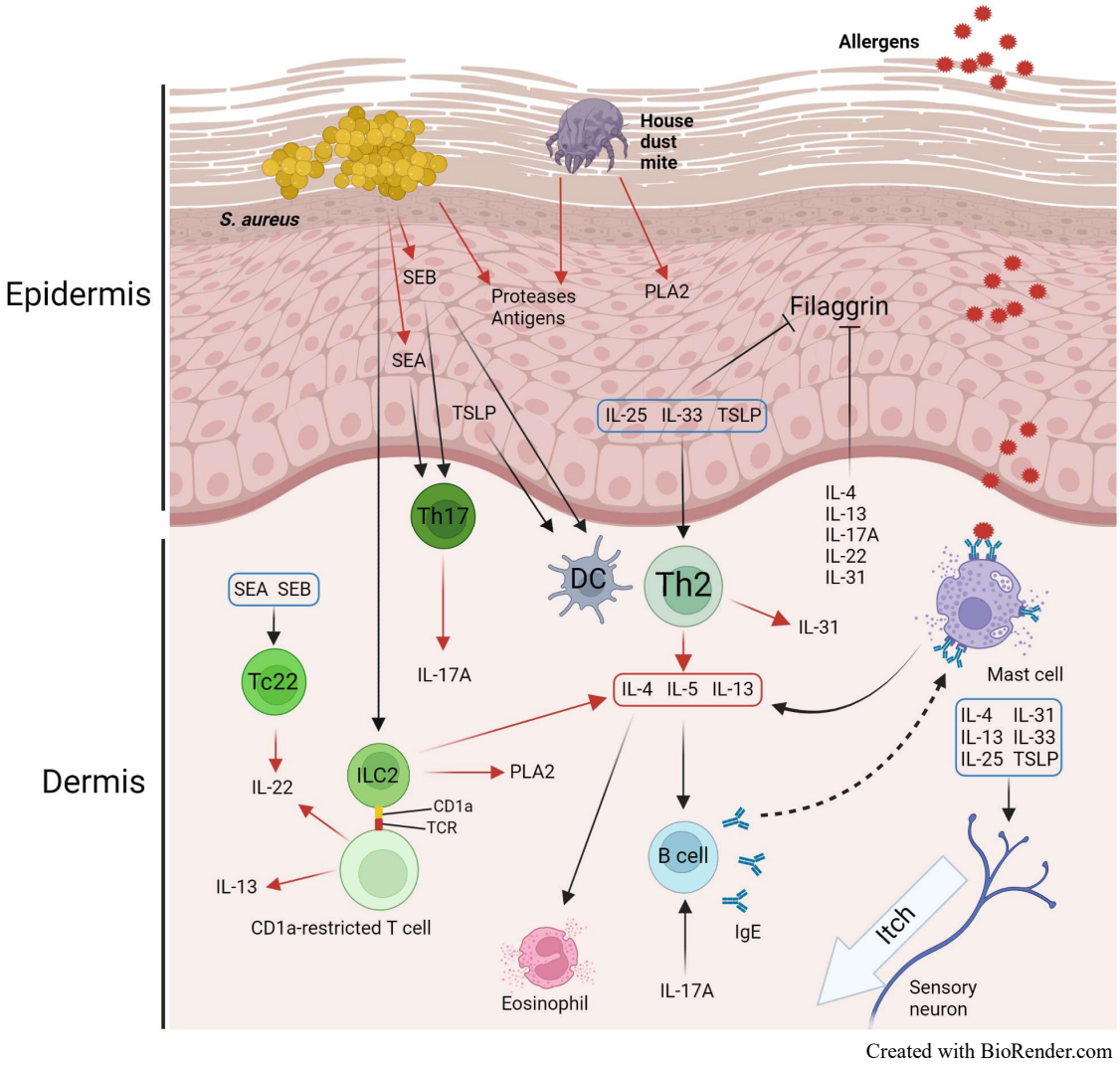


**Figure. 5. Skin inflammation in atopic dermatitis.** Acute atopic dermatitis is characterized by persistent inflammation which is dominated by the type 2 response. Pathogens and allergens may also activate type 17 and type 22 responses. Inflammation impairs the epidermal barrier which

results in increased penetration of pathogens and allergens into deeper epidermal tissues and the dermis; this exacerbates the persistent inflammation. Pathogens and allergens are sources of proteases which increase epidermal permeability. Inflammatory mediators stimulate sensory neurons to mediate itch. DC, dendritic cell; IgE, immunoglobulin E; IL, interleukin; ILC2, group 2 innate lymphoid cell; PLA2, phospholipase A2; SE, staphylococcal enterotoxin; Tc, cytotoxic T cell; TCR, T cell receptor; Th, helper T cell; TSLP, thymic stromal lymphopoietin.

Furthermore, Th2 cytokines also contribute to the disruption of the epidermal barrier in AD; IL-4 and IL-13 have been shown to downregulate genes which are crucial for the maintenance of epidermal integrity such as *FLG*, *LOR* and *IVL*[194–196]; these cytokines inhibit keratinocyte differentiation and induce their hyperproliferation[196,197]. Apart from impacting skin barrier-relevant proteins, IL-4 and IL-13 impair the barrier integrity by altering lipid production through downregulation of fatty acid elongase enzymes ELOVL3 and ELOVL6 by keratinocytes[198]. These enzymes may be at least partly responsible for the decrease of the number of lipid species with long chain fatty acids incorporated into their structure; this, in turn could lead to lower density of lipid packing in *stratum corneum* and epidermal barrier disruption[198,199]. IL-13 is also known to induce matrix metalloproteinase (MMP) 9 (MMP-9) in keratinocytes; MMP-9 can cleave the components of the basement membrane and therefore, facilitate infiltration of immune cells into the epidermis[200]. IL-13 has been shown to inhibit MMP-13 production by dermal fibroblasts which results in a decrease in collagen break down leading to fibrosis; this might contribute to lichenification, a symptom typical for chronic AD[201].

IL-4, IL-13 and IL-31 produced by Th2 cells contribute to the intense itch experienced by AD patients[131,165]. Studies showed the presence of IL-4Rα, IL-13Rα1 and IL-31RA receptors in human sensory neurons which suggests that all three cytokines can trigger pruritus by direct stimulation of the nervous system[202,203]. Further evidence on the involvement of those receptors in pruritus comes from clinical trials demonstrating that IL-4Rα or IL-31RA blocking by monoclonal antibodies alleviate this symptom[204,205].

Type 2 cytokines also play a key role in antibody-mediated responses; IL-4, IL-5 and IL-13 induce antibody class switching by B cells resulting in increased production of antigen-specific IgE antibodies[74]. Indeed, AD patients, especially those experiencing moderate-to-severe symptoms present with antigen-specific IgE antibodies against various food and airborne allergens, microbes as well as keratinocyte-derived

autoantigens; this further exacerbates the disease[206,207]. It has also been shown that more severe disease is accompanied by IgE-mediated sensitization to a greater variety of antigens compared to moderate AD[206].

While AD pathogenesis points mainly at the type 2 axis, we now know that the disease is far more complex. It has also been shown that the abundance of IL-17 and IL-23 in the peripheral blood of individuals suffering from AD correlates with the severity of disease[208]. Moreover, some studies, especially those involving AD patients of Asian descent suggest greater involvement of Th17- and Th22 cell mediated responses in the disease[209]. Data also suggest that the type 17 cytokine profile is characteristic for early AD onset in pediatric patients[161]. However, it is important to note that the increase in IL-17 and IL-22 levels observed in some patients can also be secondary to skin infections common in AD patients, e.g., staphylococcal enterotoxins SEA and SEB stimulate IL-22 production by cytotoxic subset of T cells (Tc22)[210]. *S. aureus* can also enhance production of T cell-derived IL-17[211–213]. Moreover, IL-17 levels in the serum of AD patients have been found to positively correlate with the abundance of IgE immunoglobulins[208]; this could be due to IL-17 inducing IgE production by B cells[73]. AD patients have shown an increased sensitization to allergens such as house dust mite (HDM); Those allergens can elicit T cell responses of not only Th2-, but also Th17- or Th2/Th17-skewed phenotype[206,214]. IL-22 expression is upregulated in the lesional skin of AD patients[215]. It has been shown that HDM enhances secretion of this cytokine by T cells and increases expression of its receptor subunit IL-22Rα in HaCaT cells[216]. Activation of IL-22/IL-22R signaling leads to increased secretion of CCL17 by keratinocytes[216]; this in turn enhances T cell migration[216]. IL-22 is known to stimulate proliferation and migration of keratinocytes but inhibits their differentiation[215]. These features can be attributed to the inhibition of expression of several proteins key for epidermal differentiation by IL-22: K1, K10, IVL, LOR and filaggrin[215].

While the involvement of the type 1 responses in AD remains controversial as the relationship between Th1 and Th2 responses is antagonistic, it is suggested that the chronic phase of the disease can be associated with an increased infiltration of AD skin by Th1 cells[217]. A study of nearly 250 AD patients with moderate-to-severe disease showed a decrease in markers of the type 2 and type 22 axis in the skin with age while markers typical for type 1 and type 17 axes were increased[218]. A study by Bakker *et al.* which investigated blood sera of AD patients with severe disease adds to the complexity

of this disorder; it identified a significant group of patients (18.5% of the total cohort) which showed high levels of type 1 and 17 cytokines in addition to type 2 cytokines[219]; the mean age of this group was the lowest compared to other groups[219]. Despite increasing evidence expanding our understanding of AD pathophysiology beyond the type 2 responses, attempts at exploiting the other pathways for treatment have been largely unsuccessful[220,221].

#### 1.4.1.3. Antigen-presenting cells

In AD, a population of inflammatory dendritic epidermal cells (IDECs) is recruited to the epidermis[222]. These cells express surface proteins such as CD1a, CD1b, MMR, FcεRI and low-affinity IgE receptor FcεRII, costimulatory markers CD80 and CD86 as well as MHC class I and II; however, they differ from LCs in their lack of langerin expression[222]. While LCs are crucial for maintenance of homeostasis in the healthy skin, IDECs mainly function by exacerbating the inflammatory environment in skin diseases, including in AD[222]. IDEC epidermal infiltration, along with LC activation, is a key component of the immune response occurring upon exposure of AD skin to allergens[223].

Both LCs and IDECs in AD skin are characterized by increased activation state as evidenced by upregulation of CD80 and CD86 costimulatory markers[224]. In addition to the high expression of costimulatory markers, LCs in the skin of AD patients are also enriched in the FcεRI receptor[225,226].

The keratinocyte-produced alarmin, TSLP has been found to influence the blood dendritic cell function by causing these cells to induce CD4+ T cells to produce high amounts IL-4, IL-5 and IL-13 but very little IFNy and IL-10; this suggests the acquisition of the Th2 phenotype by the T cells[227]. Similar observations were made in case of LCs which responded to TSLP stimulation by induction of Th2 cells and secretion of the TARC/CCL17 chemokine that attracts T cells of the type 2 secretory profile[228]. It has been shown that type 2 cytokines induce monocyte differentiation into dendritic cells specializing in redirecting T cell responses towards the Th2 phenotype, thus creating a positive feedback loop that favours type 2 responses and could contribute to inflammation in AD[229,230].

Plasmacytoid dendritic cells (pDCs) are the major producers of type I interferons (IFNα and IFNβ) and are therefore important in mounting immune response to viral infections[222]. The reduction of pDC numbers is AD skin and their impaired ability to produce type I interferons could be partially responsible for the susceptibility of AD patients to viral infections[222,231]. Moreover, due to the antagonistic relationship between type I interferons and Th2 cytokines, the lack of the former could contribute to the increased expression of the latter in AD skin [222,232]. Contrary to this, a study by Guttman-Yassky *at al.* showed an increased infiltration of pDCs in AD skin lesions compared to healthy skin[233]. Those pDCs also produced CCL22 which attracts Th2 cells[233].

#### 1.4.1.4. The role of CD1a in atopic dermatitis

CD1a is a lipid antigen-presenting protein belonging to the group of CD1 molecules[234]. One of the sources of the CD1a ligands are the products of digestion of diacyl glycerophosphocholine species (PCs) present in biological membranes, by phospholipase A2 (PLA2); these products are monoacylglycerophosphocholine (Lyso-PC) and fatty acids (FAs)[234]. PLA2 is highly relevant to AD; it is produced by mast cells and is present in HDM and other allergen sources[234–236]. IL-13 has been found to increase cytosolic PLA2 (cPLA2) levels in mouse macrophages and enhance activation of this enzyme in human monocytes[234,235,237,238]. Interestingly, PLA2 produced by mast cells was detected in their sEVs[235]; the enzyme contained within those vesicles was active in generating lipid CD1a antigens[235]. Mounting evidence suggests a role for CD1a, in this disease. Specifically, IL-13-producing CD1a-restricted T cell population responding to HDM was shown to be increased in the peripheral blood and skin of AD patients compared to healthy controls[234]. This observation was attributed to HDM-derived PLA2 that generated CD1a lipid neoantigens which activated CD1a-restricted T cells[234]. CD1a is also expressed on the surface of ILC2s; these reside mainly in the skin and mucosa where they maintain tissue homeostasis and produce, among others, type 2 cytokines[88]. CD1a expression in ILC2 is increased by TSLP or IL-33 suggesting that the capacity for CD1a-restricted T cell activation by those cells can be higher in AD skin[88]. CD1a antigens can be generated by the ILC2-expressed PLA2 and this expression can be further enhanced by TLR2 and TLR4 stimulation by *S. aureus*, which often colonizes the skin of AD patients[88]. Hardman

*et al.* also showed that CD1a-reactive T cells stimulated by ILC2s presenting lipid antigens produce IL-13 and IL-22[88].

### 1.4.2. Genetic component in atopic dermatitis

Genetic predispositions are important susceptibility factors for AD[239]. It has been shown that children are at greater risk of developing the disease if family history of atopy is reported[240,241]; the risk increases approximately 3-fold in case of one parent suffering from AD and to 5-fold if both parents are affected[242]. Moreover, the analysis of multiple association studies found that nearly up to a quarter of dizygotic twins develop AD together while in monozygotic twins this number rises to about half [243].

#### 1.4.2.1. *FLG*

The greatest known genetic factor that predisposes an individual to AD is the loss-of-function (LoF) mutation (null mutation) in the gene encoding profilaggrin (*FLG)* which is located within the epidermal differentiation complex (EDC) on chromosome 1q21.3[239,244–246]; EDC comprises a complex of genes expressed during keratinocyte differentiation in the upper layer of the epidermis[244]. It is estimated that up to 40% European patients with moderate-to-severe disease carry a LoF *FLG* mutation[247]. In addition to AD, *FLG* mutations are also associated with other allergic type disorders such as asthma, allergic rhinitis and food allergy[244,248,249]. It has been identified that many AD patients not necessarily carry null *FLG* mutations but exhibit a reduced number of the filaggrin repeats encoded by their functional *FLG* gene copy which further shows that insufficient amount of fully functional profilaggrin/filaggrin protein can lead to the incidence of AD[250]. In case of homozygotes and compound heterozygotes for *FLG* null mutations there is a total lack of the filaggrin protein. While the exact mutations vary between ethnic populations two dominant account for about 80% of mutations in AD in the Northern European population; R510X and 2282del4[245,251]. These mutations which result in a premature termination of protein translation allowing for expression of only the N-terminus of profilaggrin while no complete filaggrin repeats are synthesized[27]. In contrast, a study comparing *FLG* mutations in European and Singaporean Chinese AD patients found that the latter exhibited a larger variety of mutations and none of these

were as dominant[252]. While three mutations: 3321delA, 6850del8 and S1515X, not detected in the Irish population, accounted for around half of all the mutations in the Singaporean Chinese patient cohort, R501X was found in only 1% of the patients compared to 39% of the Irish cohort[252]. These data reflect the results of other studies showing the uniqueness of certain *FLG* mutations in particular ethnicities and varying prevalence of the mutations[132–135]. It is known that AD involving *FLG* mutations has a distinct phenotype, presents with more severe and persistent symptoms and is often associated with a greater risk of developing asthma or allergic sensitization in comparison to AD with no identified mutation[251]. Moreover, AD patients carrying *FLG* mutations are at higher risk of developing skin infections with S. *aureus* and herpes simplex virus[33,231]. *FLG* null mutations also predispose patients to sensitization to allergens and asthma[253–255]. The incidence of the 'allergic march' among AD patients is heavily correlated with *FLG* null mutations which increase the risk of the development of asthma and food allergy even though filaggrin is not expressed by the cells of the respiratory system or the gut[251]. Moreover, *FLG* LoF mutations have been found to predispose children to peanut allergy[248,256].

#### 1.4.2.2. Filaggrin insufficiency and the immune response

The role of filaggrin in modulation of innate and adaptive immune responses has been documented by multiple studies. *FLG* knockdown promotes higher keratinocyte reactivity to stimulation by TLR1, 2, 3 and 9 agonists as well as TNFα; this was manifested by the increase of IL-1α production by filaggrin-insufficient keratinocytes compared to filaggrin-expressing cells[257]. Additionally, TLR3 stimulation of *FLG* knockdown HaCaT cells showed enhanced IL-1β, IL-6 and TSLP secretion compared to filaggrin-expressing cells[257]. The amount of TSLP protein in the epidermal layer of a 3D skin model was higher in the filaggrin knockdown model compared to the filaggrin-expressing model in steady state[257]. TLR3 stimulation increased TSLP abundance in both models but more of this protein was present under filaggrin-insufficient conditions[257]. Sakai *et al.* investigated the effect of filaggrin insufficiency on the production of proinflammatory cytokines in stratified primary keratinocytes[258]; the results showed increased production of IL-1α, IL-8, IL-18 and GM-CSF in *FLG* knockdown cells[258]. Elias *et al.* performed a proteomic analysis of a 3D skin model in which a knockdown of

*FLG* was induced[259]; this resulted in alterations in the expression of proteins involved in innate and adaptive immunity[259]. Filaggrin was found to inhibit PLA2 and, as a result downstream CD1a-dependent T cell responses which further highlights the significance of CD1a-dependent T cell activation in the AD skin where filaggrin insufficiency is common[234]. Filaggrin insufficiency is also involved in regulation of sensitivity to irritants; filaggrin-insufficient mice (flaky tail mouse model expressing a form of profilaggrin which is not processed to monomers) developed prominent inflammation to a topical irritant at a dose which failed to produce a response in healthy mice[260]. Mutant mice exhibited increased serum IgE levels and a higher number of prostaglandin D2 receptor-expressing cells; both of these features are hallmarks of a Th2 response[260]. Increased sensitization to allergens such as peanut, Fel d 1 (cat dander allergen) and Der p 1 (HDM-derived allergen) have been reported in children carrying *FLG* null mutations[261,262]. Type 17 responses also seem to be affected since filaggrin insufficiency is associated with increased frequency of Th17 cells in the circulation of affected individuals[263]. Moreover, a study in an another mouse model (filaggrin *null* mice) showed that application of *S. aureus* on the skin elicits a Th17-type response, which was LC-dependent; such response was not observed in healthy mice[264]. Filaggrin insufficiency also impacts antigen-specific T cell responses; Marwah *et al.* showed that keratinocytes expressing reduced levels of filaggrin were superior in an antigen-dependent stimulation of HDM-derived antigen-specific T cells compared to filaggrin-expressing cells; the cytokine milieu resulting from this interaction promoted reduction in barrier quality[265]. Filaggrin insufficiency also affects APCs because LCs derived from patients with *FLG* loss-of-function mutations show increased expression of the CD83 maturation marker[266]. Moreover, the *cis*-UCA decreases the MHC class II expression well as CD40 and CD86 costimulatory molecules by dendritic cells[266]. Furthermore, *cis*-UCA impairs dendritic cell maturation in response to stimulation by lipopolysaccharide (LPS)[266]. Additionally, *cis*-UCA-treated dendritic cells induce higher proportion of regulatory T lymphocytes compared to untreated cells[266].

Allergic AD *milieu* also impacts the abundance of filaggrin in the epidermis; IL-4 and IL-13 have been found to downregulate the expression of the protein as well as impact additional epidermal barrier genes[267]; similar effect was observed for IL-17A, IL-22, IL-25, IL-31, IL-33, TNF-α and TSLP[172,268–272]. As a result AD patients without *FLG* null mutations also suffer from the effects of filaggrin insufficiency in their skin; e.g.,

transepidermal water loss (TEWL), a hallmark of a disrupted skin barrier, affects AD patients suffering from moderate-to-severe disease to a similar extent irrespective of their *FLG* mutation status[251]. Moreover, decreased quantity of filaggrin breakdown products, PCA and UCA is found in non-lesional epidermis of AD patients without *FLG* mutations; this further implies the involvement of AD *milieu* in downregulation of filaggrin[273].

#### 1.4.2.3. Mutations in additional genes in AD patients

Mutations in a number of genes encoding epidermal barrier-related proteins, but also those relevant to the immune response which encode for PRRs, cytokines, chemokines or cell membrane receptors, have been implicated in AD (Table 2.)[239,274]. However, heterogeneity in AD pathophysiology between populations can be observed; multiple candidate genes and their role in disease pathogenesis and its severity are yet to be explored.

| Genes | | | | | |
|---|---|---|---|---|---|
| **Barrier-related** | **Cytokine-related** | **Chemokine-related** | **PRR-related** | **Other immune response/ inflammation-related** | **Other** |
| *CSTA, **FLG**, KIF3A, KLK7/ SCCE, LAMA3, OVOL1, SPINK5, SPRR3, TMEM79 /MATT* | *CSF2, GATA3, IFNG, IFNGR1, IL4, IL4RA, IL6, IL6R, IL7R, IL9, IL9R, IL10, IL10RA, IL12A, IL12B, IL12RB1, IL12RB2, IL13, IL13RA1, IL17A, IL18, IL31, IL33, IRF2, PHF11, SOCS3, ST2/IL1RL1, STAT6, TGFB1, TNFA, TSLP, TSLPR/CRLF2* | *CCL5, CCL11, CCL22* | *CARD12/ NLRC4, CARD15/ NOD2, CD14, MAL/TIRAP, NOD1, TLR2, TLR4, TLR6, TLR9, TOLLIP* | *CMA1, CTLA4, CYSLTR1, DEFB1, FCER1A, FCER1B/ MS4A2, HNMT, HRH4, TAP1, TIM1/HAVCR1, TIM4/ TIMD4* | *BFL1/ BCL2A1, GSTP1, LELP1, VDR* |

**Table 2. Genes the mutations of which have been linked to atopic dermatitis.** Summarized from Al-Shobaili *et al.*[274], Bin *et al.*[275] , Marenholz *et al.*[276] and Gao *et al.*[277].

**Barrier-related genes:** *CSTA,* cystatin A; *FLG*, filaggrin; *KIF3A*, kinesin family member 3A; *KLK7/SCCE*, kallikrein related peptidase 7/stratum corneum chymotryptic enzyme; *LAMA3*, laminin subunit alpha 3; *OVOL1*, ovo like transcriptional repressor 1; *SPINK5*, serine peptidase inhibitor Kazal type 5; *SPRR3*, small proline rich protein 3; *TMEM79/MATT*, transmembrane protein 79/mattrin. **Cytokine-related genes:** *CSF2*, colony stimulating factor 2; *GATA3*, GATA binding protein 3; *IFNG*, interferon gamma; *IFNGR1*, interferon gamma receptor 1; *IL4*, interleukin 4; *IL4RA*, interleukin 4 receptor subunit alpha; *IL6*, interleukin 6; *IL6R*, interleukin 6 receptor; *IL7R*, interleukin 7 receptor; *IL9*, interleukin 9; *IL9R*, interleukin 9 receptor; *IL10*, interleukin 10; *IL10RA*, interleukin 10 receptor subunit alpha; *IL12A*, interleukin 12A;

*IL12B*, interleukin 12B; *IL12RB1*, interleukin 12 receptor subunit beta 1; *IL12RB2*, interleukin receptor subunit beta 2; *IL13*, interleukin 13; *IL13RA1*, interleukin 13 receptor subunit alpha 1; *IL17A*, interleukin 17A; *IL18*, interleukin 18; *IL31*, interleukin 31; *IL33*, interleukin 33; IRF2, interferon regulatory factor 2; *PHF11*, plant homeodomain finger protein 11; *SOCS3*, suppressor of cytokine signaling 3; *ST2/IL1RL1*, serum stimulation-2/interleukin 1 receptor like 1; *STAT6*, signal transducer and activator of transcription 6; *TGFB1*, transforming growth factor beta 1; *TNFA*, tumor necrosis factor alpha; *TSLP/CRLF2*, thymic stromal lymphopoietin/cytokine receptor like factor 2. **Chemokine-related genes:** *CCL5,* C-C motif chemokine ligand 5; *CCL11*, C-C motif chemokine ligand 11; *CCL22*, C-C motif chemokine ligand 22. **PRR-related genes:** *CARD12/NLRC4*, caspase recruitment domain containing protein 12/NLR family CARD domain containing 4; *CARD15/NOD2*, caspase recruitment domain containing protein 14/nucleotide binding oligomerization domain containing 2; *CD14*, cluster of differentiation 14; *MAL/TIRAP*, MyD88-adapter-like/TIR domain containing adaptor protein; *NOD1*, nucleotide binding oligomerization domain containing 1; *TLR2,* toll like receptor 2; *TLR4*, toll like receptor 4; *TLR6*, toll like receptor 6; *TLR9,* toll like receptor 9; *TOLLIP*, toll interacting protein. **Other immune response/inflammation-related:** *CMA1*, chymase 1; *CTLA4***,** cytotoxic T-lymphocyte associated protein 4; *CYSLTR1*, cysteinyl leukotriene receptor 1; *DEFB1*, defensin beta 1; *FCER1A*, IgE Fc receptor subunit alpha; *FCER1B/MS4A2*, IgE Fc receptor subunit beta/membrane spanning 4-domains A2; *HNMT*, histamine N-methyltransferase; *HRH4*, histamine receptor H4; *TAP1*, transporter 1, ATP binding cassette subfamily B member; *TIM1/HAVCR1*, hepatitis A virus cellular receptor 1/T-cell immunoglobulin mucin receptor 1; *TIM4/TIMD4*, T-cell immunoglobulin mucin receptor 4/ T-cell immunoglobulin and mucin domain containing 4. **Other:** *BFL1*/*BCL2A1*, BCL2 related protein A1; *GSTP1*, glutathione S-transferase Pi 1; *LELP1*, late cornified envelope like proline rich 1; *VDR*, vitamin D receptor.

Downregulation of barrier-related genes other than *FLG* has been reported in AD[239]; transcriptomic profiling revealed reduced expression of genes encoding IVL (*IVL*), LOR (*LOR*) or late cornified envelope protein 2B (*LCE2B*) in lesional AD skin[275]. Downregulation of genes encoding corneodesmosin (*CDSN*) and claudin-1 (*CLDN1*), components of cell-to-cell adhesion complexes has also been reported in AD skin lesions[275]. Regarding mutations in the epidermal barrier-relevant genes identified in AD patients, a polymorphism in the small proline-rich protein 3 (*SPRR3*) gene, one of the EDC genes, has been identified in a European cohort of patients[276]. Individuals carrying gene mutations resulting in deficiency of desmoglein 1 a key desmosomal protein were found to suffer from severe dermatitis and multisensitization to allergens[275]. Transmembrane protein 79 (TMEM79) is crucial for lamellar body secretion and, therefore epidermal barrier formation[275]. A meta-analysis of AD cases pointed to an association between a specific single nucleotide polymorphism (SNP), rs6694514 in the *TMEM79* gene with this disease[278]. An association was also identified in a meta-analysis of genetic association studies for the ovo-like transcriptional repressor 1 (*OVOL1)* gene; OVOL1 is a transcription factor which induces *FLG* expression[279,280]. LEKTI is encoded by *SPINK5* gene[275]. LEKTI is crucial for regulation of serine proteases that facilitate desquamation of *stratum corneum*[281]. Impaired function of this protein results in increased proteolytic activity in the epidermis and therefore, dysfunctional epidermal

barrier[281]. Polymorphisms in *SPINK5* have been identified in Caucasian and Japanese AD patients[281]. Moreover, polymorphisms in this gene were associated with increased severity of the disease and incidence of food allergy in Japanese children suffering from AD[281].

Since TLRs are crucial for innate recognition of pathogens, mutations in their genes in AD could alter immune response to common bacteria, fungi or viruses colonizing the skin of AD patients. While multiple TLR-encoding genes such as *TLR1*, *TLR6* and *TLR9* have been implicated, it is *TLR2* and *TLR4* that have shown to be linked to AD in the largest number of studies[239,274]. Specifically, the R753Q mutation in the *TLR2* gene has been found in patients with severe AD[282]. Increased susceptibility to *S. aureus* infection and elevated total serum IgE were also identified in AD patients carrying this mutation[282]. TLR2-stimulated monocytes of patients with mutated *TLR2* exhibited increased production of IL-6 and IL-12[283]. Furthermore, a meta-analysis combining results from 9 different studies identified the rs5743708 SNP in *TLR2* that increases the probability of developing AD among the Caucasian population[284]. A positive correlation has been shown between TLR2 stimulation and FcεRI expression in AD patients but not healthy donors, suggesting that *S. aureus* can further exacerbate allergic inflammation in people suffering from the disease[284]. Interestingly, rs2252226 polymorphism in the gene encoding the α chain of the FcεRI protein and rs4696480 in the *TLR2* gene are, together, associated with a more severe disease while having no effect separately[285]. The rs4986790 *TLR4* polymorphism has been found to increase the risk of AD incidence but it did not correlate with disease severity in a meta-analysis of studies involving Caucasian populations[284]. A study by Shi *et al.*, revealed that out of four *TLR4* polymorphisms identified in Chinese Han children the rs11536891 was predictive of the disease onset while the rs7869402 SNP was shown to confer high risk of severe AD[286].

Among keratinocyte-produced alarmins which promote type 2 response, *TSLP* variants have been associated with AD[275,287]. Additional *TSLP* polymorphisms have been linked to the disease in patients with a history of disseminated herpes simplex virus skin infections (ADEH)[287]. An association between the TSLP receptor gene (*TSLPR/CRLF2*) variants and AD was also identified[287]. Moreover, *IL33* polymorphisms have been implicated as risk factors of AD[277].

Genes encoding multiple components of the type 2 response have been also associated with AD[275]; of these *IL4*, *IL13,* the IL-4 receptor (IL-4R) gene *IL4R* and the α 1 subunit of the IL-13 receptor (IL-13Rα1) gene *IL13RA1* have been implicated[275]. An association was also found for *STAT6* which encodes a protein involved in the IL-4R signaling pathway[275]. Polymorphisms in the *IL31* gene have also been identified[275]. Additionally, a role for variants in the gene encoding the alpha chain of FcεRI (*FCER1A*) in AD has been described[275,288].

The predominance of the type 2 response in AD may, to some degree, be explained by the impairment of other response types. Certain variants of the *IL12B* and *IL12RB1* genes which are components of type 1 immunity were associated with AD risk in a Japanese population[275]. *IL18* is a gene important for both type 1 and 2 responses; its variants have been associated with AD in German and South Korean populations[289,290]. Moreover, polymorphisms of immune inhibitory *SOCS3* and *IL10* genes were associated with AD in European and Korean patients, respectively[275].

### 1.4.3. Skin infections in atopic dermatitis

Infections are a common feature of the skin of AD patients and exacerbate the inflammation and barrier damage in this organ[291]. Lesional skin of around 70-90% of patients is colonised by *S. aureus*, which is the most common pathogen infecting AD skin[291,292]. *S. aureus* disrupts epidermal integrity by producing V8 serine protease that increases epidermal peeling by disrupting keratinocyte tight junctions; *S. aureus* also produces exfoliative toxins A and B which degrade the key protein involved in the formation of desmosomes, desmoglein-1[293,294]. Staphylococcal α-hemolysin (Hla) forms a complex with ADAM10 protease which results in the cleavage of E-cadherin, necessary for the formation of adherens junctions[295]. *Stratum corneum* is further compromised by the ceramidase enzyme secreted by the bacteria which breaks down lipids crucial for the maintenance of the barrier impermeability[292].

There is evidence that *S. aureus*-derived lipoteichoic acid (LTA) contributes to colonization of the nasal mucosa by the bacteria by a mechanism involving indirect induction of the secretion of IL-10, by the skin immune cells[296]. *S. aureus* also induces PLA2 expression; apart from generation of the CD1a ligands, the key function of this enzyme is the production of proinflammatory mediators such as prostaglandins,

leukotrienes and thromboxane A2[52,88]. *S. aureus* colonizing AD skin secretes large amounts of δ-toxin which induces of mast cell degranulation and stimulates the production of IgE and IL-4; therefore it contributes to the allergic *milieu* in AD skin[297]. Moreover, phenol-soluble modulins (PSMs) produced by *S. aureus* recruit leukocytes and cause the release of IL-18 by keratinocytes[292]. Additionally, PSMs stimulate keratinocytes which then induce secretion of IL-17 by T lymphocytes and ILC3 cells[292]. *S. aureus*-derived enterotoxins A, B, C and D are extremely potent immune activators because they function as superantigens; some of these toxins also influence DCs to promote induction of the Th2 cell phenotypes[225,298]. Epicutaneous sensitization with SEB results in the induction of inflammatory response characterized by the production of IL-4 but not IFNy mRNA and elevated serum IgE suggesting that enterotoxins promote typical type 2 cytokine-dominated allergic inflammation[299]. Furthermore, the enterotoxins can negatively impact the inhibitory properties of regulatory T cells (Tregs) and act as adjuvants on allergen-specific T cell responses[225,300]. SEB has also been shown to stimulate IL-4 secretion by allergen-specific T cells and HLA-DR expression in keratinocytes which increases the ability of the latter to present allergen-derived antigens to T cells[301]; similar pattern in keratinocyte HLA-DR expression has also been observed in response to SEA[302]. The increased levels of IL-4 in AD skin create environment favourable for the spread of bacteria. Specifically, the cytokine impedes neutrophil proliferation and migration, which are required for efficient *S. aureus* clearance[303].

Some AD patients also suffer from a disseminated infection with the HSV-1 virus, eczema herpeticum (EH). EH is a very serious complication associated with high morbidity, often resulting in encephalitis and meningitis; the eye may also be affected which can lead to blindness[231]. It has been estimated that as many as 5% of patients exposed to the virus suffer from EH[287]. Evidence suggests that AD skin is more susceptible to HSV-1 penetration; the epidermal barrier defect enables the infection to reach the cells of the deeper epidermal layers beyond *stratum corneum*[304]. A study in an EH mouse model implicated decreased activity of natural killer (NK) cells as a risk factor for the incidence of severe skin lesions caused by HSV-1[305]. The common R501X *FLG* mutation and some variants of *TSLP* have been associated with increased risk of EH in AD patients[287]. Interferons are known for their important role in mounting immune responses to viral infections; mutations in the gene encoding interferon regulatory factor (IRF) 2 (*IRF2*) or reduced expression of IRF3 and IRF7 have been determined to be risk

factors for HSV-1 infection among the AD patients[306,307]. Reduced IFNy production as well as mutations in genes encoding this cytokine and the ligand-binding chain of its receptor, *IFNG* and *IFNGR1*, respectively, are also risk factors[307,308]. Increased expression and activity of the immune inhibitory indoleamine 2,3-dioxygenase 1 (IDO1) enzyme in LCs have also been implicated[309]. Staphylococcal Hla promotes HSV-1 infection in keratinocytes hence it might increase the risk of EH also in AD patients with *S. aureus* infection[307].

Sensitization to *Candida albicans* (*C. albicans*), a fungus which constitutes a part of healthy skin microbiome has been reported among AD patients[310–312]. This is mirrored by higher *C. albicans*-specific IgE antibody levels in the sera of AD patients compared to healthy controls[310,311]. This pathogen is recognized by TLRs, of which TLR2 and TLR4 play the main role; additionally, CLRs such as dendritic cell-specific ICAM-3-grabbing non-integrin 1 (DC-SIGN), dectin-1 or MMR are involved. Recognition of *C. albicans* by PRRs induces secretion of IL-1β, IL-6 and IL-23 by APCs; these cytokines promote differentiation of CD4+ T cells into the Th17 lineage[313]. Indeed, *C. albicans* has been identified as a unique inducer of type 17 immune response as other fungi stimulated mainly IFNy production suggesting the predominance of type 1 response[314]. The importance of type 17 response in *C. albicans* infection is further evidenced by the increased risk of developing candidiasis by individuals undergoing therapy with IL-17 inhibitors[315].

*Malassezia* spp. are commensal yeast that constitute the main fungal component of the microbiome of healthy skin[316]. Most *Malassezia* species lack fatty acid synthase genes so they depend on fatty acids which are present in the environment they inhabit[316]. Hence, they tend to localize to skin areas in which large amounts of lipid-rich sebum is produced; these are mainly head and neck[316]. Among *Malassezia* species *M. sympodialis* was the most prevalent in the skin of AD patients in Polish, Canadian and Korean studies[317]. However, a different study in Japanese AD patients found *M. furfur* to be the most prevalent in lesional skin followed by *M. globosa* and *M. sympodialis*[317]. While the diversity of *Malassezia* spp. remains largely unchanged in AD compared to healthy skin, a Japanese study detected the presence of a greater number of species in AD patients[316,318]. Contrary to healthy individuals, AD patients are sensitized to *Malassezia* spp. as evidenced by the presence of *Malassezia* spp.-specific IgE in their serum[131,316]. Interestingly, these antibodies are found more commonly in the adult than in child AD

patients[316]. This could be due to low sebum amounts in child skin, while the secretion of sebum increases during puberty and persists in the adult life; low sebum creates unfavourable growth conditions for the yeast[316]. The abundance of *Malassezia* spp.-specific IgE in the AD patient serum correlated with the severity of the disease in adults but not in children[316]. However, colonization of the scalp by *Malassezia* spp. in infants, children and adults promotes the development of seborrheic dermatitis[319]; this chronic inflammatory skin disease affects primarily the scalp and other body areas with high abundance of sebaceous glands such as face or trunk[319]. The disorder presents with an itchy rash and flaky scales in affected skin areas[319]. Currently, 14 *Malassezia*-derived allergens have been described; 10 from *M. sympodialis,* 3 from *M. furfur* and 1 from *M. globosa*[316,320]. An *in vitro* study found that *M. sympodialis* produces and releases more of the Mala s 12 allergen in pH 6.1, resembling the less acidic AD skin surface compared to the more acidic pH of 5.0 or 5.5 of healthy skin[316,321]. Some of the identified allergens are enzymes manganese superoxide dismutase (MnSOD) or thioredoxin which share a high degree of homology with their human counterparts[316]. Hence, T cells which respond to bacterial enzymes are cross-reactive to the corresponding human proteins which results in an autoreactive immune response[316,320]. *Malassezia*-derived MnSOD induced maturation of DCs and release of IL-6, IL-8, IL-12p70 and TNF-α by those cells[316,320]. The MGL_1304 fungal protein is an allergen derived from *M. globosa* which induces mast cell degranulation and production of IL-4 by basophils[316,320]. Other *Malassezia*-derived allergens include mitochondrial malate dehydrogenase, glucose-methanol-choline oxioreductase, peroxisomal membrane protein, or cyclophilin[320]. CD34+ progenitor cell-derived mast cells from AD patients released more IL-6 upon exposure to *M. sympodialis* compared to cells generated from healthy donors[316]. *Malassezia* species are recognized by monocyte-derived dendritic cells by one of the PRRs, which leads to the secretion of IL-1β by those cells[322]. TLR2 was shown to interact with *M. furfur*[323]. Specifically, the yeast engaged this PRR in human keratinocytes which resulted in an increased expression of *IL8* and *DEFB4B;* the latter encodes the microbicidal human β-defensin-2 peptide[323]. Moreover, the exposure of keratinocytes to the pathogen upregulated keratinocyte *TLR2* expression[323]. In terms of the characteristics of T cell response to *Malassezia* spp., *in vitro* stimulation of PBMCs from AD patients exhibiting IgE reactivity with *M. furfur* resulted in production of type 2 cytokines[324]. However, another study found that memory CD4+ cells from healthy individuals and AD patients exhibited a type 17 phenotype upon stimulation with *Malassezia* spp.[325]. A subset of those

memory CD4+ T cells derived from AD patients and stimulated with *Malassezia* spp. produced more IL-17A and IL-22 compared to the same subset isolated from healthy donors[325].

### 1.4.4. The role of extracellular vesicles in atopic dermatitis

Surprisingly, little is known about the impact of sEVs on the pathogenesis of AD[90]. Zhu *et al.* focused on a different EV population, i.e., m/lEVs; which, derived from the plasma of pediatric AD patients inhibited proliferation of keratinocytes and stimulated their death by apoptosis *in vitro*[326]. Moreover, expression of *TSLP* and *IL33* by m/lEV-exposed keratinocytes was increased as determined by quantitative polymerase chain reaction (qPCR)[326]. Interestingly, this treatment also led to more abundant expression of keratin 10, which is characteristic for terminally differentiated keratinocytes, and keratin 6, which is normally elevated in hyperproliferative keratinocytes at the same time[326].

Given the scarcity of the data concerning this subject, new studies investigating the role of sEVs in AD are crucial to obtain a more complete picture of this complex disease; all the evidence showing the major keratinocyte involvement in this disease or allergic disorders associated with this condition present keratinocytes as prime targets for investigation of sEVs they produce in AD pathogenesis.

There is some evidence showing the potential for certain sEV populations to be used in the treatment of AD[90]. A study in an AD mouse model suggested that sEVs produced by mesenchymal stem cells derived from adipose tissue (ASC-sEVs) alleviate multiple aspects of AD-related inflammation; reduced numbers of mast cells and mature APCs in the skin and eosinophils in circulation were observed[327]. This was accompanied by decreased expression of AD cytokines such as TNFα, IL-4, IL-23 and IL-13 in lesional skin as well as reduced serum IgE levels[327]. Moreover, sEV treatment improved the clinical score of disease severity[327]. Building on these results, the same group has subsequently shown that ASC-sEVs can additionally reduce TEWL and improve hydration of *stratum corneum*[328]. Moreover, ASC-sEVs stimulated synthesis and improved the delivery of ceramides and dihydroceramides to *stratum corneum*; these are crucial for the maintenance of epidermal integrity[328]. The study also demonstrated improved expression pattern of genes related to inflammatory response, skin barrier formation, lipid metabolism and cell cycle in lesional skin upon sEV treatment[328]. Collectively, both studies suggest that ASC-sEVs could exert their therapeutic function

through modification of an array of pathways related to both skin inflammation and maintenance of epidermal integrity[327,328]. Moreover, preliminary clinical data suggests that human ASC-sEVs can reduce facial redness, resulting from the treatment of AD patients with dupilumab; this is a monoclonal antibody targeting the IL-4Rα subunit of the IL-4 receptor [329].

## 1.5. Functions of small extracellular vesicles produced by keratinocytes

Keratinocytes, despite their previously discussed involvement in immune responses are non-immune cells. While sEVs from immune cells have an established role in regulating immune response, there is plenty of evidence that vesicles secreted by cells of the non-immune origin also influence inflammation and immune processes, including those that act in the context of allergy[90].

It is known that functional state of cells affects the functionality of EVs produced by them[89]. Crucially, keratinocyte differentiation state impacts the proteome of keratinocyte-derived sEVs as determined by Chavez-Muñoz *et al.*[330]. Proteomic analysis performed in this study revealed that differentiation alters the content of chaperones, cytoskeletal proteins and proteins involved in cell metabolism, cell adhesion or the complement cascade[330].

The role of keratinocyte-derived sEVs ($KC_{sEVs}$) in immune response is still very poorly studied which urgently calls for more research in this area[90]. A study by Kotzerke *et al.* investigated the functionality of murine $KC_{sEV}$ in an animal model of mice sensitized to ovalbumin (OVA); such model allows to study antigen-specific responses[331]. $KC_{sEVs}$ were shown to carry OVA internalized by keratinocytes[331]. These sEVs induced upregulation of CD40 co-stimulatory markers by mouse bone marrow-derived dendritic cells and enhanced their production of IL-6, IL-10 and IL-12[331]. This suggests that $KC_{sEVs}$ convey a signal that induces dendritic cell maturation[331]. Nevertheless, these dendritic cells were unable to induce OVA-specific T cell response in this model[331]. $KC_{sEVs}$ derived from HaCaT keratinocytes could, however, induce CD4+ and CD8+ T cell proliferation when keratinocytes were exposed to the SEB enterotoxin and IFNγ *in vitro*[332]. These $KC_{sEVs}$ also expressed MHC class I and MHC class II, which suggests that they might be capable

of antigen presentation[332]. Another study showed the ability of $KC_{sEVs}$ to stimulate immune cells and demonstrated that pathology altered the functionality of these vesicles[333]. Specifically, human primary keratinocytes were exposed to psoriasis-like inflammatory *milieu* and sEVs secreted by those cells induced production of IL-6, IL-8, and TNFα by human peripheral neutrophils *in vitro*[333]. This was accompanied by the release of neutrophil extracellular traps (NET) by these cells[333]. In contrast, $KC_{sEVs}$ from untreated keratinocytes failed to elicit those effects[333]. Furthermore, sEVs isolated from the epidermis of a psoriasis-like but not healthy mice exacerbated skin inflammation in imiquimod-induced psoriasis-like mouse model [333].

A role for $KC_{sEVs}$ in protection of skin against UVB radiation has been proposed by two studies[334,335]. Specifically, evidence suggests that human primary keratinocyte sEVs stimulate melanin production in human primary melanocytes by increasing the activity of the tyrosinase (TYR), an enzyme involved in the production of this pigment[334,335]. These effects were attributed, at least partially, to miRNA species carried by the vesicles[334]. Additionally, Shi *et al.* showed increased melanocyte proliferation upon treatment with $KC_{sEVs}$[335]. Involvement of miRNA species was also proposed in this process; however, this hypothesis was not experimentally validated[335].

$KC_{sEVs}$ have been implicated in the regulation ECM remodeling through their effect on skin fibroblasts[336]. Specifically, Chavez-Muñoz *et al*. proposed a mechanism involving stimulation of dermal fibroblast matrix metalloproteinase-1 (MMP-1) by primary human $KC_{sEVs}$ carrying stratifin[336]. MMP-1 is known to degrade collagen, a major component of ECM[337]. Since stratifin-stimulated MMP-1 expression in fibroblasts is known to facilitate wound healing, $KC_{sEVs}$ may potentially aid this process[338]. Interestingly, differentiated human primary $KC_{sEVs}$ were more abundant in stratifin compared to sEVs derived from undifferentiated cells[330]. While the role of stratifin carried by $KC_{sEVs}$ in wound healing has not been experimentally validated, two additional studies directly implicate the involvement of $KC_{sEVs}$ in this process through separate mechanisms[339,340]. Bo *et al.* revealed that sEVs derived from keratinocytes differentiated from human induced pluripotent stem cells (iPSC-$KC_{sEVs}$) accelerate re-epithelialization of the second-degree burn wounds in mice[339]. The mechanism of this process was examined *in vitro* and was likely mediated by sEV miRNA-dependent enhanced migration of keratinocytes and endothelial cells[339]. In a different study, sEVs were isolated from murine keratinocytes present on the wound edge and those in uninjured skin;  their role in wound healing was

then investigated *in vivo*[340]. Interestingly, the wound-edge $KC_{sEVs}$ were taken up by macrophages more efficiently compared to those from uninjured skin[340]. This was due to the differences in the composition of N-glycans in the two $KC_{sEV}$ populations which affected their CLR-mediated uptake by macrophages[340]. Moreover, wound-edge $KC_{sEVs}$ polarized macrophages into the pro-resolving phenotype that facilitated the wound healing process[340]. Inhibition of miRNA loading into $KC_{sEVs}$ resulted in a persistent accumulation of proinflammatory macrophages in the wound[340]. Moreover, it also compromised the impermeability of re-epithelialized skin and impaired full restoration of the expression of profilaggrin, loricrin and cell-to-cell adhesion proteins in the tissue[340]. Overall, the study suggests that $KC_{sEV}$ miRNA is crucial for an efficient wound healing process[340]. An interesting approach was taken in the investigation of the differences between wound-edge $KC_{sEVs}$ produced by healthy and diabetic mice[341]. This is of interest since persistent inflammation impedes wound healing in diabetes[341]. Charge detection mass spectrometry (CDMS) was employed to analyze the mass and charge of intact $KC_{sEVs}$; this allowed for identification of physical differences between examined sEV populations[341]. Between 10-20 separate subpopulations distinguished based on their mass and charge were identified among samples from both diabetic and non-diabetic mice[341]. The former contained fewer high mass $KC_{sEV}$ subpopulations which may indicate differences in the cargo content of those vesicles[341].

# 2. Aims and Objectives

Atopic dermatitis (AD) is an inflammatory skin disease of multifactorial pathogenesis. While immune cells play a key role in the pathology, keratinocytes also actively participate in the immune responses and majorly contribute to skin inflammation in AD as outlined in the Introduction. Even though many keratinocyte-derived immune mediators have been described, hardly any studies exploring the role of small extracellular vesicles (sEVs) produced by those epidermal cells ($KC_{sEV}$) in their communication with the immune system have been published.

A decrease in the abundance of epidermal profilaggrin and its processing products is associated with a number of inflammatory disorders. It is, however, unknown whether profilaggrin-related products can be packaged into $KC_{sEVs}$ and, if so, delivered to the circulation and distant tissues in this form.

**Research presented in this thesis aimed to elucidate whether keratinocyte-derived sEVs carry out immune-related function under the atopic dermatitis-relevant conditions.**

The aim was broken down into the following objectives:

1. Investigation of the influence of keratinocyte differentiation and AD-relevant pathogens on $KC_{sEV}$ interaction with dendritic cells;
2. Assessment of the impact of filaggrin insufficiency on the ability of $KC_{sEV}$ to modulate antigen-specific T cell responses;
3. Identification of profilaggrin/filaggrin in sEVs derived from keratinocytes and blood plasma of healthy individuals and AD patients;
4. Investigation of the effect of *S. aureus* on profilaggrin/filaggrin incorporation into $KC_{sEV}$ cargo

Data obtained during the research are contained within 3 manuscripts (**P1**-**P3)**; **P1** has been published in the *Frontiers in Immunology* journal while **P2** is currently undergoing review and **P3** is in revision in the *Journal of Extracellular Vesicles*. All three manuscripts are included in this thesis and represent the work performed to achieve the objectives.

# 3. Copies of manuscripts

**3.1.** **Kobiela A.*,** Frąckowiak J. E.*, Biernacka A., Hovhannisyan L., Bogucka A. E., Panek K., Paul A. A., Łukomska J., Wang X., Giannoulatou E., Królicka A., Zieliński J., Deptuła M., Pikuła M., Gabrielsson S., Ogg G. S., Gutowska-Owsiak, D. Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors. *Frontiers in Immunology* **13**, 884530 (2022).
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# Exposure of Keratinocytes to *Candida Albicans* in the Context of Atopic *Milieu* Induces Changes in the Surface Glycosylation Pattern of Small Extracellular Vesicles to Enhance Their Propensity to Interact With Inhibitory Siglec Receptors

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*Candida albicans (C. albicans)* infection is a potential complication in the individuals with atopic dermatitis (AD) and can affect clinical course of the disease. Here, using primary keratinocytes we determined that atopic *milieu* promotes changes in the interaction of small extracellular vesicles (sEVs) with dendritic cells and that this is further enhanced by the presence of *C. albicans*. sEV uptake is largely dependent on the expression of glycans on their surface; modelling of the protein interactions indicated that recognition of this pathogen through *C. albicans*-relevant pattern recognition receptors (PRRs) is linked to several glycosylation enzymes which may in turn affect the expression of sEV glycans. Here, significant changes in the surface glycosylation pattern, as determined by lectin array, could be observed in sEVs upon a combined exposure of keratinocytes to AD cytokines and *C. albicans*. This included enhanced expression of multiple types of glycans, for which several dendritic cell receptors could be proposed as binding partners. Blocking experiments showed predominant involvement of the inhibitory

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Siglec-7 and -9 receptors in the sEV-cell interaction and the engagement of sialic acid-containing carbohydrate moieties on the surface of sEVs. This pointed on ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) as potential enzymes involved in the process of remodelling of the sEV surface glycans upon *C. albicans* exposure. Our results suggest that, in combination with atopic dermatitis *milieu*, *C. albicans* promotes alterations in the glycosylation pattern of keratinocyte-derived sEVs to interact with inhibitory Siglecs on antigen presenting cells. Hence, a strategy aiming at this pathway to enhance antifungal responses and restrict pathogen spread could offer novel therapeutic options for skin candidiasis in AD.

**Keywords: Candida albicans, glycosylation, extracellular vesicle, keratinocyte, siglec, atopic dermatitis (AD), immune evasion, exosomes**

## INTRODUCTION

*Candida albicans* (*C. albicans*) is a pathogen which can colonise the skin of atopic dermatitis (AD) patients, contributing to exacerbation of clinical symptoms (1, 2). Suspected mechanisms beyond the spread of the pathogen suggest that the exposure to *C. albicans* in the context of atopic inflammation promotes complex cytokine responses, with a pronounced involvement of Th17 cells (3–5), as confirmed by increased candidiasis risk in patients undergoing anti-IL-17 (6) therapy, in whom these cells are lacking. In effect, IgE-mediated hypersensitivity may follow (5), as a consequence of class-switching events (7) involving the antibodies directed against the yeast (1, 8). In addition, reduced lymphocyte proliferation upon *C. albicans* stimulation was observed in early AD studies (9); all this may compound the pathology.

*C. albicans* can be recognised by numerous innate receptors (10); epidermal keratinocytes, which form the uppermost layer of the skin and naturally come in contact with *C. albicans* are involved in the innate response directed against the fungus. Specifically, keratinocytes sense the invasion through pattern recognition receptors (PRRs) (11), i.e., Toll-like receptors (TLR) -2, -4 and -9, C-type lectins (dectin-1, DC-SIGN, mannose receptor), galectin-3 as well as NOD-like receptor NLRP3; some of those receptors are only expressed in activated keratinocytes and not in the steady state (11–14). Interestingly however, *C. albicans* has not been reported to be directly recognised by Siglec-type receptors which are abundantly expressed by the Langerhans cells. It has been documented that keratinocytes respond to the fungal threat by secretion of immune cell-attracting cytokines and chemokines (11).

In addition to the soluble factors, keratinocytes also secrete a very different kind of mediators, i.e. membranous organelles known as extracellular vesicles (EVs); of those, the fraction of exosome-enriched small EVs (sEVs) seems to be involved in long-distance communication. sEV membrane can either non-specifically fuse with the membrane of the recipient cell or participate in receptor-ligand interaction; both may result in the sEV uptake (15, 16). In addition, binding itself can impact processes occurring in the target cell (17, 18). Keratinocytes secrete sEVs (19–21) containing antigens that the cells are exposed to (19) and mediators which promote response against pathogens (22). Only one study so far investigated keratinocyte response to infection in the EV context; the authors showed that sEVs may be carriers for the staphylococcal enterotoxin and stimulate polyclonal T cell responses (22). Little is known on the role of keratinocyte-derived sEVs in the defense against other skin pathogens, including in atopic skin disease which predisposes to difficult to treat infections.

Here, we focused on the primary events at the initiation of the immune response, i.e. the process of sEV interaction with immune cells. We investigated the adhesiveness of sEVs secreted by keratinocytes during their differentiation process as well as the modifying effect of the AD *milieu* and exposure of the cells to common skin pathogens, i.e. *Candida albicans* and *Staphylococcus aureus*. Using lectin array we next profiled carbohydrate moieties present on the surface of the adhesive sEVs and identified glycosylation patterns which could be correlated with the increase in the propensity of sEVs to interact with dendritic cells (DCs). Modelling of those carbohydrate patterns onto DC receptors identified potential binding partners; these were validated experimentally. We found that Siglec-7 and Siglec-9 blockade reduced interaction of keratinocyte-derived sEVs, suggesting the role for those receptors in the process of information transfer between keratinocytes and antigen presenting cells, with relevance to the setting of allergic skin inflammation and *C. albicans* infection. Further analysis of the carbohydrate moieties suggested ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) as enzymes likely contributing to the changes on the sEV surface. Hence, targeting either this sialyltransferase or inhibitory Siglecs during sEV-cell interaction could be explored as a novel therapeutic strategy to enhance antifungal response in the patients.

## RESULTS

### *Atopic* Dermatitis Inflammatory Milieu Promotes Changes *in sEVs Secreted by Proliferatory and* Differentiated Keratinocytes Leading *to* Their Differential Interaction With Denritic Cells

Keratinocyte gene expression is heavily remodelled during the process of differentiation and this affects all organelles (23).

Keratinocyte-derived extracellular vesicles, and especially exosome-enriched sEVs may provide both an efficient source of antigenic information and contain innate mediators (24–26); their uptake may alert residing skin immune cells. Previous studies documented secretion of sEVs by epidermal murine and human keratinocytes (19, 20, 27, 28); however, little is known about functional effects of keratinocyte differentiation state on the fate of those vesicles and their downstream effect on immune responses to pathogens. Hence, we set out to investigate if the differentiation advancement of keratinocytes may have any effects on the process of sEV interaction with antigen presenting cells. To begin, we isolated primary normal human epidermal keratinocytes (NHEK) from skin samples. Cultures were brought to near-confluence; at this point we performed "calcium switch" on the cells, by either keeping them in the proliferation (low calcium; 0.06 mM) or differentiation-promoting (high calcium; 1.5 mM) medium. We harvested conditioned media after 72h, isolated sEV fractions (100K pellet) using ultracentrifugation method (**Figure 1A**) and assessed their size and morphology (**Figures 1B, C**). 100K pellets contained cup-shaped vesicles enriched in exosomal markers (CD63, CD9 and syntenin), while being negative for calnexin (**Figure 1D**), as expected (29).

Next, we set out to test for differentiation-dependent changes in the capacity of NHEK-derived sEVs to transfer the pathogen-dependent signals to antigen presenting cells. To this end, we generated dendritic cell models (iMDDC, immature dendritic cells and mMDDC, mature dendritic cells) and subjected them to the PKH67-labelled sEVs, to allow fluorescent tracking of their interaction with the cells by flow cytometry. To this end, since the technique itself does not allow to discriminate between the sEV binding and uptake, for the purpose of this study we interpret positive signal as the 'interaction' between sEVs and recipient cells. However, it is highly likely that the uptake may occur given the nature of these interactions. Here we found that both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ interacted with mature and immature dendritic cells; the signal observed was higher for the latter, as expected given the efficient phagocytic ability of those cells. However, although we observed a trend towards more effective interaction of $sEV_{0.06NHEK}$, this difference was not significant, suggesting that in the healthy skin this interaction is not dependent on the differentiation status of the secreting steady-state keratinocytes (**Figures S1A–F**). Nevertheless, we anticipated that keratinocytes might communicate *via* sEVs differently when activated through specific conditions, such as

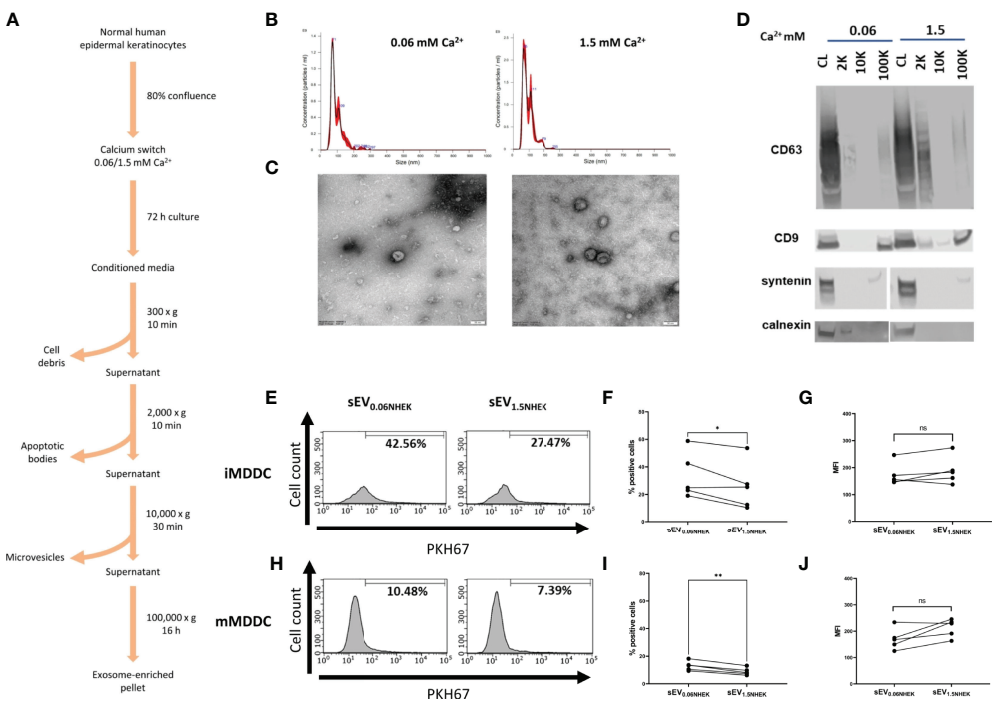



**FIGURE 1** | Atopic dermatitis *milieu* alters properties of keratinocyte-derived small extracellular vesicles (sEVs) which leads to their differential interaction with dendritic cells. **(A)** Protocol for sEV isolation from NHEK-conditioned medium; **(B)** size distribution of NHEK-derived sEVs (100K pellet) by Nanoparticle Tracking Analysis (NTA); **(C)** electron microscopy pictures confirming enrichment of cup-shaped exosomes within 100K pellet sEV fraction; scale bar: 50nm; **(D)** marker expression pattern in 2K, 10K and 100K fractions during isolation, confirming enrichment of exosomal makers in the sEV fraction (100K pellet); **(E)** iMDDC interaction with $sEV_{NHEK}$ secreted under AD cytokine stimulation; example FACS data; **(F)** iMDDC interaction with $sEV_{NHEK}$ under AD cytokine stimulation; % positive cells data pooled from n = 5 donors; **(G)** iMDDC interaction with $sEV_{NHEK}$ under AD cytokine stimulation; MFI data pooled from n = 5 donors; **(H)** mMDDC interaction with $sEV_{NHEK}$; example FACS data; **(I)** mMDDC % positive cells data pooled from n = 5 donors; **(J)** mMDDC interaction with $sEV_{NHEK}$ under AD cytokine stimulation; MFI data pooled from n = 5 donors; *p < 0.05; **p < 0.01 (paired t-test). ns, not significant.

inflammation in AD. To test this we subjected the cells to the "AD cytokine cocktail" (containing IL-4, IL-13, IL-22 and TSLP) at the time of the calcium switch. Here, we observed that $sEV_{0.06NHEK}$ were interacting more than $sEV_{1.5NHEK}$ when produced by keratinocytes in the "AD inflammatory context"; the difference was not very pronounced, i.e. only 23% and 30% reduction for the imMDDC and mMDDC models, respectively; yet observed as significant and consistent for different donors (**Figures 1E–J**).

## *Keratinocyte-Derived sEVs* Are Enriched in Glycoproteins Involved in Adhesion

Next, to better understand how keratinocyte differentiation state in combination with AD *milieu* may affect the sEVs in the context of cell adhesion, we analyzed $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ proteomes by LC-MS/MS and further profiling using Gene Ontology (GO), STRING and Reactome Pathway Database. We first started with the re-analysis of the proteomic dataset published by Chavez-Muñoz et al. (28), which contained results of proteomic profiling of exosome-enriched sEVs (sucrose cushion purified) derived from NHEKs in a very similar model to ours (0.07 and 1.8 mM calcium was used in that study, similar to our work). These results suggested that during calcium-induced differentiation steady state cells increase content of $sEV_{NHEK}$ proteins which could be assigned the 'cell adhesion' term (GO:0007155) by GO analysis, albeit this is not very pronounced (**Figure 2A**).

When we exposed sEV-secreting NHEKs to AD cytokines, we detected a greater variety of proteins in $sEV_{0.06NHEK}$ in comparison to $sEV_{1.5NHEK}$, suggesting that the differentiation process in keratinocytes leads to a shift to a more profiled sEV proteome (**Table S1**). Interestingly, all of the proteins identified in $sEV_{1.5NHEK}$ were also found in $sEV_{0.06NHEK}$. Similarly to the steady state conditions, we also observed a higher proportion of proteins assigned with the 'cell adhesion' term present in $sEV_{1.5NHEK}$ compared to $sEV_{0.06NHEK}$ (**Figure 2B**). Further STRING analysis of the 'cell adhesion'-related proteins revealed strong predicted interactions between the vast majority of those (**Figure 2C**). A similar proportion of proteins among both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ proteomes was predicted to interact with 10 or more partners within their corresponding datasets (pink circle in **Figure 2C**) and we did not observe any substantial differences between the conditions, suggesting that the presence of adhesion-relevant proteins alone in not sufficient to define the $sEV_{NHEK}$ propensity for differential interaction with a cell. However, the adhesive properties of EVs have been shown to also depend on their surface glycosylation pattern. Hence, we next assessed the content of glycoproteins which may undergo such a modification. Nevertheless, the number of glycoproteins implicated in cell or extracellular matrix (ECM) adhesion detected in both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ was similar (**Figure 2D**). Further analysis of the $sEV_{NHEK}$ proteome against Reactome Pathway Database also revealed similar extent of overrepresentation in glycosylation-related pathways in both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ (**Figure 2E** and **Figure S2A**). Interestingly, we also noted that a number of enzymes involved in protein glycosylation or N-linked carbohydrate processing during glycoprotein turnover were also present in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$ (**Figure S2B**).

## Exposure to *C. Albicans* but Not *S. Aureus* in the Context of AD Milieu Promotes sEV Cell Interaction

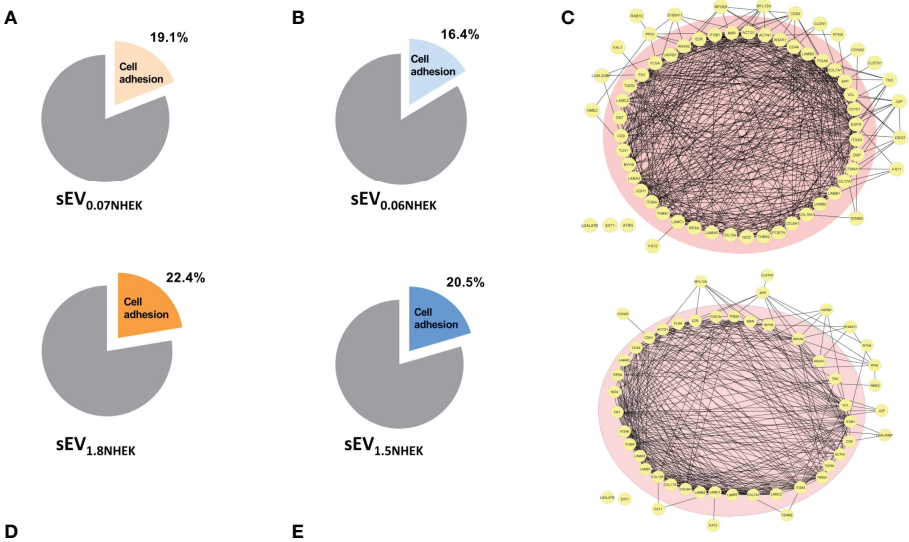
Since the previous results did not provide any strong indications on the functional differences we observed, i.e. $sEV_{NHEK}$ secreted by both steady-state and "AD *milieu*-exposed" NHEKs seemed to have similar content of adhesion-relevant proteins, including glycoproteins, we deepened our analysis by the addition of another AD-relevant factor. Specifically, given that the allergic-type AD inflammation *milieu* is often clinically overlayed with an infection by AD-related pathogens, we aimed to investigate the effect of a combined stimulation of keratinocytes by AD cytokines and either *S. aureus* or *C. albicans* on $sEV_{NHEK}$-cell interaction. Here, in the case of iMDDCs we noted increased interaction of the $sEV_{0.06NHEK}$ secreted by AD/*C. albicans*-stimulated cells in comparison to the $sEV_{0.06NHEK}$ obtained from keratinocytes treated only with the cytokines; no similar difference was noted for the $sEV_{1.5NHEK}$ interaction (**Figure 3A**). In contrast, while differential interaction was also observed between AD/*C. albicans* vs AD-control in the mMDDC model, this was noted for $sEV_{1.5NHEK}$ rather than $sEV_{0.06NHEK}$ (**Figure 3B**). Intrestingly, we did not see any of those effects with sEVs produced upon the exposure of cells to AD/*S. aureus*, suggesting that the pathways which lead to the difference in the sEV interaction may be more specifically activated by the fungus and not broadly relevant to all skin pathogens or general keratinocyte activation.

## *Pathways of* Innate Recognition Of *C. Albicans and AD* Cytokine Signalling Are Linked *to the* Glycosylation Enzyme Network in Keratinocytes

Identification of additional conditions promoting $sEV_{NHEK}$-cell interaction allowed us to further hypothesize on the mechanisms involved. Specifically, we asked whether *C. albicans* recognition by keratinocytes may affect pathways involved in glycosylation. To this end we next modelled protein networks between the pathogen recognition receptors (PRR) and glycosylation enzymes, based on the work of Schjoldager et al. (30) (**Table S2**). Given differential results between the two AD pathogens, for this analysis we only included PRRs known to be involved in the recognition of *C. albicans*, but excluded those implicated in the detection of *S. aureus*. The networks identified 11 enzymes with recognised links to PRR-mediated signalling (**Figures 3C, D**; full list, including references in **Table S3**), implying that exposure of keratinocytes to this pathogen may impact protein glycosylation.

## *Increased* Expression of Glycosylation Enzymes in the *AD* Skin Is Disease-Specific and Not Observed in Psoriasis

Next, we investigated the levels of the identified enzymes in the AD skin, by analysing publically available transcriptome




**D**

Glycoproteins

| 0.06 | 1.5 |
|---|---|
| Syndecan-1 | Syndecan-1 |
| Syndecan-4 | Syndecan-4 |
| Heparan sulfate proteoglycan 2 | Heparan sulfate proteoglycan 2 |
| Fibronectin-1 | Fibronectin-1 |
| Tenascin-C | Tenascin-C |
| Galectin-3-binding protein | Galectin-3-binding protein |
| Glypican-1 | Glypican-1 |
| Integrin ITGA3 | Integrin ITGA3 |
| Integrin ITGA6 | Integrin ITGA6 |
| Integrin ITGB1 | Integrin ITGB1 |
| Integrin ITGB4 | Integrin ITGB4 |
| Thrombospondin-1 | Thrombospondin-1 |
| Thrombospondin-2 | Thrombospondin-2 |
| E-cadherin | E-cadherin |
| Agrin | Agrin |
| CD44 | CD44 |
| Desmoglein-3 | |

**E**

Glycosylation-related Reactome pathway analysis

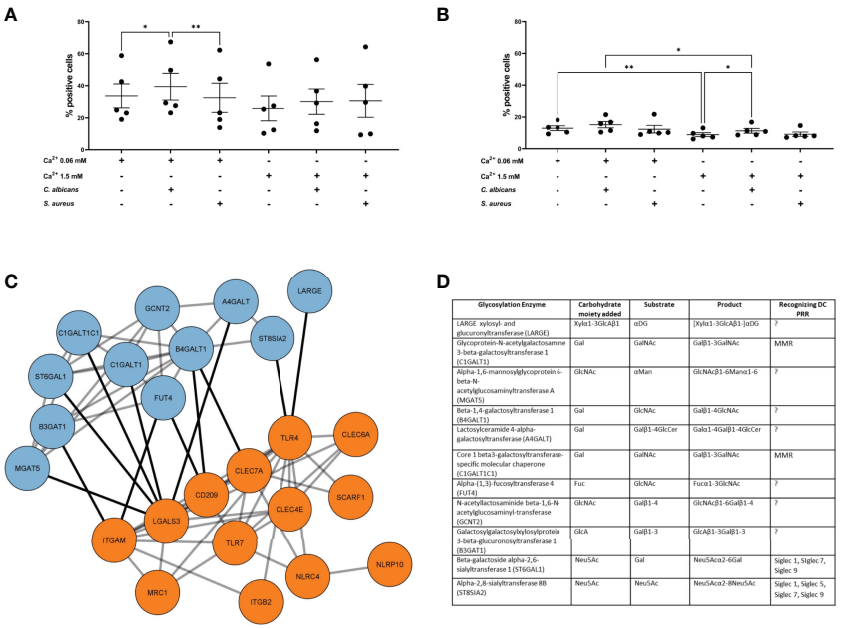
| Ca2+ concentration (mM) | Pathway identifier | Pathway name | FDR |
|---|---|---|---|
| 0.06 | R-HSA-446203 | Asparagine N-linked glycosylation | 1.334E-4 |
| | R-HSA-3000178 | ECM proteoglycans | 1.477E-4 |
| | R-HSA-532668 | N-glycan trimming in the ER and Calnexin/Calreticulin cycle | 1.364E-3 |
| | R-HSA-3560782 | Diseases associated with glycosaminoglycan metabolism | 3.435E-3 |
| | R-HSA-879415 | Advanced glycosylation endproduct receptor signaling | 0.037 |
| 1.5 | R-HSA-3000178 | ECM proteoglycans | 1.407E-6 |
| | R-HSA-3560782 | Diseases associated with glycosaminoglycan metabolism | 2.445E-4 |
| | R-HSA-532668 | N-glycan trimming in the ER and Calnexin/Calreticulin cycle | 5.231E-3 |
| | R-HSA-3781865 | Diseases of glycosylation | 6.072E-3 |
| | R-HSA-1630316 | Glycosaminoglycan metabolism | 0.023 |

**FIGURE 2** | Keratinocyte-derived sEVs are enriched in glycoproteins involved in adhesion in steady state and in AD *milieu*. **(A)** Enrichment of adhesion proteins involved in cell adhesion in $sEV_{NHEK}$ secreted in steady-state keratinocytes; Reactome - Gene Ontology terms re-analysis of the data available from Chavez-Muñoz et al. (28); **(B)** enrichment of adhesion proteins involved in cell adhesion in $sEV_{NHEK}$ secreted by NHEKs exposed to AD *milieu* as identified by Reactome - Gene Ontology terms analysis; **(C)** interaction network for sEV adhesion-relevant proteins identified in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$; **(D)** cell adhesion-relevant glycoproteins identified by mass spec in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$; **(E)** Reactome-Gene Ontology identified term enrichment for the proteins in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$; FDR, False Discovery Rate; N.B. classical exosomal glycoprotein markers are included in the supplementary data (**Figure S2D**); mass spectrometry data based on n=3 biological replicates.

profiling datasets published by He et al., Leung et al. and Esaki et al. (31–33). This analysis revealed differential expression for 7 out of 11 glycosylation enzymes listed in **Figure 3D**. Specifically, while the identified proteins differed between the studies, we consistently noticed a positive change in expression (upregulation) for all the differentially regulated genes in AD; this was observed both in the epidermal samples obtained by tape stripping (He et al. and Leung et al.) and those harvested by laser-capture microdissection (Esaki et al.), increasing our confidence in the physiological relevance of the obtained data. Here we noted several enzymes differentially expressed, however, only FUT4 and ST6GAL1 were detected as upregulated in at least two of those datasets (**Figure 4A**). Furthermore, a comparison of the levels of the enzymes in AD vs psoriatic epidermis suggested a degree of disease-specificity, with B4GALT1, FUT4 and ST6GAL1 found expressed at levels significantly higher than in psoriasis. Interestingly, of all the enzymes, the highly upregulated expression of ST6GAL1 in lesional AD epidermis compared to the healthy epidermis was the most consistent and significant across all the three datasets analyzed. However, the single cell dataset that we sourced from the Skin Cell Atlas (34) was somehow different, showing increased expression in both those inflammatory skin diseases (**Figures S5A, B**), including in proliferating, undifferentiated and differentiated keratinocytes (**Figures 4B**, **S5B**).

## *Upregulation of* Glycosylation Enzymes in the *AD* Skin Is Driven by Atopic Milieu and Not by Filaggrin Insufficiency

Next, having gathered substantial AD-relevant evidence, we aimed to provide additional insights into the causes leading to the expression changes we next attempted to identify links




**D**

| Glycosylation Enzyme | Carbohydrate moiety added | Substrate | Product | Recognizing DC PRR |
|---|---|---|---|---|
| LARGE xylosyl- and glucuronyltransferase (LARGE) | Xylα1-3GlcAβ1 | αDG | [Xylα1-3GlcAβ1-]αDG | ? |
| Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GALT1) | Gal | GalNAc | Galβ1-3GalNAc | MMR |
| Alpha-1,6-mannosylglycoprotein i-beta-N-acetylglucosaminyltransferase A (MGAT5) | GlcNAc | αMan | GlcNAcβ1-6Manα1-6 | ? |
| Beta-1,4-galactosyltransferase 1 (B4GALT1) | Gal | GlcNAc | Galβ1-4GlcNAc | ? |
| Lactosylceramide 4-alpha-galactosyltransferase (A4GALT) | Gal | Galβ1-4GlcCer | Galα1-4Galβ1-4GlcCer | ? |
| Core 1 beta3-galactosyltransferase-specific molecular chaperone (C1GALT1C1) | Gal | GalNAc | Galβ1-3GalNAc | MMR |
| Alpha-(1,3)-fucosyltransferase 4 (FUT4) | Fuc | GlcNAc | Fucα1-3GlcNAc | ? |
| N-acetyllactosaminide beta-1,6-N-acetylglucosaminyl-transferase (GCNT2) | GlcNAc | Galβ1-4 | GlcNAcβ1-6Galβ1-4 | ? |
| Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1 (B3GAT1) | GlcA | Galβ1-3 | GlcAβ1-3Galβ1-3 | ? |
| Beta-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) | Neu5Ac | Gal | Neu5Acα2-6Gal | Siglec 1, Siglec 7, Siglec 9 |
| Alpha-2,8-sialyltransferase 8B (ST8SIA2) | Neu5Ac | Neu5Ac | Neu5Acα2-8Neu5Ac | Siglec 1, Siglec 5, Siglec 7, Siglec 9 |

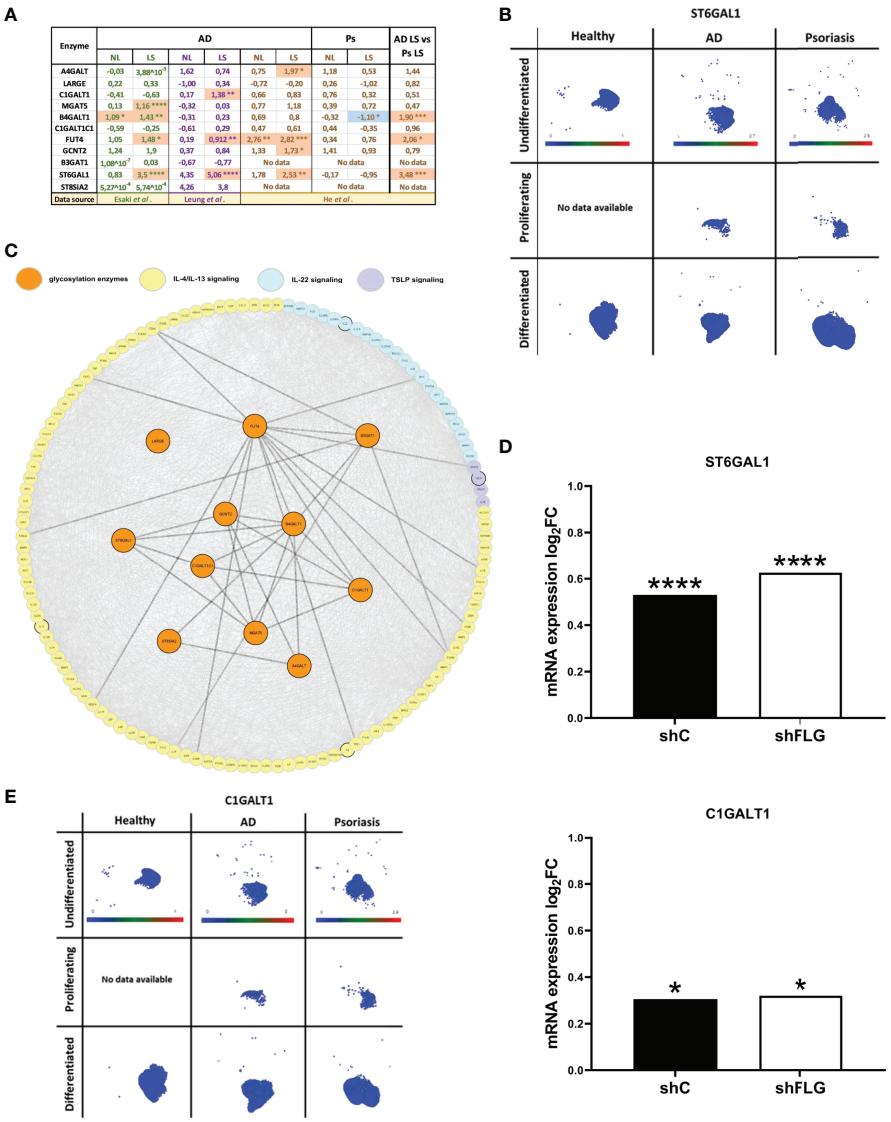
**FIGURE 3** | AD *milieu* and *C albicans* exposure affects $sEV_{NHEK}$-cell interaction by interfering with glycosylation enzyme network in keratinocytes. **(A)** iMDDC interaction with $sEV_{NHEK}$ under combined AD cytokine/pathogen stimulation; data pooled from n=5 donors; **(B)** mMDDCs interaction with $sEV_{NHEK}$ under combined AD cytokine/pathogen stimulation; data pooled from n = 5 donors; *p < 0.05; **p < 0.01 (A-B: one-way ANOVA), means and SEM are shown; N.B. some comparisons with significant p-values are not labelled for the clarity of the graph; **(C)** STRING analysis of the protein networks between *C. albicans*-stimulated signalling from pattern recognition receptors in human keratinocytes (PRRs; in orange) and linked glycosylation-relevant enzymes (in blue); **(D)** Carbohydrate moiety/substrate/product-specificity of glycosylation enzymes identified in the network linked to *C. albicans*-specific PRRs (full list, including references in **Table S3**); DC, Dendritic cell; PRR, Pattern recognition receptor; PAMP, Pathogen associated molecular pattern; LacNAc, N-acetyllactosamine; GalNAc, N-acetylglucosamine; GlcNAc, N-acetylglucosamine; Fuc, Fucose; Gal, Galactose; Man, Mannose; Neu5Ac, N-acetylneuraminic acid; MMR, Macrophage mannose receptor; MGL, Macrophage galactose type lectin; DCIR, Dendritic cell immunoreceptor; BDCA2, Blood dendritic call antigen 2; CLEC, C-type lectin domain family; Siglec, sialic acid-binding immunoglobulin-type of lectin.

between the enzymes and signalling networks of AD cytokines (**Figure 4C**). This highlighted several connections to their downstream pathways which may suggest potential modulatory effect on the glycosylation network. To this end, we noticed that those connections were present mainly downstream from the IL-4/IL-13 pathway. To this end, we identified a transcriptomic dataset which reported an increase in expression of ST6GAL1 in normal keratinocytes (reconstructed into an organotypic model) upon IL-4/IL-13 treatment (35), further suggesting that atopic cytokines may affect this pathway.

Downregulation of filaggrin (FLG) in AD skin is one of the hallmarks of the disease; the extensive impact of this downregulation is a reflection of multifaceted role FLG carries out in the skin (36, 37), also at the keratinocyte biology level. Hence, it is plausible that FLG insufficiency itself could also affect the expression of the glycosylation enzymes; in which case the effect in the skin would not be exclusive to the *milieu* but could potentially require the underlying FLG insufficiency background providing synergestic effect. To test this, we used filaggrin-insufficient keratinocytes (shFLG), in which FLG expression was reduced by shRNA interference (38–40) and the control (shC) line; we assessed mRNA expression in both lines upon exposure to IL-4/IL-13. We determined that two of the enzymes, i.e. ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) were upregulated by this treatment (**Figure 4D**); expression of both of those enzymes was increased in AD epidermis as detected before in AD skin. Importantly, however, there was no difference between shC and shFLG cells, ruling out that FLG status is prerequisite to the observed effect. Given that C1GALT1 is a core extension enzyme and its activity in generating O-linked glycans is prerequisite to the action of the ST6GAL1 (as a capping enzyme), we also investigated the distribution of its expression reported by Skin Atlas. Here, we noted that C1GALT1 levels are pronouncedly upregulated in both AD and psoriatic keratinocytes, with no visibly significant difference between the two conditions (**Figure 4E**), which is in line with the analysis of the skin transcriptomic data (**Figure 4A**). This may suggest that the expression of the two enzymes may be regulated differently with the involvement of either both C1GALT1 and ST6GAL1 or exclusively ST6GAL1 for AD and psoriasis, respectively.

## *sEVs* Secreted Under Exposure to *C. Albicans* and *AD* Cytokines Express Altered Surface Glycosylation Pattern

Next, to investigate the glycosylation pattern on the surface of sEVs which were characterised by increased cell interaction, we proceeded with the identification of the sEV membrane-exposed




| Enzyme | AD | | | | Ps | | AD LS vs Ps LS |
|---|---|---|---|---|---|---|---|
| | NL | LS | NL | LS | NL | LS | |
| A4GALT | -0,03 | 3,88^10^7 | 1,62 | 0,74 | 0,75 | 1,97 * | 1,18 | 0,53 | 1,44 |
| LARGE | 0,22 | 0,33 | -1,00 | 0,34 | -0,72 | -0,20 | 0,26 | -1,02 | 0,82 |
| C1GALT1 | -0,41 | -0,63 | 0,17 | 1,38 ** | 0,66 | 0,83 | 0,76 | 0,32 | 0,51 |
| MGAT5 | 0,13 | 1,16 **** | -0,32 | 0,03 | 0,77 | 1,18 | 0,39 | 0,72 | 0,47 |
| B4GALT1 | 1,09 * | 1,43 ** | -0,31 | 0,23 | 0,69 | 0,8 | -0,32 | -1,10 * | 1,90 *** |
| C1GALT1C1 | -0,59 | -0,25 | -0,61 | 0,29 | 0,47 | 0,61 | 0,44 | -0,35 | 0,96 |
| FUT4 | 1,05 | 1,48 * | 0,19 | 0,912 ** | 2,76 ** | 2,82 *** | 0,34 | 0,76 | 2,06 * |
| GCNT2 | 1,24 | 1,9 | 0,37 | 0,84 | 1,33 | 1,73 * | 1,41 | 0,93 | 0,79 |
| B3GAT1 | 1,08^10^7 | 0,03 | -0,67 | -0,77 | No data | | No data | | No data |
| ST6GAL1 | 0,83 | 3,5 **** | 4,35 | 5,06 **** | 1,78 | 2,53 ** | -0,17 | -0,95 | 3,48 *** |
| ST8SIA2 | 5,27^10^4 | 5,74^10^4 | 4,26 | 3,8 | No data | | No data | | No data |
| Data source | Esaki et al. | | Leung et al. | | He et al. | | | | |

**FIGURE 4** | Glycosylation enzymes are upregulated in the epidermis of AD patients and keratinocytes exposed to AD *milieu*. **(A)** The expression of glycosylation enzymes in the epidermis of AD and Ps patients; analysis of the transcriptome profiling data available as datasets in Esaki et al., Leung et al. and He et al. (values in the table show expression $log_2$FC compared to healthy epidermis); *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; (moderated t-test for Esaki et al. and He et al., multiple unpaired t-tests for Leung et al.); NL, non-lesional epidermis; LS, lesional epidermis; **(B)** UMAP plots of single cell expression of ST6GAL1 enzyme in keratinocyte subpopulations in the skin; data available through Human Developmental Cell Atlas; **(C)** protein network links between AD-relevant cytokine pathways and the previously identified 11 glycosylation-relevant enzymes; **(D)** mRNA expression of glycosylation enzymes differentially regulated upon exposure to IL-4/IL-13 stimulation in filaggrin-insufficient (shFLG; knockdown) keratinocytes and control (shC) keratinocytes (log2 fold change shown); combined data for n=3 biological replicates; *p < 0.05; ****p < 0.0001 (moderated t-test); **(E)** UMAP plots of single cell expression of C1GALT1 enzyme in keratinocyte subpopulations in the skin; data available through Human Developmental Cell Atlas; ST6GAL1, β-Galactoside α-2,6-Sialyltransferase 1; C1GALT1, Core 1 β,3-Galactosyltransferase 1.

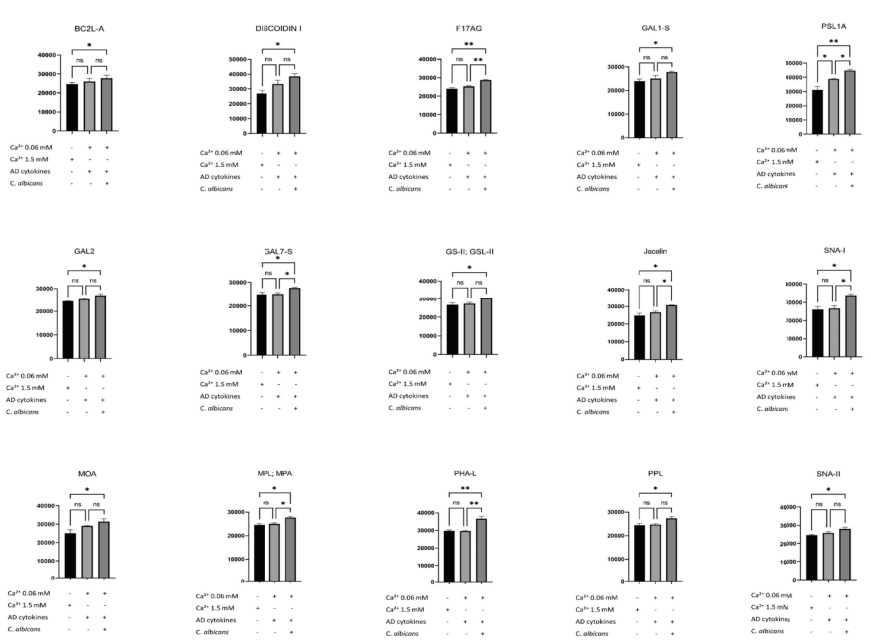
carbohydrate moieties by lectin array. Lectin array is a useful tool for glycosylation pattern identification; lectins on the slide have binding specificity towards defined carbohydrate moieties which allows identification of glycosylation of the bound molecules (either soluble or displayed on sEV (41)). To dissect the differences in the functional outcomes, we selected conditions on a spectrum of the interaction characteristics, i.e. AD/*C. albicans* $sEV_{0.06NHEK}$ vs AD cytokines $sEV_{0.06NHEK}$; we also included the condition which resulted in the lowest level of this interaction observed in our experiments, i.e. $sEV_{1.5NHEK}$ (at steady state). We observed substantial binding of sEVs to the array for 17 out of 70 lectins reporting significant changes between the conditions (**Figure 5A**, **Figure S2C**); we identified enrichment rather than *de novo* appearance of any additional carbohydrate patterns on sEVs secreted upon the combined AD/*C. albicans* stimulation. The results were filtered to identify lectins for which sEV binding to the array followed the functional results of the cell interaction (the highest for the AD/*C. albicans* $sEV_{0.06NHEK}$, the lowest for

$sEV_{1.5NHEK}$ and intermediate for AD cytokines $sEV_{0.06NHEK}$). This yielded 15 lectins binding glycans enriched in sEVs characterised by increased interaction; the binding from the majority (almost 90%) of the lectins with differential outcomes represented the trend, indicating unidirectional alterations in the sEV glycan profile. The resulting panel of the identified carbohydrate moieties was next matched to the innate carbohydrate recognition receptors on the antigen presenting cells, yielding several potential binding partners suitable for experimental validation (**Figure 5B**; full list, including references in **Table S4**).

## *Siglec-7 and Siglec-9* Receptors Are Involved in the Interaction Between Keratinocyte-Derived sEVs and Antigen Presenting Cells

Since the binding to the array revealed a considerable level of detected glycans on $sEV_{NHEK}$, we reasoned that these

**A**




**B**

| Lectin | Carbohydrate Specificity (PAMP) | DC PRR |
|---|---|---|
| BC2L-A | High mannose | MMR, Dectin 2, DC-SIGN, Langerin, CLEC2, Mincle, DCIR, BDCA2 |
| Discoidin 1 | GalNAc | Langerin, MGL |
| | Galβ1-3GalNAc | MMR |
| | GalNAcβ1-3Gal | MGL |
| | αGalNAc (Tn antigen) | MGL |
| | Gal | MGL, MMR |
| | GlcNAcβ1-3Gal | Langerin |
| | LacNAc | BDCA, DCIR, Galectin 1 |
| F17AG | GlcNAc | Langerin, BDCA2 |
| | pNP-GlcNAc | ? |
| | GlcNAcβ1-3Gal | Langerin |
| GAL1S | β-galactoside | Galectin |
| | Gal | MGL, MMR |
| | LacNAc | Galectin 1 |
| | branched-LacNAc | Galectin |
| Gal2 | GalNAcα1-3Gal (Blood group A) | MGL, DC-SIGN |
| | Lac | BDCA2, CLEC2B |
| | branched-LacNAc | Galectin |
| Gal7s | Gal | MGL, MMR |
| | Galβ1-3GlcNAc | DC-SIGN |
| GS1, GSL2, BSL1 | αGal | MMR, MGL |
| | α3GalNAc | MGL |
| Jacalin | Galβ1-3GalNAc | MMR |
| MOA | Galα1-3Gal | MMR |
| | Galα1-3Galβ1-4GlcNAc | DC-SIGN |
| | Galα1-3Fucα1-2Galβ1-4GlcNAc (Blood group B) | DC-SIGN, Langerin |
| MPL, MPA | Gal | MMR, MGL |
| | GalNAc | MGL |
| | Galβ1-3GalNAc | MMR |
| PHA-L | Man | MMR, Langerin, DC-SIGN, BDCA2 |
| | Gal | MGL, MMR |
| | GlcNAc | Langerin, DCIR2, BDCA2 |
| | Galβ1-4GlcNAcβ2-6GlcNAcβ1-2Manα3Manα3 | ? |
| PPL | GalNAc | MGL |
| | Gal | MGL, MMR |
| | α/βGalNAc | MGL, |
| PSL1a | Neu5Acα2-6 linkages [α2-6 Sialic acid] | Siglec 1, Siglec 2, Siglec 3, Siglec 5, Siglec 7, Siglec 8, Siglec 9, Siglec 10 |
| | Neu5Acα2-6Galβ1-4GlcNAc | Siglec 1, Siglec 9 |
| Sna1 | Neu5Acα2-6Gal | Siglec 1, Siglec 7, Siglec 9 |
| | NeuAcα2-6Galβ1-4Glc | Siglec 9 |
| | Neu5Acα2-6GalNAc | Siglec 2 Siglec 3, Siglec 5, Siglec 7, Siglec 9 |
| | GalNAc | MGL |
| | Lac | BDCA2, CLEC2B |
| | GalNeu5Acα2-6Gal | ? |
| Sna2 | Gal | MMR, MGL |
| | GalNAc | MGL |
| | Neu5Acα2-6Lac | Siglec 1, Siglec 2, Siglec 3, Siglec 5, Siglec 7, Siglec 8, Siglec 9, Siglec 10 |
| | GalNAcα1-3Gal | MGL, DC-SIGN |

**FIGURE 5** | Lectin array-based identification of carbohydrate moieties on the adhesive $sEV_{NHEK}$ map to potential receptors on antigen presenting cells. **(A)** Lectin binding to carbohydrate moieties on the surface of $sEV_{NHEK}$; data summarizes biological duplicates with n=3 keratinocyte donors; *p < 0.05; **p < 0.01; **(B)** carbohydrate moieties enriched in the more adhesive sEVs as identified by lectin array, together with potential binding receptors on antigen presenting cells as identified by the literature search (full list, including references in **Table S4**). ns, not significant.

glycosylation patterns may also promote interaction between sEVs and antigen presenting cells in the steady state. Hence, to identify the receptor route involved, we next performed receptor blocking experiments, using IL-4/GM-CSF-differentiated THP-1 cells, serving as antigen presenting cells and N/TERT-1 immortalised keratinocytes (42, 43) as an efficent sEV source (**Figure S3**). Given the previously identified carbohydrate moieties involved, we focused on the C-type Lectin Receptors (CLRs), i.e. Langerin (CD207), Macrophage Mannose Receptor (MMR; CD206), Dendritic Cell-Specific Intercellular adhesion molecule (DC-SIGN; CD209). In addition, we also investigated Sialic acid-binding Immunoglobulin-like Lectins known to recognize sialic acid. Specifically, we included Siglec-2, Siglec-7 and Siglec-9, as these receptors could be matched to the recognized carbohydrate moieties detected by the array but they differ in specificity and affinity to the same glycans (44). In this model differences could be observed in the expression level for the selected receptors during the differentiation process; a positive population was present for each of the markers and further increased during THP-1 differentiation (**Figure S4A**). Of those, DC-SIGN expression was the highest and seen for nearly all of the cells; on the other hand, Langerin was the least abundant marker, as expected, but also showed an increase in expression. However, the differences in the outcome of the blocking experiments with specific antibodies could not be simply explained by the variation in the receptor expression levels. Specifically, while we observed no clear effect of the anti-MMR, -Langerin, -DC-SIGN (**Figure S4B**) and Siglec-2 antibodies, blocking with Siglec-7- and Siglec-9-specific antibodies significantly decreased cell interaction of either $sEV_{0.06NHEK}$ or both $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$, respectively (**Figure 6A**); the most profound effect was observed upon Siglec-9 blockade. We also observed the p-value approaching significance (p=0.063) for the anti-Siglec-7 blocking of the $sEV_{1.5NHEK}$ interaction; as well as (a non-significant) trend for anti-Siglec-2 blokade. Siglec-7 and Siglec-9 are known inhibitory receptors which decrease PRR-dependent activation of the cell upon sialic acid binding (45, 46). Their expression within the epidermis is almost exclusively confined to the population of Langerhans cells (**Figure 6B**). In the whole skin samples those receptors have broader and elevated expression in inflammatory skin diseases, i.e. AD and psoriasis (Ps). However, the expression is, still mainly confined to the myeloid cell populations, as identified by single cell analysis available through Skin Cell Atlas (34). This includes macrophages, monocyte-derived DCs, LCs, etc., which can serve as antigen presenting cells in the skin (**Figure 6C**; **Figure S5A**). Combined, these results suggest that sialic acid moieties could provide a specific targeting motif directing keratinocyte-derived sEVs to antigen presenting cell populations in the tissue.

### *Changes in* Glycosylation Pathways Are Implicated *in* Sialylation Pattern *on* Keratinocyte-Derived *sEVs and* Define Their Interaction With *Siglec-7 and Siglec-9* Receptors

Finally, we set out to determine the specific enzyme(s) implicated in the observed process of sEV surface glycan remodelling. Indeed, the ST6GAL1 enzyme identified by us earlier within the glycosylation enzyme network linked to the *C. albicans*-stimulated PRR signalling (**Figure 3C**) and most consistently upregulated in AD skin and keratinocyte cultures by IL-4/IL-13 (**Figure 4**) was an immediate match to the glycosylation pattern observed, as it catalyses the reaction of the addition of sialic acid *via* an α2-6 linkage (**Figure S6A**). The associated GO terms for this enzyme reflected as enrichment of the term GO: 0005515 "Enables protein binding" (**Figure S6B**). We were not able to match other enzymes which we found upregulated by AD cytokines or in patient epidermal samples, including FUT4 enzyme, highly differentially expressed and consistent between the three studies. Likewise, C1GALT1 also does not seem to be of the primary importance in our model given that it despite that it produces Galβ1-3GalNAc linkage detected in the array; however, this linkage is recognised by the MMR receptor (47), blockade of which did not affect the strength of sEV-cell interactions (**Figure S4B**).

Finally, with the aim of identifying $sEV_{NHEK}$ proteins that could be targeted by glycosylation changes we carried out further analysis, focusing on the direct interactomes of ST6GAL1 and C1GALT1 (**Figure 6D**). This revealed links to the other glycosylation-relevant proteins. In addition, both enzymes link to several mucins as expected given their inclusion in the mucin formation pathways. In addition, a node connection between ST6GAL1 and galectin-3 (LGALS3) can be also seen. This suggests potential substrates for the enzymes, displayed on the $sEV_{NHEK}$ surface; unfortunately, none of those were found in our mass spectometry dataset, thus not providing us with any indications regarding the identity of the potential targets.

## DISCUSSION

Small extracellular vesicles (sEVs) produced by virtually all living cells, including those of non-immune origin have been shown to participate in immune responses, both innate and adaptive (48, 49). sEV-specific role depends on their capacity to directly interact with cells, where receptor-ligand interactions could lead to a downstream effect. This cellular interaction is also critical initial event enabling sEV uptake and intracellular transfer of sEV cargo, enriched in antigens and innate signalling molecules or other immune-relevant compounds. Experiments with various cell lines indicated that sEV glycan composition, which is important during cellular adhesion of sEVs, is dependent on the cellular source. Importance of glycans as potential targeting motifs for the recipient cell was previously indicated (50).

To this end, our results suggest that the exposure of keratinocytes to stimulation relevant to atopic dermatitis (AD), i.e. allergic inflammatory *milieu* and *C. albicans* may induce relevant changes in the sEV surface glycosylation patterns and are translated into differential functional outcomes; here we showed certain increase in the propensity for sEV interaction with dendritic cells. By carbohydrate moiety identification with lectin array we subsequently determined that these alterations include enhanced expression of forms of sialic acid. Modelling

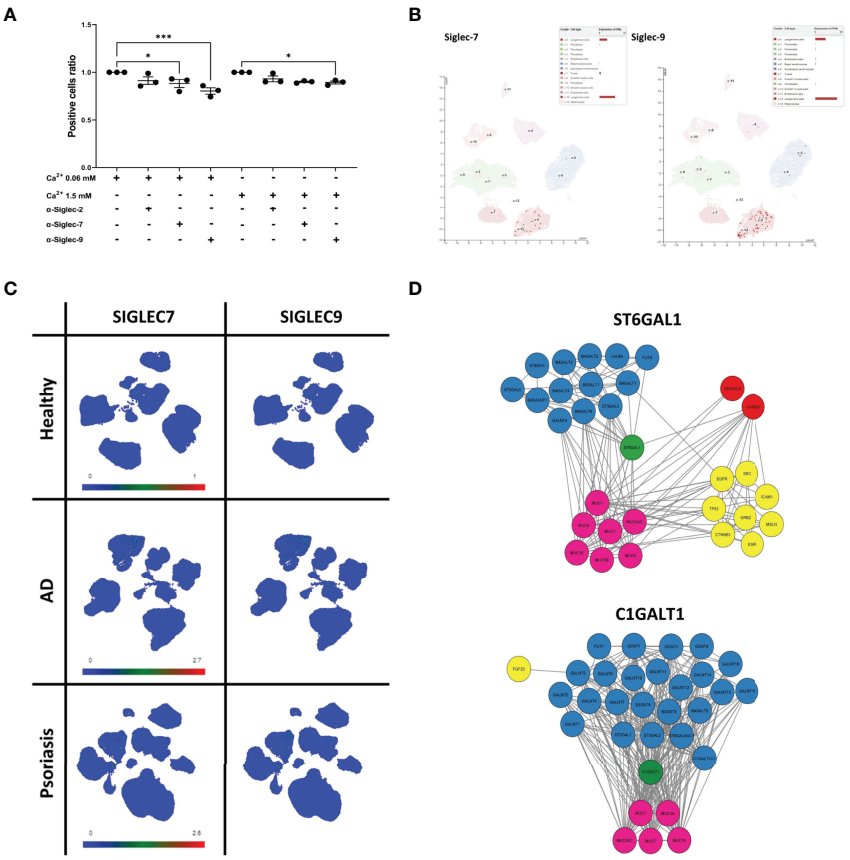



**FIGURE 6** | Sialyltransferase ST6GAL1 is implicated in sialylation pattern on keratinocyte-derived sEVs and defines their cell interaction propensity *via* Siglec-7 and Siglec-9 receptors. **(A)** Identification of binding receptors for $sEV_{N/TERT-1}$ cell interaction by blocking experiments with anti Siglec-type receptors on differentiated THP-1 cells; combined data for n=3 biological replicates; **(B)** single-cell RNA expression levels of Siglec-7 and Siglec-9 in the epidermis of the skin; data available through ProteinAtlas; **(C)** UMAP plots of single cell expression of SIGLEC7 and SIGLEC9 receptors in the skin; data avalabale through Human Developmental Cell Atlas; **(D)** protein interaction network of ST6GAL1 and C1GALT1 enzymes obtained *via* STRING analysis. * - p<0.05, *** - p<0.001.

indicated that the effect downstream of the PRR signalling induced by *C. albicans* on the activity of sialyltransferase ST6GAL1 could provide a plausible explanation for this effect. Sialic acid-containing motifs were abundant on keratinocyte-derived sEVs and enriched further in vesicles characterised by enhanced propensity for cell interaction (41).

Innate recognition of sialic acid is mediated by a group of Sialic acid-binding Immunoglobulin-like Lectins (Siglecs) and we determined that the cell interaction of keratinocyte-derived sEVs in our model is Siglec-7 and Siglec-9-mediated. Furthermore, the dependence on either both Siglecs or exclusively Siglec-9 for the undifferentiated vs. differentiated keratinocytes as sEV sources, respectively, mirrors the difference observed by us earlier at the functional level. Specifically, higher interaction efficiency could be observed for $sEV_{0.06NHEK}$, for which the interaction seems to be aided by both of these receptors. Being two members of the CD33-related Siglec family both Siglec-7 and Siglec-9 are considered "endocytic" receptors (51, 52); therefore the sEV binding may promote their efficient uptake by the cells expressing those receptors. From the first glance this could appear as benefiting the host, since provision of *C. albicans* antigens and other stimulatory signals within the cargo should promote T cell responses. However, it has been shown that signalling through Siglec-7 and Siglec-9, which contain tyrosine-based inhibition motif (ITIM) within their intracellular domains dampens proinflammatory responses by inhibiting NF-κB-dependent TLR4 signalling pathway (53); as a result, stimulation of these "inhibitory Siglecs" provides strong negative signal. Interestingly, data published by Varchetta et al. (54) stands in contrast to the studies implicating the inhibitory role of Siglec-7 in immune response, suggesting a potential role of sialic acid-*independent* stimulation of this receptor in triggering the release of proinflammatory cytokines by monocytes. In this particular study Siglec-7 was activated by either antibody-mediated crosslinking or zymosan, a yeast cell wall-derived particle devoid of any forms of sialic acid. Here, since we propose a mechanism critically dependent on the sialic acid engagement of Siglec-7, the study might be less relevant in the context of our findings. However, it brings up a potentially important question regarding the possibility of different

functional outcomes upon the activation of the Siglec-7 pathway depending on the chemical composition of the ligand. Furthermore, while we did not establish the content of the $sEV_{NHEK}$ cargo, these may contain RNA species, incl. regulatory, proteins and other effector molecules which could potentially also contribute to the effect.

While *C. albicans* expresses several PRR agonists, it does not seem to express enough sialic acid to stimulate Siglec receptors (10) and switch off the NF-κB signalling by Siglec-dependent inhibition. Besides, as a predominantly intracellular pathogen, *C. albicans* may not have any effective means to directly interact with the membrane-expressed Siglecs on the antigen presenting cells. Hence, the proposed mechanism could potentially increase the chances of the pathogen to achieve the induction of tolerogenicity, if *C. albicans* exploit the host's enzymatic machinery to induce sialic acid coating on sEVs. It has been also demonstrated that antigen sialylation results in the inhibition of Th1 and Th17 cells and induction of Treg subsets (55); given the Th1/Th17-dominated effective antifungal response, such an effect would similarly benefit *C. albicans*. Intrestingly, the inhibitory Siglec pathways seem to be hijacked by numerous PRRs-stimulating pathogens escaping immunosurveillance (46). To our best knowledge we are the first to report a possibility of remodelling of the host sEV surface glycosylation by a pathogen which could also constitute a potentially attractive and resource-saving immune evasion strategy. However, the relevance of this mechanism would have to be confimed in the dedicated immune evasion *in vivo* studies.

In the context of the skin disease, this mechanism may potentially have an important effect on the Siglec-7 and Siglec-9 expressing Langerhans cells (LCs). LCs switch between immunomodulation and immunactivation by integration of incoming stimuli (56); in the healthy skin this would mean tolerogenic phenotype upon encounter of non-threatening signals and proinflammatory one during skin infection. By preventing LC activation with inhibitory signalling, *C. albicans*-induced sEV sialylation could target those cells predominantly, given the LC-exclusive expression of these Siglecs in the epidermis. Deeper in the tissue, these receptors are also expressed by additional myeloid cells, all of which could both present antigens and react to innate stimuli, e.g. macrophages or dendritic cells. Hence, sialic acid-dependent exosome/sEV-mediated activation inhibition could have a potential to affect clearance of *C. albicans* from the AD skin and lead to its enhanced spreading.

Recently, the inhibitory Siglec-type receptors were proposed as a novel class of immune checkpoints targeting myeloid cells with inhibitors suggested for clinical application (45). In addition, in the cancer setting T cells may also express inhibitory Siglec-7 and Siglec-9, meaning that they could be directly targeted; in agreement with this, sialic acid-dependent exosome/sEV-mediated direct T cell inhibition was also previously shown (57). Interestingly, candidiasis itself increases risk of many malignancies (58); hence, the question that needs further addressing is if *C. albicans*-remodelled sEVs could suppress anticancer response and promote tumour growth.

Mammalian proteins involved in the glycosylation processes show great diversity (30). In our study, we identified 11 enzymes which could be linked to the innate response to *C. albicans* in keratinocytes. Of those, ST6 β-Galactoside α-2,6-Sialyltransferase 1 (ST6GAL1) and Core 1 β,3-Galactosyltransferase 1 (C1GALT1) seemed to be involved in the changes in the glycosylation pattern induced by the fungus on keratinocyte-derived sEVs, i.e. both enzymes are upregulated in AD and Ps and expressed in the majority of skin cell populations, including all subopulations of (proliferating, undifferentiated and differentiated) keratinocytes. It is important to note that Siglec-7 may also have additional specificities i.e also recognise 2-8 and 2-3-linked moieties (59, 60) (these were not identified by our lectin array screen) as well as detecting more complex epitopes, beyond isolated glycans (61). Interestingly, recent genome-wide CRISPR-aided screen highlighted the importance of additional enzymes (62) for generaton of Siglec-7 ligands, including C1GALT1. This makes sense as C1GALT1 is a core extension enzyme acting at the beginning of the synthesis pathway which generates core of the O-glycans exposed on the sEV surface, hence required as an anchor for the exposed ST6GAL1-generated moieties added subsequently. Recent study by Büll et al (61) indicated that the core extension feature prevails over the capping glycan features and the binding is completely abolished in the C1GALT1C1 (C1GLAT1-specific chaperone; COSMC) knockout. Hence, given the role of O-glycosylation for Siglec-7 recognition, it seems that the changes in the expression of this enzyme that we noted upon analysis of data from both the epidermal samples and cytokine-stimulated keratinocytes (normal (35) and FLG insufficient) may also critically contribute to the observed effect. It is unclear, however, if the direct product(s) of the enzyme (T-antigen) may be detected by Siglec-7; further glycan modifications by sequential enzymatic action of additional capping enzymes are likely required for the recognition; this is where ST6GAL1 may execute its role in our system as moiety exposed as a part of complex Siglec-7-recognized epitopes; additional role of sulfation has to be also considered given that a group of carbohydrate sulfotransferases (CHSTs) has been implicated in the binding affinity for both Siglecs we found important for sEV-cell interactions (61, 63). Some moieties may be of course less involved, e.g. our negative blocking data for MMR receptor which has high affinity to Gal1β-3GalNAc glycans (47) suggest considerably lower importance of this linkage in our model. In our study we were not able to match our data to enzymes implicated in the binding of Siglec-9 ligands, ST3GAL4/6 (61). Overall, our results support the notion on the complexity in the Siglec system; e.g. in our experiments we only observed slight disruption of the cellular interaction with Siglec-2 blokage (not significant, despite shared receptor specificity to the 2-6 linkage). This may be dependent on the breadth of the accepted ligand pool which is constricted for this Siglec and the differences in affinities of specific glycans containing the linkage in comparison to that of Siglec-7 and -9 as shown in detail by Blixt et al. (44).

Interestingly, ST6GAL1 seems to be an important enzyme during influenza infection, since the virus uses sialic acid-containing glycans as cellular entry points (64). It has been

shown that ST6GAL1 expression also correlates with poor tumour prognosis (65) and affects multiple mechanisms related to cancer (66), suggesting that the immune effect is not limited to infection. ST6GAL1 was also recently shown as enzymatically active cargo of both exosome-like sEVs and exomeres, capable of transferring sialyltransferase activity to receipient cells and inducing expression of sialylated proteins on the cell membrane (67). As for the C1GALT1 enzyme, it has also been implicated in cancer; however, the role is less clear-cut, with contradicting data on the pro-/antitumorogenic effect (68). The enzyme has also been linked to IgA nephropathy by deposition of galactose-deficient IgA1 (Gd-IgA1) circulating in the patients with *C1GALT1* mutation (69).

The exact identity of the sEV-expressed proteins which may be modified by the ST6GAL1 enzyme is not known, although we identified several proteins present within keratinocyte-derived sEVs which are likely to undergo such modification. Literature indicates some potential examples in sEVs, e.g. in a study in ovarian cancer-derived vesicles galectin-3-binding protein (LGAL3BP), was previously identified as a sialoprotein (70). LGAL3BP is a known sialic acid-dependent ligand for CD33-related Siglec family (71) (including Siglec-7 and Siglec-9), so could be potentially important in the sEV-mediated communication in the skin. Similarly, β1 integrins (72), also present in our samples, could be similarly modified; lipid molecules which may also be sialylated, as shown for ganglioside GD3 delivering a direct T cell inhibitory signal *via* sialic acid (57). With respect to successful adhesion and delivery of the inhibitory signal, also the spatial distribution of sialylated proteins on the surface of keratinocyte-derived sEVs could potentially affect the outcome, especially if the proteins segregate into rafts or microdomains on the surface of sEVs to mediate specific interaction by formation of so called "glycosynapses" (73); however, we did not determine this in this study.

In summary, our study showed that in the context of AD *C. albicans* promotes sialic acid-enriched glycosylation pattern on the host sEVs to increase their interaction with inhibitory Siglec receptors. We may predict potential future applicability of targeting this glycosylation-sEV-Siglec-dependent pathway as a novel adjuvant therapy in skin candidiasis in AD patients; however, we cannot exclude potential applicability also beyond the skin.

# MATERIALS AND METHODS

## Samples

Ethical approvals for the study were obtained from the Independent Bioethics Committee for Scientific Research at Medical University of Gdansk, ethical approval numbers: NKBBN/559/2017-2018 and NKBBN/621-574/2020. Perioperative skin samples (2-3 $cm^2$) were obtained from the individuals undergoing surgery at the Department of Surgical Oncology, Medical University of Gdansk, Poland. Until isolation, the material was stored in PBS (Sigma-Aldrich, St. Louis, MO, USA), with 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA), in 4°C. Buffy coats were obtained as a byproduct from blood donations coming from healthy donors at the Regional Blood Centre in Gdansk.

## Keratinocyte Isolation and Culture

Skin samples were washed in PBS with 100 U/ml penicillin + 100 μg/ml streptomycin, subcutaneous adipose tissue was removed and the samples were cut into small pieces. Epidermis was removed from the dermis after 2-3 hour incubation in dispase (12U/mL, Corning, NY, USA) at 37°C and digested in a 0.25% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) with trypsin inhibition with EpiLife Medium supplemented with EpiLife™ Defined Growth Supplement (EDGS) (Thermo Fisher Scientific, Waltham, MA, USA), antibiotics and 10% FBS (Sigma-Aldrich, St. Louis, MO, USA). Keratinocytes were seeded in a collagen IV-coated dishes (Corning, NY, USA) in EpiLife medium supplemented with EDGS, 100 U/ml penicillin + 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS. The next day, the medium was changed to a serum-free EpiLife with EDGS and antibiotics with regular changes every 2 days of culture at 37°C, 5% $CO_2$. For experiments, pooled NHEK cultures from n=3-4 donors were used. N/TERT-1 keratinocytes obtained as a kind gift from Prof Jim Rhinwald were cultured in Keratinocyte SFM medium (Thermo Fisher Scientific, Waltham, MA, USA) + 25 μg/mL bovine pituitary extract (Thermo Fisher Scientific, Waltham, MA, USA) + 0.2 ng/mL Epidermal Growth Factor (Thermo Fisher Scientific, Waltham, MA, USA) + 0.4 mM $Ca^{2+}$. ShC and shFLG cells were grown in Dulbecco's Modified Eagle's Medium (DMEM-high glucose, Sigma-Aldrich, St. Louis MO, USA). Media used for EV isolation contained no animal products or were supplemented with EV-depleted FBS.

## Calcium Switch and AD-Relevant Treatments

Upon reaching 80% confluence in a serum-free EpiLife with EDGS supplemented with antibiotics (free from animal products), cells were washed and cultured for 3 days as undifferentiated (in 0.06 mM $Ca^{2+}$) or differentiated (in 1.5 mM $Ca^{2+}$) cells in the following conditions: untreated or treated with atopic dermatitis (AD) cytokine cocktail (20 ng/mL IL-4, and 10 ng/mL IL-13, IL-22 and TSLP each; Peprotech, London, UK). For pathogen treatment addition of 75 ng/mL of a selected AD-relevant inactivated pathogens; *Candida albicans* (prick test; Inmunotek, Madrid, Spain) and *Staphylococcus aureus* (heat-killed) was applied.

## Heat-Killed Bacteria

1 ml of overnight culture of *S. aureus* "Newman" (2.4 x $10^9$ CFU/mL) was centrifuged at 1700 x g. The cell pellet was washed with PBS and centrifuged 1700 x g, 5 min (2x) and resuspended in 1 ml PBS, followed by heat treatment with shaking (80°C, 30 min, 1000 rpm). The resulting suspension of heat-killed bacteria was cooled on ice and protease inhibitors (final concentration: 1 μM of E-64, 0.5 μg/mL of pepstatin A and 5 μM of leupeptin) were added after heat treatment in order to retain their stability and stored in -20°C.

## EV Isolation

The exosome-enriched fraction of sEVs (100K pellet) secreted by keratinocytes was isolated by the differential ultracentrifugation protocol as in **Figure 1A**. Briefly, conditioned medium was first centrifuged at 300 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific, Waltham, MA, USA) for 10 min to remove the cellular debris, followed by 2,000 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific, Waltham, MA, USA) for 10 min to remove soluble proteins and apoptotic bodies (AP; 2K pellet). The supernatant was ultracentrifuged at 10,000 x g (maximum rotation speed) for 30 min (OptimaTM L-90K or OptimaTM LE-80K ultracentrifuge, Beckman Coulter, Brea, CA, USA) to isolate microvesicles (MVs; 10K pellet). The supernatant was then ultracentrifuged at 100,000 x g (maximum rotation speed) for 16 h to pellet exosome-enriched fraction (exosome-enriched sEVs; 100K pellet). The 100K pellet was washed in PBS by additional spin and stored at -20°C for further use.

sEVs were labelled using the PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane Labeling (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. In brief, sEVs corresponding to 6 x $10^6$ keratinocytes for a given culture condition were resuspended in 100 µl of Diluent C and incubated with 5 µM PKH67 for 5 minutes. The labelling reaction was quenched by the addition of 2x volume of EV-depleted complete RPMI medium (Sigma-aldrich, St. Louis, MO, USA) (supplemented with 10% EV-depleted FBS and 100 U/ml penicillin + 100 µg/ml streptomycin), and samples were then washed in PBS (100,000 x g (maximum rotation speed), 16 h, 4°C). Labelled sEVs were resuspended in EV-depleted complete RPMI medium and used directly for MDDC interaction assessment.

## Western Blot

Cell lysates were prepared in RIPA buffer (Cell Signalling Technology, Danvers, MA, USA) supplemented with the cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA), vortexed well and centrifuged for 15 min at 4°C in 13,000 rpm. The supernatant was collected, and 4X Bolt™ LDS Sample Buffer (Thermo Fisher Scientific, Waltham, MA, USA) (10x diluted) was added. The same amount of the loading buffer was added to the EV samples in PBS (10x diluted). The samples were heated for 10 min at 80°C. EV samples (an equivalent of EVs isolated from 1.71 mln cells per well) were then loaded onto the Bolt™ 4 to 12% Bis-Tris precast gel (Thermo Fisher Scientific, Waltham, MA, USA) and ran for 30-60 min at 150V and then transferred onto nitrocellulose membranes (iBlot™ 2 Transfer Stacks, Thermo Fisher Scientific, Waltham, MA, USA) in the iBlot transfer system (iBlot™ 2 Gel Transfer Device, Thermo Fisher Scientific, Waltham, MA, USA). Next, the membranes were blocked in 5% fat-removed powdered milk (Carl Roth, Karlsruhe, Germany) in PBS for an hour on the shaker, and next incubated with primary antibodies overnight at 4°C on the shaker (all primary Abs were diluted 1:250, only CD63 was 1:500 diluted). The next day membranes were washed 3x for 5 min in PBS-T (PBS + 0.5ml/l Tween 20) and secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) in PBS-T (1:25,000) were added. After 30 min of incubation with the secondary antibodies the membranes were washed 3x for 5 min in PBS-T and once in PBS, paper-dried and scanned using the Odyssey CLx Near Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

## EV Characterisation

Electron microscopy imaging was carried out as a paid service by the University of Gdansk Electron Microscopy Facility. Briefly, samples were adsorbed onto formvar/carbon-coated copper grids size 300 mesh (EM Resolutions, Sheffield, UK), stained with 1.5% uranyl acetate (BD Chemicals Ltd.), and imaged by Tecnai electron microscope (Tecnai Spirit BioTWIN, FEI, Hillsboro, OR, USA). Nanoparticle Tracking Analysis was carried out using NS300 NanoSight NTA (Malvern Panalytical, Malvern, UK), the EV samples were diluted 1000x in PBS.

### Dendritic Cell Models

PBMCs were separated from buffy coat samples with Lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada). $CD14^+$ cells were isolated by the MojoSort™ Human CD14 Selection Kit (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. Cells were seeded in 24-well plates in complete RPMI medium (supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin + 100 µg/ml streptomycin) at a density of 1 x $10^6$ cells in 1 ml of medium per well. Cells were cultured for 7 days at 37°C and 5% $CO_2$ in the presence of 50 ng/mL (500 U/mL) GM-CSF and 200 ng/mL (1000 U/mL) IL-4 for the generation of immature monocyte-derived dendritic cells (iMDDCs). Cytokine-supplemented medium was replaced on day 2 and 4 of the culture. To generate mature monocyte-derived dendritic cells (mMDDCs) 1 µg/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) was added on day 6 of the culture.

### EV Cell Interaction Assessment and Blocking

On day 7, iMDDCs and mMDDCs were washed, resuspended in EV-depleted complete RPMI medium and seeded on 96-well round-bottom plates at a density of 0.066 x $10^6$ cells/well. Cells were then incubated for 4 h in a total volume of 100 µl/well with PKH67-labelled sEVs obtained from 1 x $10^6$ of keratinocytes cultured as previously described. Cells were then washed with PBS (10 min, 300 x g), fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and the cell interaction of sEVs by MDDCs was analyzed by flow cytometry using the Millipore Guava EasyCyte Flow Cytometer (Merck Millipore, Burlington, MA, USA).

For the blocking experiment with a THP-1-based model, N/TERT-1-derived sEVs were used. sEVs were isolated by differential centrifugation as described before and quantified using NanoSight NS300 NTA (Malvern Panalytical, Malvern, UK). Before use sEVs were resuspended in Diluent C and labelled with PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich, St. Louis, MO, USA), for a mock control Diluent C alone was used for labelling. THP-1 cells were differentiated by culturing them in the presence of 1000 U/mL IL-4 (PeproTech, London, UK) and 50 ng/mL GM-CSF (PeproTech, London, UK) for 7 days. On days 2 and 4 of the

culture the whole medium was replaced; fresh medium was supplemented with cytokines as before. On day 7 cells were collected, washed, and treated with CD206, CD207, CD209, Siglec-2, Siglec-7 or Siglec-9 antibodies (Biolegend, San Diego, CA, USA) at 10 μg/mL for 1 hour at 37°C. Next, cells were washed twice with PBS and exposed to either PKH67-labelled N/TERT-1-derived sEVs or mock control, and incubated for 4 hours at 37°C. Cells were then washed, fixed Bioligand in 4% Formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and analyzed using the Millipore Guava EasyCyte Flow Cytometer (Merck Millipore, Burlington, MA, USA).

### Lectin Array

Lectin array 70 product (GA-Lectin-70-14) was purchased from RayBiotech (Peachtree Corners, GA, USA) and the assay was run according to the manufacturer's protocol, with an anjustment of using PHK67-labelled sEVs directly as the sample source with the omission of flouorophore-conjugated detection antibody. The array slide was imaged with the Amersham Typhoon RGB scanner (Cy2 525BP20 filter) (Marlborough, MA, USA) at adjusted PMT voltages (intensities).

### Mass Spectrometry

After lysis of sEVs with 1% SDS and cysteine residues' reduction with dithiothreitol, samples were processed in a standard Multi-Enzyme Digestion Filter Aided Sample Preparation (MED-FASP) procedure with cysteine alkylation by iodoacetamide and consecutive proteolytic digestion by LysC, trypsin, and chymotrypsin. Peptides obtained after each digestion were separately desalted on a C18 resin in a STAGE Tips procedure, and subsequently measured in the data-dependent acquisition mode on a Triple TOF 5600+ mass spectrometer (SCIEX, Farmingham, MA, USA) coupled with an Ekspert MicroLC 200 Plus System (Eksigent Technologies, Redwood City, CA, USA). All measurement files were subjected to joint database search in the MaxQuant 1.6.2.6a against the Homo sapiens SwissProt database (version from 09.11.2020). Resulting intensities were normalized using Total Protein Approach and protein concentrations in pmol/mg were calculated. Concentrations were imported into Perseus software and log2-transformed, data was restricted to 50% valid values, missing values were imputed from normal distribution and all values were normalized by z-score. T-tests between the test groups were conducted, and the results with p-value lower than 0.05 were considered to be statistically significant. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier: PXD031729.

### Microarray

For the microarray study, shC and shFLG cells were grown to 80% confluence and then exposed to IL-4 and IL-13 (Peprotech, London, UK) at 50 ng/mL for the incubation time of 24 h. RNA was extracted with RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The microarray was performed by ServiceXS (Leiden, Netherlands) on an Illummina HT12v4 BeadArray platform (Illumina, San Diego, CA, USA) and the data were normalized using lumi (74) and analysed with LIMMA (75). The microarray dataset has been deposited to the Gene Expression Omnibus (GEO) repository and assigned the accession number: GSE203409.

### Analysis

Data was analysed by Graph Pad Prism version 7 with one-way ANOVA (Holm-Sidak correction); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Cell adhesion-related proteins in sEVs were identified using the Gene Ontology tool, available at http://geneontology.org/ (76, 77). Interactions between the proteins of interest were identified using the STRING (78) database available in the Cytoscape 3.8.2 software (https://cytoscape.org/) (79) *via* the stringApp. Glycoproteins and glycosylation-relevant enzymes within NHEKs sEVs MS dataset were identified by literature search. Glycosylation-related pathways were identified using the Reactome Pathway Database (https://reactome.org/). For the STRING analysis protein lists were subjected to STRING database analysis (78). Generated networks were obtained with confidence mode of display of network edges. As a source of interactions between proteins we used "textmining", "experiments" and "databases" only with medium confidence interaction score (0.4) applied. Networks were not further expanded. Graphical adjustment was done using Cytoscape software platform. Single cell data on protein expression in skin population was obtained from the Human Developmental Cell Atlas available at https://developmentcellatlas.ncl.ac.uk/.

Transcriptomic data from Esaki et al. (GSE120721) was analyzed using the GEO2R tool available through the Gene Expression Omnibus (GEO) database (80, 81).

In datasets analyzed for the expression of glycosylation enzymes, i.e. Esaki et al., Leung et al. and He et al. p-values were adjusted using the Benjamini & Hochberg method.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below:

https://www.ebi.ac.uk/pride/archive/, PXD031729.

https://www.ncbi.nlm.nih.gov/geo/, GSE203409.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Independent Bioethics Committee for Scientific Research at Medical University of Gdansk. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AKo, JF, ABi, LH, ABog performed experiments, analysed data and contributed to the writing and figure preparation. AKr and MD performed experiments. JL performed data analysis. JZ provided surgical samples. SG, GSO, MP interpreted the data and participated in manuscript writing. DG-O provided funding, planned experiments and analysed the data, wrote the first and

subsequent paper drafts. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.884530/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Supplementary figures

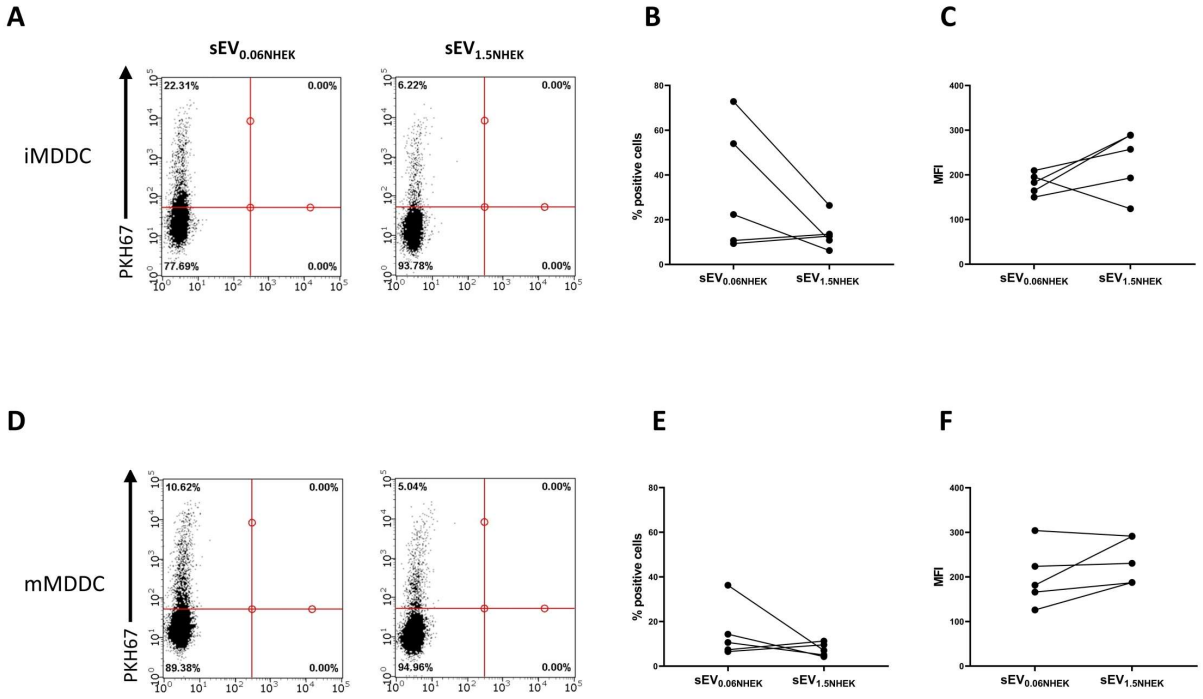
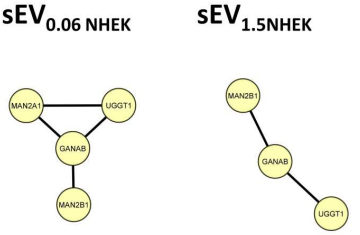



**Figure S1.** A) Interaction of $sEV_{NHEK}$ with iMDDCs; example FACS data; B) interaction of $sEV_{NHEK}$ with iMDDCs % positive cells; data pooled from n=5 donors; C) interaction of $sEV_{NHEK}$ with iMDDCs MFI; data pooled from n=5 donors; D) interaction of $sEV_{NHEK}$ with mMDDCs; example FACS data; E) interaction of $sEV_{NHEK}$ with mMDDCs % positive cells; data pooled from n=5 donors; F) uptake of $sEV_{NHEK}$ by mMDDCs MFI; data pooled from n=5 donors (paired t-test).

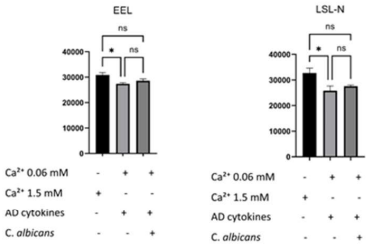
A

| Ca2+ concentration (mM) | Pathway identifier | Pathway name | FDR |
|---|---|---|---|
| 0.06 | R-HSA-3781865 | Diseases of glycosylation | 0.058 |
| | R-HSA-5173214 | O-glycosylation of TSR domain-containing proteins | 0.111 |
| | R-HSA-1630316 | Glycosaminoglycan metabolism | 0.171 |
| | R-HSA-110330 | Recognition and association of DNA glycosylase with site containing an affected purine | 0.225 |
| | R-HSA-110328 | Recognition and association of DNA glycosylase with site containing an affected pyrimidine | 0.225 |
| | R-HSA-3906995 | Diseases associated with O-glycosylation of proteins | 0.259 |
| | R-HSA-975578 | Reactions specific to the complex N-glycan synthesis pathway | 0.305 |
| | R-HSA-5173105 | O-linked glycosylation | 0.594 |
| | R-HSA-975576 | N-glycan antennae elongation in the medial/trans-Golgi | 0.611 |
| | R-HSA-446219 | Synthesis of substrates in N-glycan biosythesis | 0.899 |
| | R-HSA-446193 | Biosynthesis of the N-glycan precursor (dolichol lipid-linked oligosaccharide, LLO) and transfer to a nascent protein | 0.942 |
| 1.5 | R-HSA-879415 | Advanced glycosylation endproduct receptor signaling | 0.071 |
| | R-HSA-446203 | Asparagine N-linked glycosylation | 0.093 |
| | R-HSA-5173214 | O-glycosylation of TSR domain-containing proteins | 0.119 |
| | R-HSA-3906995 | Diseases associated with O-glycosylation of proteins | 0.218 |
| | R-HSA-110330 | Recognition and association of DNA glycosylase with site containing an affected purine | 0.221 |
| | R-HSA-110328 | Recognition and association of DNA glycosylase with site containing an affected pyrimidine | 0.246 |
| | R-HSA-5173105 | O-linked glycosylation | 0.477 |

B




C

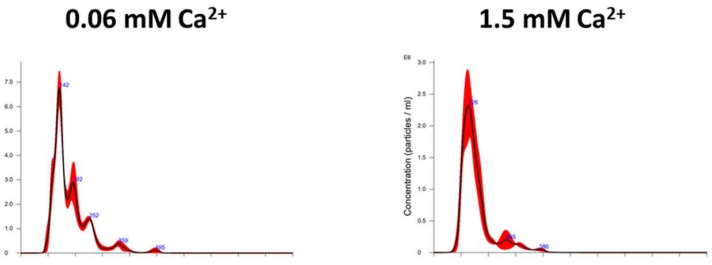



D

| 0.06 | 1.5 |
|---|---|
| CD9 | CD9 |
| CD82 | CD82 |
| CD63 | |

**Figure S2.** A) Additional Reactome-identified pathways for proteins identified in $sEV_{NHEK}$ (non-significantly enriched); B) STRING network of glycosylation enzymes found within $sEV_{NHEK}$ cargo; C) additional sEV lectin binding identified as significant with a 70 lectin array; D) Classical exosomal glycoprotein markers in $sEV_{0.06NHEK}$ and $sEV_{1.5NHEK}$.

A




B

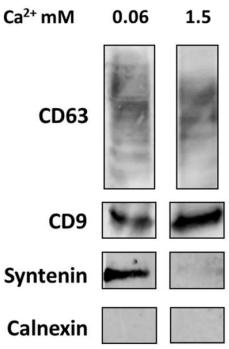



**Figure S3.** A) Size distribution of N/TERT1-derived sEVs (100K pellet) by Nanoparticle Tracking Analysis (NTA); B) marker expression pattern in the N/TERT1-derived 100K fraction, confirming enrichment of exosomal makers in the sEV fraction (100K pellet); pellets from both conditions were probed on the same membrane which had to be cut due to the sample alignment on the gel. N.B.: Size difference between N/TERT-1 and NHEK-derived sEVs but corresponding WB markers.

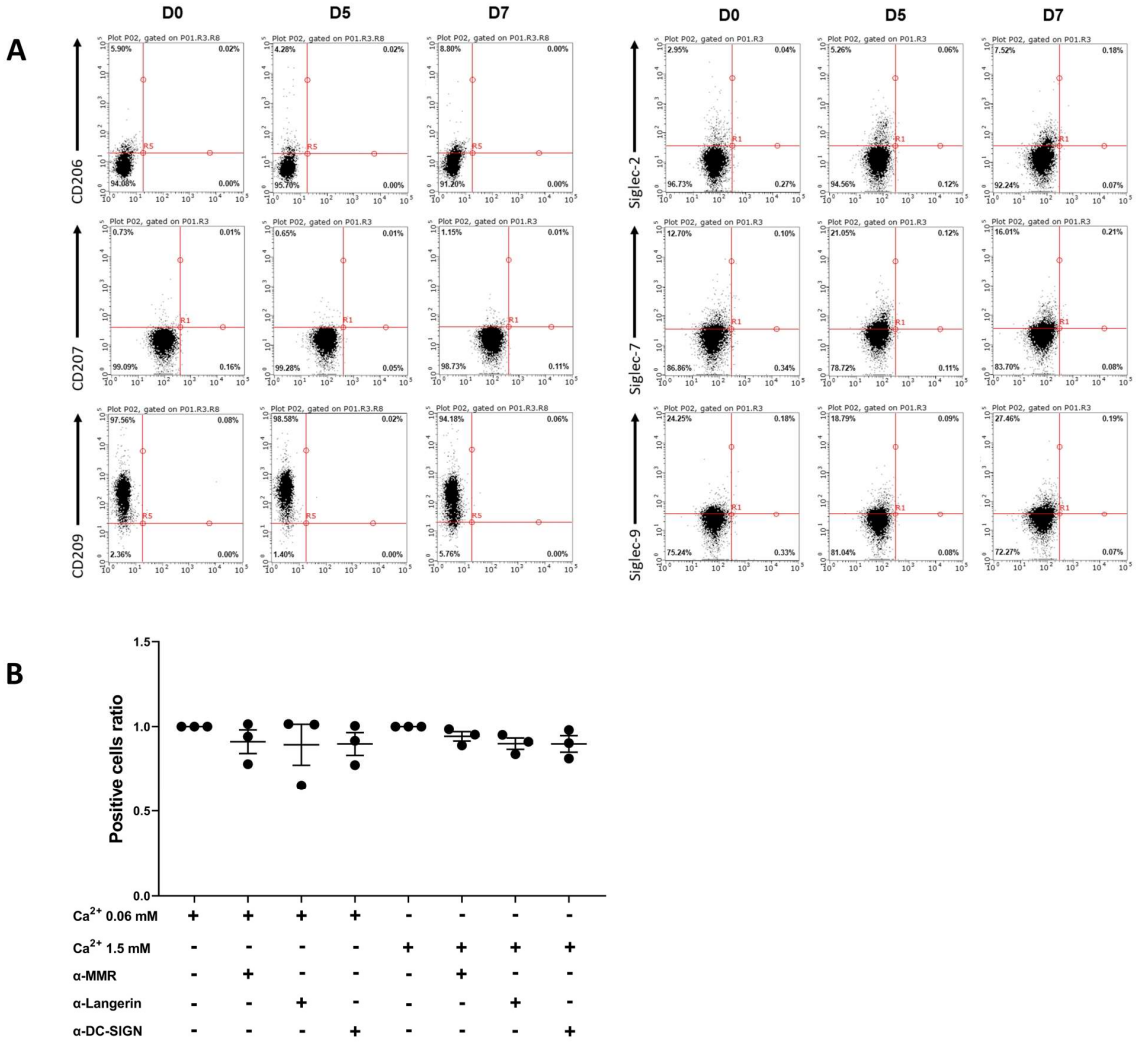



**Figure S4**. A) FACS data on receptor expression on the differentiated THP-1 model; B) identification of binding receptors for $sEV_{N/TERT-1}$ cell interaction by blocking experiments with anti-MMR, anti-Langerin and anti-DC-SIGN antibodies in the model of differentiated THP-1 cells and N/TERT-1-derived sEVs; combined data from n=3 biological replicates.

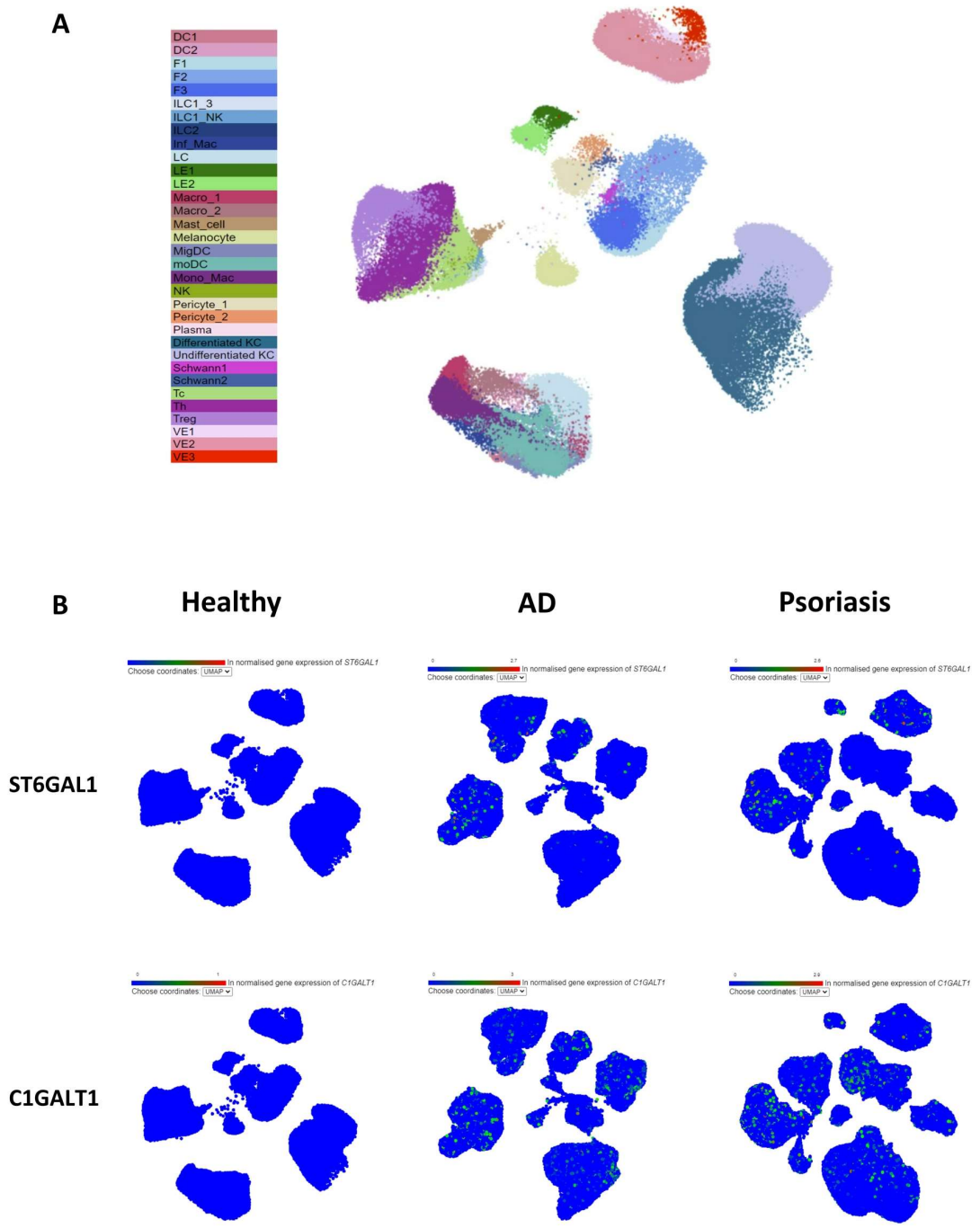
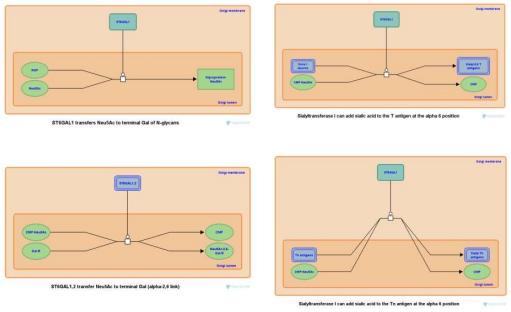



**Figure S5.** A) Visualisation of the location of cellular population according to UMAP; proliferating keratinocytes can be visualised within the undifferentiated population; B) expression of ST6GAL1 and C1GALT1 enzymes; single cell data obtained for all skin populations from Human Developmental Cell Atlas; UMAP shown.

A



B

ST6GAL1

| GO ID | Qualified GO term |
|---|---|
| GO:0003835 | Enables beta-galactoside alpha-2,6-sialyltransferase activity |
| GO:0005515 | Enables protein binding |
| GO:0008373 | Enables sialyltransferase activity |
| GO:0016740 | Transferase activity |
| GO:0016757 | Glycosyltransferase activity |
| GO:0042803 | Enables protein homodimerization activity |

**Figure S6.** A) Enzymatic reactions catalysed by ST6GAL1; obtained from Reactome; B) Gene Ontology terms associated with ST6GAL1 as identified by Reactome.

## Additional supplementary material:

**Table S1.** Mass spectrometry analysis of proteins in $sEV_{NHEK}$ secreted by AD cytokine-exposed NHEKs.

**Table S2.** List of proteins included in the STRING/Cytoscape analysis.

**Table S3.** Full table of carbohydrate moiety/substrate/product-specificity of glycosylation enzymes identified in the network linked to C. albicans-specific PRRs, including references.

**Table S4.** Full table of carbohydrate moieties enriched in the “more adhesive” sEVs as identified by lectin array, together with matched potential binding receptors on antigen presenting cells as identified by the literature search, including references.

**3.2.** **Kobiela A.**, Hewelt-Belka W., Frąckowiak J. E., Kordulewska, N., Hovhannisyan L., Bogucka A. E., Etherington R., Piróg A., Dapic I., Gabrielsson S., Brown S. J., Ogg G. S., Gutowska-Owsiak D. Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation. **Under review in *The Journal of Extracellular Vesicles* (2023).**

**Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation**

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## Abstract

The promoting effect of *FLG* loss-of-function mutations on the development of atopic dermatitis (AD) signifies the role of filaggrin in the formation of a protective skin barrier; *FLG* mutations are also linked to asthma, food allergy and allergic rhinitis despite the absence of the protein in the affected tissues (lungs, intestines, and the majority of the nasal mucosa). AD patients suffer from chronic inflammation and recurrent skin infections; inflammation often precedes the appearance of spatially distant allergic manifestations. Here we show that exosome-enriched small extracellular vesicles (sEVs) secreted by filaggrin-knockdown keratinocytes are extensively remodelled as a consequence of the abnormal keratinocyte differentiation process. This alteration modulates the sEV capacity to promote type 1 and type 2 CD1a-dependent T cell responses by direct effects on self-lipid neoantigen generation; both modulating the amount of permissive (stimulatory) and non-permissive (inhibitory) CD1a ligands released from the sEV membranes by phospholipase A2. We found that this aberrant sEV lipid composition reflects a generalised cellular lipid bias with downregulation of multiple enzymes of lipid metabolic pathways, observed both in filaggrin knockdown keratinocytes *in vitro*, and in the skin of AD patients. Provision of modulatory ligands by sEVs secreted on a filaggrin insufficiency background, impeding both homeostatic autoreactive and protective antimicrobial CD1a-mediated type 1 and enhancing type 2 T cell responses provides basis for reduced tissue integrity and pathogen clearance and perpetuates inflammation in AD skin as well as in distant tissues to which sEVs are transferred by systemic circulation.

**Keywords:** CD1a, sEV, exosome, T cell, atopic dermatitis, filaggrin, allergic inflammation

## Introduction

Loss-of-function mutations in the *FLG* gene encoding critical skin barrier protein, filaggrin provide the strongest genetic predisposition for atopic dermatitis (AD) and linked allergic respiratory and gut manifestations[1–4]. Filaggrin, expressed almost exclusively in the skin, has been identified as an essential factor supporting epidermal barrier formation, from the structural function through to control of keratinocyte life cycle and terminal differentiation[5–9]; this is reflected in abnormal functional properties of filaggrin-insufficient cells[6,8,9]. Importantly, the protein exerts a critical role in skin immunity supporting low pH of the *stratum corneum* and regulating Toll-like receptor-mediated signalling[10–12] as a part of innate defence. Filaggrin also

modulates dendritic cell capacity to present antigens[13,14] and T cell responses induced by activated keratinocytes[15], it also directly inhibits CD1a lipid neoantigen generation by phospholipase A2 (PLA2)[14]. While this confirms the role of filaggrin in local immune tolerance, the link between *FLG* mutations and the development of allergic manifestations at distant tissues and organs is puzzling, given the almost exclusively epidermis-restricted expression pattern of this protein and its absence from the lungs and gut[16,17].

Small extracellular vesicles (sEVs), enriched in exosomes, are secreted organelles falling within the 50-150nm size range, released by all nucleated cells, including keratinocytes[18–23]. Due to the unique biogenesis pathway, exosome-enriched sEVs acquire distinct characteristics enabling them to penetrate between cells and enter the systemic circulation without damage. This, together with a set of cell type-dependent membrane receptors and specific molecular cargo, provides the basis for their involvement in long-distance communication between spatially separated tissues and body organs.

Here, we showed that filaggrin insufficiency alters the way in which keratinocytes communicate with the immune system by sEVs; this specifically affects CD1a-mediated immune responses. First, through integration of data from a 2D *in vitro* system with 3D organotypic models and AD patient study, we determined that filaggrin insufficiency extensively impacts the keratinocyte sEV compartment, resulting in secretion of qualitatively altered sEVs, characterised by a remodelled lipid profile. This change reduces the capacity of sEVs to constitute a source of lipid neoantigens stimulating homeostatic CD1a-restricted autoreactive T cell responses; sEVs are in turn enriched in non-permissive (inhibitory) CD1a ligands, resulting in a reduction in the interferon-γ (IFN γ) cell responses and promoting type 2 bias. We determined that the altered lipid profile of the sEVs produced on the filaggrin insufficiency background is a consequence of the dysregulation of the lipid metabolic pathways, consequential of aberrant keratinocyte differentiation, including downregulated expression of key enzymes determining lipid chain length and saturation, apparent both *in vitro* and in AD skin.

Loss of protective and homeostatic CD1a-restricted T cell activation with a concomitant type 2 bias contributes to allergic skin inflammation and diminished responses supporting tissue integrity and antimicrobial control locally in the skin, further augmenting allergic inflammation perpetuated by AD pathogens. However, since sEVs are transferred distally, vesicles secreted in the skin may similarly act in distant organs (including lungs and gut), thus likely compounding the progression of allergic manifestations in those body sites.

## Results

*Filaggrin insufficiency in keratinocytes affects the exosomal/sEV compartment*

Apart from the widespread disturbances of the structural components resulting in the cardinal features of AD epidermis, isolated filaggrin insufficiency in keratinocytes also affects additional, seemingly unrelated functions in those cells. Here we hypothesized that the exosomal/sEV compartment is also disturbed, influencing the message conveyed between keratinocytes and the immune cells in the disease context. Since primary keratinocytes are very inefficient sEV producers, harvesting a sufficient quantity from cells obtained from atopic skin punch biopsy is not feasible; hence, the model of choice in this study is a filaggrin knock-down keratinocyte line which we previously established by shRNA interference[5,6,15]. We found extensive changes in the mRNA expression pattern between shC and shFLG cells, with a pronounced difference in expression of keratinocyte-specific genes (Fig. 1A) and we further proceeded with data analysis using the FunRich tool[24]. The advantage of using this tool is that apart from the standard gene ontology (GO) terms for compartmental localisation, it also integrates datasets available within the Vesiclepedia[25], i. e., a database of proteins specifically enriched within extracellular vesicles. This gives better insights into the changes relevant to the exosomal/sEV compartment and indeed, the analysis determined that, amongst many compartments affected, the exosomal/sEV compartment is most significantly altered (Fig. 1, B to D). Specifically, over 40% of the differentially regulated genes encoded proteins known to be associated with exosomes/sEVs; interestingly, the change was most pronounced for the upregulated genes but less for those downregulated. The dataset filtered for enrichment within this compartment was subsequently carried through into the enrichment analysis of GO terms for biological process and molecular functions, as well as Reactome pathways (by Panther tool[26] and Reactome Knowledgebase[27], respectively). This identified extensive differences between filaggrin sufficient and insufficient cells at the mRNA level, showing changes in several biological processes related to the immune cell activation, molecular function of cell adhesion and molecule binding and pathways for immune cell activation (predominantly in innate immunity) and stress response (Fig.1, E to G and table S1). Next, we also compared shC and shFLG cells at the protein level, using mass spectrometry (Fig. 1H). This yielded similar FunRich outcomes, although we also identified significant downregulation in the exosome-relevant proteins on this examination (Fig. 1, I to K). GO term enrichment returned findings aligning with those from the mRNA data (Fig. 1, L to N and table S2) with respect to the immune activation and cell adhesion; in addition, processes related to exocytosis, secretion and cellular export showed up as the most prominent in this dataset.

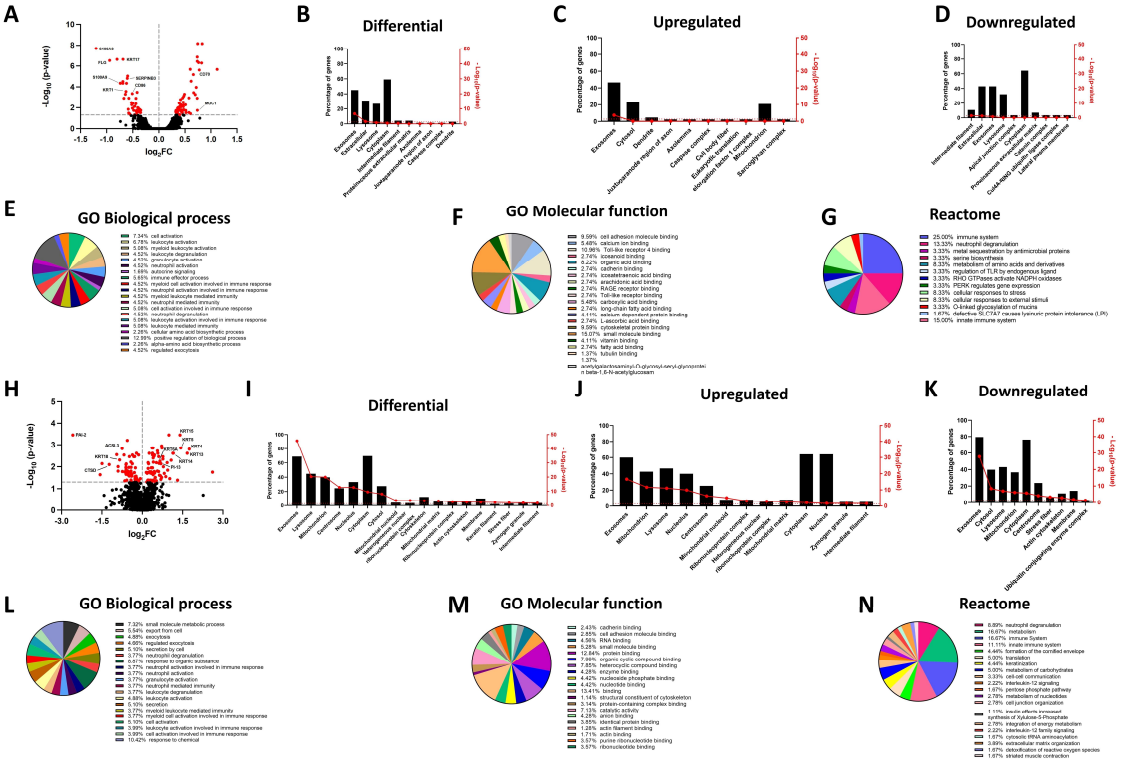


**Figure 1. mRNA and protein expression signature related to the proteins enriched in the exosomal/EV compartment is altered in filaggrin-insufficient keratinocytes.** (**A**) Volcano plot depicting mRNA expression changes in shFLG keratinocytes; n=3 biological replicates; moderated t-test; FC values were $log_2$-transformed and p-values were $log_{10}$-transformed; differentially expressed genes with p<0.05 in red; symbols of selected epidermal barrier- and immune response-related genes are shown; (**B** to **D**) FunRich analysis showing differential expression of genes encoding proteins enriched within cellular compartments; (B) total, (C) upregulated and (D) downregulated in shFLG; (**E** to **G**) Gene Ontology and Reactome terms related to genes encoding proteins identified in exosomes/sEVs by FunRich, differentially expressed in shFLG keratinocytes; analysis by Panther tool; enrichment in GO terms related to: (E) biological process, (F) molecular function and (G) Reactome terms; (**H**) Volcano plot depicting protein expression changes in shFLG keratinocyte cultures; n=4 biological replicates; Benjamini-Hochberg FDR; FC values were $log_2$-transformed and p-values were $log_{10}$-transformed; differentially expressed proteins with p<0.05 in red; symbols of selected epidermal barrier-, lipid metabolism- and immune response-related proteins are shown; (**I** to **K**) FunRich analysis showing differential expression of proteins enriched within cellular compartments; (I) total, (J) upregulated and (K) downregulated in shFLG; (**L** to **N**) Gene Ontology and Reactome terms related to proteins identified by FunRich in exosomes/sEVs, differentially expressed in shFLG keratinocytes; analysis by Panther tool; enrichment in GO terms related to (L) biological process, (M) molecular function and (N) Reactome terms; FC, fold change.

While our 2D monolayer model overcomes the limitation of insufficient exosomal/sEV yields in comparison to the 3D systems or skin samples, to ensure that the differential outcome we observe is also relevant at the level of complex epidermal tissues, we also analysed extensive

proteomic data from the filaggrin-insufficient organotypic epidermal model published by Elias et al.[9] (Fig. 2, A to F and table S3) as well as the transcriptome dataset obtained from the skin samples of AD patients[28] (Fig. 2, G to L and table S4). The results of the analysis were in a strong agreement with those obtained for the monolayer experiments, i.e., confirming that filaggrin insufficiency leads to significant alterations within the exosomal/sEV compartment in 3D tissues amongst other cellular compartments (lysosomes, cytosol/cytoplasm as well as nucleolus and mitochondria; Fig. 2, G to I). Despite some differences likely consequential to the complexity of the stratified epidermis, the GO terms related to the biological processes of exocytosis/cellular export and immune cell activation were also enriched in both datasets; binding and cellular adhesion was clearly identifiable, and pathways related to the immune system, cellular metabolism and stress response were also prominent (Fig. 2, J to L and table S4). These results provided cross validation and increased confidence in our cellular model.

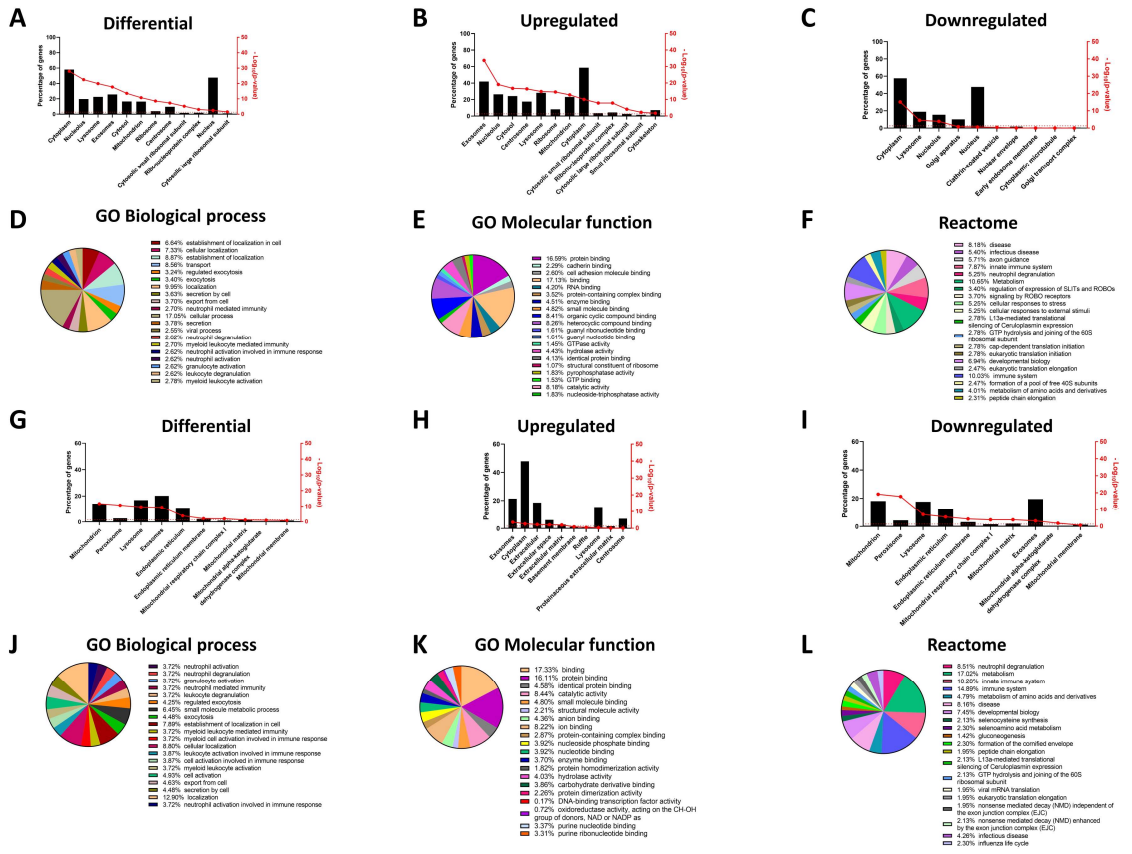


**Figure 2. Cellular compartments are remodelled at the protein level in epidermal organotypic cultures and at the gene level in the skin of atopic dermatitis patients.** (**A** to **C**) Differential expression of proteins enriched in cellular compartments of siFLG organotypic cultures by FunRich tool; (A) total, (B) upregulated and (C) downregulated in siFLG; (**D** to **F**) Gene Ontology and Reactome terms related to the proteins identified by FunRich in exosomes/sEVs, differentially expressed in siFLG organotypic cultures; analysis by Panther tool; enrichment in

GO terms related to (D) biological process, (E) molecular function and (F) Reactome terms. (**G** to **I**) FunRich analysis showing enrichment of differentially expressed genes encoding proteins within cellular compartments in AD skin; (G) total, (H) upregulated and (I) downregulated in AD skin; (**J** to **L**) Gene Ontology and Reactome terms related to the FunRich-identified proteins enriched in exosomes/sEVs, encoded by genes differentially expressed in AD skin; analysis by Panther tool; enrichment in GO terms related to (J) biological process, (K) molecular function and (L) Reactome terms.

As for the GO terms related to antigen presentation, only the analysis of the *FLG* knock-down organotypic skin model revealed relevant terms; specifically, the terms associated with general as well as MHC class I-specific antigen processing and presentation were enriched; similar terms were identified by the Reactome pathways (fig. S1). Altogether, we identified extensive alterations in the keratinocyte exosomal/sEV compartment as a consequence of filaggrin insufficiency, including those with relevance to immunological processes.

*Exosomes/sEVs secreted by filaggrin sufficient and insufficient keratinocytes display similar size and marker characteristics*

We next isolated exosome-containing sEV fractions from the conditioned keratinocyte media by the ultracentrifugation protocol (Fig. 3A). Vesicles were examined by electron microscopy and Nanoparticle Tracking Analysis (NTA) and we confirmed the characteristic cup shape and size distribution (Fig. 3, B and C), demonstrating exosome enrichment. We did not observe any substantial differences with respect to the vesicle sizes or secretion level between the shC and shFLG cells (fig. S2). 100K pellets fractionated on a sucrose/iodixanol gradient, contained high levels of exosomal markers CD9, CD63 and syntenin-1 in the top fractions (fractions 1-5; Fig. 3D) but not in the lower fractions (fractions 6-10) which suggested no significant contamination of small microvesicles (MVs), which display lower but still detectable CD9/CD63 levels[29,30], indicating that the 100K pellet contained relatively pure exosomal population. However, we did not find any substantial differences in the expression of the markers between the sEVs obtained from shFLG cells ($shFLG_{sEV}$) in comparison to those from shC cells ($shC_{sEV}$).

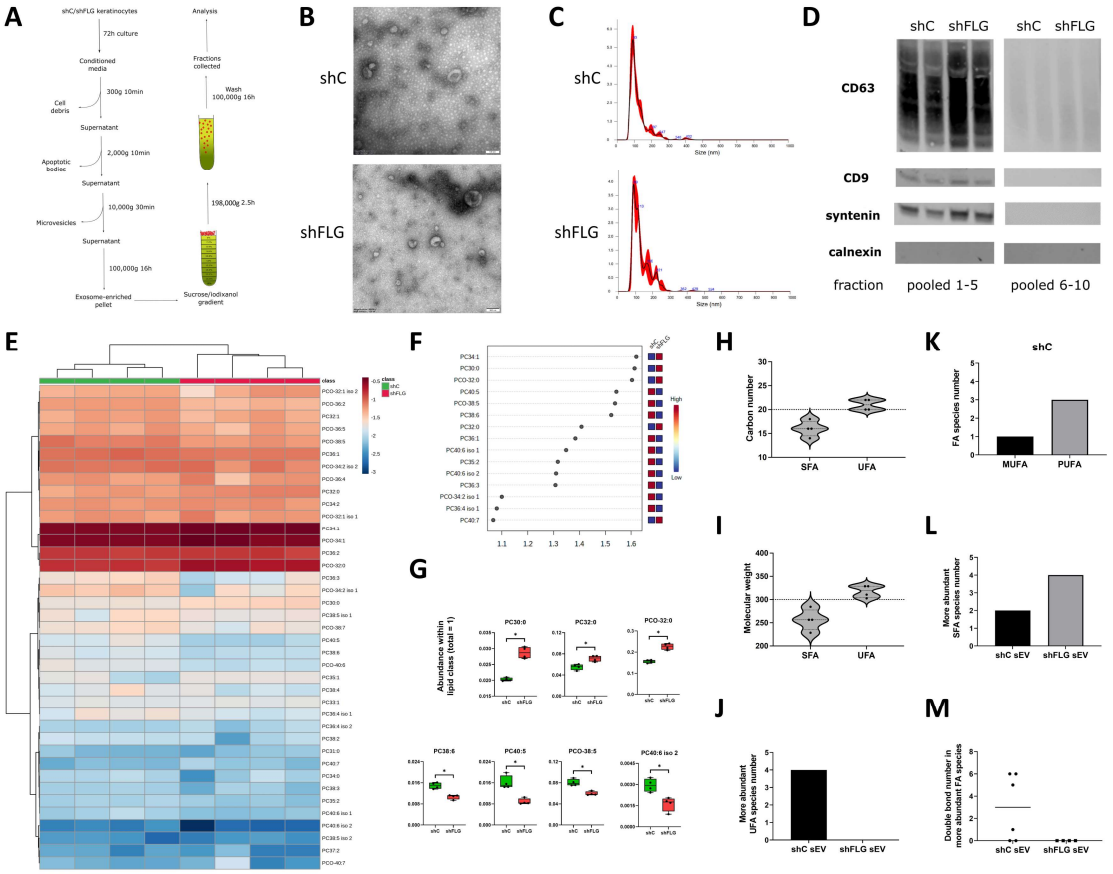


**Figure 3. Filaggrin insufficiency alters the sEV composition of PLA2-digestible lipids.** (**A**) A protocol for isolation of extracellular vesicles by ultracentrifugation; exosome-enriched sEVs are pelleted as 100K fraction and purified by density gradient; (**B**) Electron microscopy pictures of sEVs preparations; representative of n=3; (**C**) Size distribution of purified sEVs by Nanoparticle Tracking Analysis (NTA); representative example shown; (**D**) Enrichment of exosomal markers in purified sEVs; Western blot; representative blot, n=2; pooled fractions 1-5 are purified exosome-enriched sEV; pooled fractions 6-10 are smaller microvesicles; (**E** to **G**) Lipidomic analysis of PLA2-digestible lipid species in sEVs; (E) heatmap of the detected lipids; (F) lipid species most affected by filaggrin accordingly to the PLS-DA analysis; the variance importance for the projection values (VIP) were used to sort lipids accordingly to their contribution to PLS-DA model; (**G**) boxplots showing lipid species significantly different in abundance; combined data from n=4 biological replicates; unpaired t-test, FDR correction; *, p<0.05; (**H** and **I**) Fatty acid composition of sEV phospholipids differing in abundance by (H) a chain length or (I) molecular weight; dotted line shows the length and mass benchmarks for highest CD1a-dependent response; (**J**) Number of the more abundant UFA species in sEVs; (**K**) Breakdown of UFA species from (J) in $shC_{sEV}$ by degree of unsaturation; (**L**) Number of the more abundant SFAs in sEVs; (**M**) Number of double bonds in the more abundant FA species in sEVs; PLA2, phospholipase A2; VIP, variable importance in projection; FA, fatty acid; UFA, unsaturated fatty acid, SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; PC, diacylglycerophosphocholine; PCO, ether-linked glycerophosphocholine.

*Exosomes/sEVs secreted by filaggrin insufficient keratinocytes do not impact responses to peptides or whole proteins*

Subsequently, we proceeded to define the capacity of $shC_{sEV}$ and $shFLG_{sEV}$ to affect antigen presentation to T cells. To this end, we first tested the effect of the vesicles on dendritic cells but did not observe any differential outcomes regarding the expressed surface markers in either immature or mature monocyte-derived dendritic cells (moDCs) (fig. S3A). Next, we proceeded with a comparison of the effect of $shC_{sEV}$ and $shFLG_{sEV}$ on specific T cell IFNy responses to peptide antigens. We used a panel of MHC class I and class II-restricted peptides derived from common pathogens and vaccination strains which most of the population has been exposed to in their lifetime and maintain memory responses (CEFT peptide pool); exosomes/sEVs were added to immature moDCs during the antigen pulsation period. The ELISpot assay experiments did not reveal any major differences in the response level between cells stimulated with CEFT in combination with $shFLG_{sEV}$ and $shC_{sEV}$ (fig. S3B). This was also the case for IFNy responses where a whole protein (CMV pp65) was provided as an antigen source (fig. S3C), suggesting that the addition of keratinocyte-derived exosomes/sEVs did not impact peptide antigen processing and class I/II loading pathways, regardless of the filaggrin status in the cells.

*Exosomes/sEVs secreted by filaggrin insufficient keratinocytes display altered lipid profile*

While we did not observe any differential outcomes from the MHC class I/class II-restricted T cells, we considered it still plausible that lipid presentation could be affected. Given that the skin is a body site highly dependent on CD1a-mediated T cell responses, and $CD1a^+$ cells are in abundance, we next followed with an assessment of the effect that sEV could exert on CD1a-mediated lipid-specific responses. We previously determined the role of phospholipase PLA2 in neoantigen generation and induction of T cell reactivity via this pathway[14,31]. We also showed that mast cell-derived exosomes/sEVs may contain active PLA2 enzyme and supply it to induce neoantigen-specific T cell responses[32]. Hence, we next investigated whether keratinocytes express considerable amounts of the enzyme that could be enclosed within exosomes/sEVs. However, our mass spectrometry data for the cell lysates suggested this was not the case (table S5); similarly, we did not detect any relevant enzymatic activity in either the keratinocyte lysates or exosomes/sEVs when testing for the PLA2 activity which detects both the secretory and cytosolic PLA2s (fig. S4A). This ruled out the possibility that PLA2 may be supplied in exosomes/sEVs secreted by keratinocytes in the steady state, as well as those filaggrin insufficient. However, since sEVs are lipid-based organelles, they could potentially provide a source of lipid ligands to CD1a-restricted T cells. Hence, we followed with mass spectrometry lipidomic profiling of $shFLG_{sEV}$ and $shC_{sEV}$, confirming that the exosomal/sEV lipid content was biased towards phospholipids, as expected. In terms of the changes in the relative content between $shFLG_{sEV}$ and $shC_{sEV}$ we found substantial alterations among PLA2-digestible lipid

classes, specifically diacyl glycerophosphocholines (PCs) and ether analogs (PCOs) (Fig. 3, E to G and fig. S4B)

*Filaggrin insufficiency background narrows the repertoire of exosome/sEV-derived lipids most suitable for CD1a binding*

As far as the CD1a-mediated presentation is concerned, the size and topology of the CD1a binding groove defines the suitability of lipids of various lengths and structural complexity to bind and form stable complexes with the molecule. To this end, Nicolai *et al.* [33] elegantly documented that ligands of around 20 carbon atoms and molecular weight ca. 300 are optimal; the majority of ligands promoting strong T cell activation fell in those ranges. Similarly, features of added structural complexity, e.g., presence of unsaturated bonds also improved CD1a-restricted T cell responses in comparison to fully saturated chains[33]. From our results increased contribution of PCs containing saturated or monounsaturated long-chain fatty acids (LCFA) and decreased content of very long chain polyunsaturated fatty acid (PUFA)-containing PCs (e.g., C22:6; docosahexaenoic acid; DHA, in PC40:6 and PC38:6) was apparent in $shFLG_{sEV}$ vs $shC_{sEV}$ (Fig. 3G). Hence, we next assessed the breadth of the potential antigenic lipid repertoire within the exosomal/sEV compartment, taking into account the phospholipid fatty acid constituents. We noted that identified unsaturated fatty acids (UFAs; including both mono- and polyunsaturated FAs; MUFAs and PUFAs) detected in exosomes/sEVs closely matched the optimal length and size in terms of the carbon number (Fig. 3H) and molecular weight (Fig. 3I) benchmarks in comparison to the saturated fatty acids (SFAs), potentially suggesting that those UFAs were more likely to impact CD1a. When lipid sources were compared, it was clear that FAs identified as more abundant in $shC_{sEV}$ represented much greater variety and were also more suitable for CD1a presentation than those in $shFLG_{sEV}$ (Fig. 3J). Strikingly, when assessing the saturation of the FA chains, we found no single UFA to be more abundant in the $shFLG_{sEV}$ (Fig. 3J); at the same time, we detected three times more PUFA over MUFA species in $shC_{sEV}$ (Fig. 3K). In contrast, the SFA content showed the reverse, i.e., we found much greater number of SFAs within the pool of more abundant FAs in $shFLG_{sEV}$ (Fig. 3L). Lastly, the number of double bonds in the FA chains also differed greatly, with no single FA more abundant in $shFLG_{sEV}$ containing those (Fig. 3M).

Altogether, our results imply that phospholipids in exosomes/sEVs secreted on the filaggrin insufficiency background may influence FAs of sufficient diversity and characteristics to constitute a repertoire of strongly binding CD1a ligands.

*Exosomes/sEVs secreted by filaggrin insufficient keratinocytes modulate CD1a-autoreactive T cell responses*

To determine if the differential content of CD1a ligands translates into differences in the T cell reactivity we next proceeded with the IFNγ ELISpot assay. Here we used a CD1a transfected K562 cell line, devoid of class I and II expression, as antigen presenting cells (K562-CD1a; fig. S4C); this model was successfully used in several studies investigating CD1a-mediated T cell responses[14,31,33–37]. While we noted some reactivity with certain donors manifesting a level of IFNγ production, this was not significant in comparison to the unpulsed control cells or between the cellular sEV source, regardless of the filaggrin status (fig. S4D). This argues that intact exosomes/sEVs derived from keratinocytes do not provide enough CD1a ligands readily available for binding, and further supports the finding of the lack of the PLA2 activity in keratinocyte-derived sEVs and is consistent with their relative stability. Hence, with the aim of liberating lipids from the sEV membranes, we next followed with the addition of bee venom PLA2 as a source of the enzymatic activity to generate lipid neoantigens; pulsing of the cells with exosomes/sEVs and PLA2 was carried out simultaneously. Interestingly, we observed that the addition of $shC_{sEV}$, together with PLA2, resulted in the induction in CD1a-specific IFNγ responses above the "PLA2 only" level (Fig. 4A), indicating that digestion of exosomes/sEVs secreted by filaggrin-sufficient keratinocytes released lipids suitable for CD1a-dependent T cell activation. In contrast, the addition of $shFLG_{sEV}$ failed to induce IFNγ T cell responses above the control level; we also measured IL-10 and IL-13 secretion in the supernatants by ELISA, but the levels produced were negligible in this system (fig. S4E).

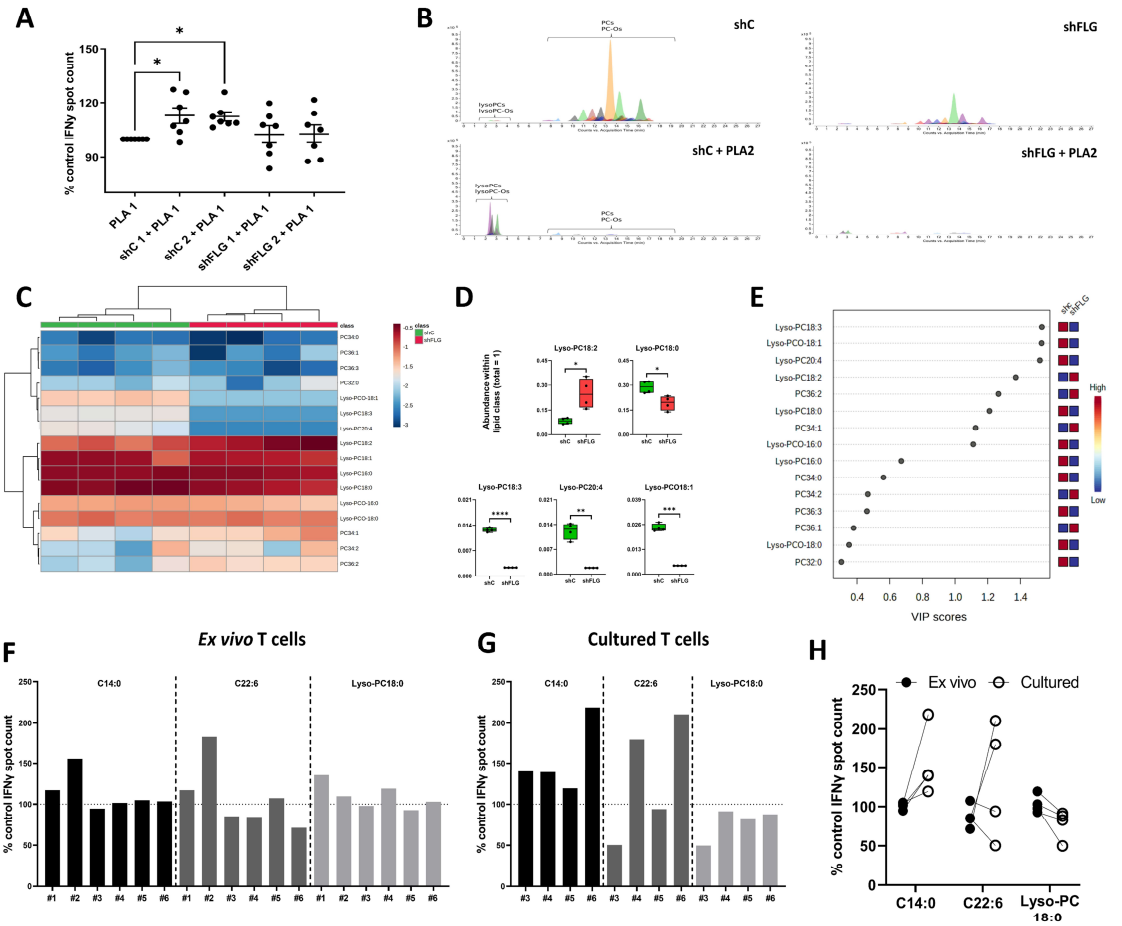


**Figure 4. shFLG$_{sEV}$ demonstrate a reduced capacity to stimulate CD1a-specific T cell responses.** (**A**) IFNy responses of T cells stimulated with K562-CD1a cells pulsed with 1 µg/ml PLA2 and sEVs from 1 or 2 million keratinocytes measured by ELISpot assay; means +/- SEM shown; data normalized to control=100%; n=7 donors; one-way ANOVA with Šídák's multiple comparisons test; (**B**) Extracted Ion Chromatograms (EICs) showing sEV lipid profile before and after digestion with 1 µg/ml PLA2 for 1h (n=4; representative data shown); (**C** to **E**) Lipidomic analysis of glycerophosphocholine-related products after sEV digestion; (C) heatmap of detected lipids; (D) boxplots showing lipid species significantly different in abundance; data from n=4 biological replicates, unpaired t-test, FDR correction; (E) lipid species most affected by filaggrin insufficiency accordingly to the PLS-DA analysis; the variance importance for the projection values (VIP) were used to sort lipids accordingly to their contribution to PLS-DA model; (**F** to **H**) IFNy responses from (F) *ex vivo* T cells stimulated with K562-CD1a cells pulsed with 10 µM of lipids overnight; n=6 donors; and (G) T cells cultured for 13 days following ELISpot, n=4 donors; means from two technical replicates for each individual donor, normalized to the control=100% are shown; (H) comparison of responses between *ex vivo* and cultured T cells from n=4 donors represented both in F and G; one-way ANOVA with Šídák's multiple comparisons test. PLA2, phospholipase A2; VIP, variable importance in projection. PC, diacylglycerophosphocholine; Lyso-PC, monoacylglycerophosphocholine; Lyso-PCO, monoalkylglycerophosphocholine; C14:0, tetradecanoic acid; C22:6, docosahexaenoic acid; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Taken together, these results suggest that while exosomes/sEVs derived from filaggrin-expressing keratinocytes contain CD1a neoantigens which can be liberated from their membranes by PLA2 digestion, filaggrin insufficiency background reduces the capacity of exosomes/sEVs to carry substrates suitable for generation of type 1 response-inducing CD1a ligands.

*Filaggrin insufficiency reduces the complexity of exosomal/sEV lipid composition and diversity of the ligands promoting homeostatic responses*

Next, to determine which lipid species may be involved in the differential outcomes we subjected exosomes/sEVs to PLA2 treatment in a cell-free assay. We observed that phospholipids in both $shFLG_{sEV}$ and $shC_{sEV}$ undergo complete digestion by the enzyme and disappear from both $shC_{sEV}$ and $shFLG_{sEV}$ samples (Fig. 4B). Low signal for PCs was detected only for the most abundant product species; these were lysoglycerophosphocholines (lysoPCs and ether analogs lyso-PCO) and the relative content of almost all the detected lyso-PCs and lyso-PCOs was much lower in the digested $shFLG_{sEV}$ in comparison to the digested control $shC_{sEV}$ (Fig. 4, C to E and fig. S5A). Lyso-PC18:0 was the most abundant species within its lipid class found in keratinocyte-derived sEVs and was also significantly decreased in $shFLG_{sEV}$; shFLGsEV were also lower in the content of all differentially abundant Lyso-PCs and Lyso-PCOs apart from Lyso-PC18:2 which showed an opposite trend (Fig. 4D).

To further define the impact of the lipids contained within the $shC_{sEVs}$ on the observed T cell reactivity, we next selected three lipids found in sEVs, with representative acyl chain lengths and molecular weights that reflected optimal and suboptimal characteristics for ligand binding to CD1a, i.e., a short chain SFA (C14:0), a long-chain PUFA (C22:6; DHA), and lysophosphatidylcholine (Lyso-PC18:0) and tested their capacity to promote IFNγ responses from peripheral blood T cells. We observed low but detectable responses to all those lipids in some of the donors already *ex vivo* (Fig. 4F). Culturing of the T cells into short-term lines augmented the responses to C14:0 and C22:6 but responses of the cultured cells to Lyso-PC18:0 were reduced (Fig. 4, G and H). Altogether, these results suggest that lipids supplied to T cells within sEV are permissive but weak ligands, corresponding to the autoantigen characteristics and demonstrating interindividual variability.

*Exosomes/sEVs secreted by filaggrin insufficient keratinocytes contain more non-permissive/inhibitory lipids capable of CD1a binding and dampening T cell responses*

In addition to the phospholipids which are classical PLA2 substrates, we determined that exosomes/sEVs also contain many lipids which are not preferential targets for PLA2-mediated enzymatic cleavage, such as ceramides and sphingolipids (Fig. 5, A to C and fig. S5B). Accordingly, we found that the relative proportion within classes of those lipids does not change upon PLA2 digestion and their relevant $shC_{sEV}$ vs $shFLG_{sEV}$ contribution remained comparable to that in the untreated samples (Fig. 5, D to F). However, while resistant to the digestion process itself, these lipids would also get liberated from exosomes/sEVs due to the perturbing impact of PLA2 on vesicular membranes and so, would be present in the lipid mixture after digestion and their impact may be important; specifically, a recent study by Cotton *et al*. identified a propensity of CD1a to preferentially bind endogenous non-permissive lipid ligands which inhibit T cell responses (CD1a blockers)[38]. Hence, we attempted to determine if any of the detected nondigestible lipids may have a potential to reduce CD1a reactivity. Indeed, we found that the keratinocyte-derived sEVs contained sphingomyelins, non-permissive ligands capable of strongly binding to CD1a[38], i.e., SMd42:1, SMd42:2 and SMd42:3 (fig. S5D) which exhibit blocking potential on T cell activation.

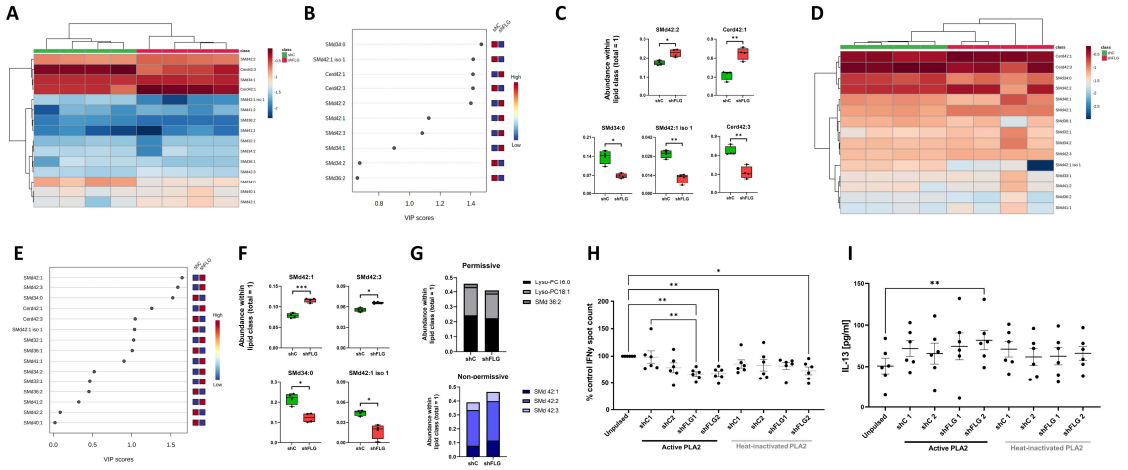


**Figure 5. Non-permissive CD1a lipid antigens are enriched in sEVs secreted by filaggrin-insufficient keratinocytes.** (**A** to **C**) Lipidomic analysis of PLA2-non-digestible lipid species in sEVs; (A) heatmap of all detected lipids; (B) lipid species most affected by filaggrin insufficiency accordingly to the PLS-DA analysis; the variance importance for the projection values (VIP) were used to sort lipids accordingly to their contribution to PLS-DA model; (C) boxplots showing lipid species significantly different in abundance; n=4 biological replicates, unpaired t-test, FDR correction; (**D** to **F**) Lipidomic analysis of PLA2-non-digestible lipid species in sEVs digested with 1 µg/ml PLA2 for 1h; (D) heatmap of all detected lipids; (E) lipid species most affected by filaggrin insufficiency accordingly to the PLS-DA analysis; the variance importance for the projection

values (VIP) were used to sort lipids accordingly to their contribution to PLS-DA model; (F) boxplots showing lipid species significantly different in abundance; n=4 biological replicates; unpaired t-test, FDR correction; (**G**) Relative amounts of permissive and non-permissive species in PLA2-digested sEVs; (**H**) IFNγ responses by T cells stimulated with K562-CD1a cells pulsed overnight with sEVs from 1 or 2 million keratinocytes digested with 1 µg/ml PLA2 for 1h; n=6 donors; data normalized to control=100%; (**I**) IL-13 secretion into culture supernatants from (H) measured by ELISA; n=6 donors; means +/- SEM are shown; one-way ANOVA with Šídák's multiple comparisons test; *, p<0.05; **, p<0.01; PLA2, phospholipase A2; VIP, variable importance in projection; SMd, sphingomyelin; Cerd, ceramide.

To obtain a clearer picture of the relative sEV content of candidate permissive and non-permissive CD1a ligands we classified lipid species based on published data[31,32,37–39]. The results of our analysis showed that $shFLG_{sEV}$ were less abundant in some permissive ligands, i.e., Lyso-PC16:0, Lyso-PC18:1 and SMd36:2 (Fig. 5G). In contrast, we observed greater enrichment of $shFLG_{sEV}$ in non-permissive ligands, i.e., inhibitory very long-chain sphingomyelins; experimentally tested SMd42:1, SMd42:2 and SMd42:3, predicted as non-permissive because of the structural features (very long chain and protruding headgroup) (Fig. 5G). Interestingly, we observed an opposite trend for the SMd42:1 isomer, slightly more abundant in $shC_{sEV}$. However, while the structure of this isomer is not known, the SMd42:1 species increased in abundance in $shFLG_{sEV}$ has the same composition as the one shown to be non-permissive by Cotton et al.[38]

With these new insights we recognized that the enrichment of lipids with inhibitory function in $shFLG_{sEV}$ could have interfered with the ELISpot assay, reducing detectable IFNγ T cell response, which might have partly depended on the self-ligands liberated from the membranes of cells exposed to PLA2 at the time of exosome/sEV pulsation; those could potentially mask some of the differential effects. Hence, we conducted another assessment of IFNγ responses, this time using exosomes/sEVs already digested by PLA2; we also included heat inactivated PLA2 controls, to confirm the active enzyme dependency. We observed that PLA2-digested $shFLG_{sEV}$ significantly inhibited IFNγ secretion from T cells while the addition of digested $shC_{sEV}$ did not result in any differential outcomes (Fig. 5H). In addition, we also noted a significant decrease in the IFNγ response to the higher $shFLG_{sEV}$ concentration even in the heat inactivated PLA2, possibly due to the spontaneous release of some inhibitory lipids from those vesicles (Fig. 5H). In contrast to the IFNγ response, we observed stimulation of IL-13 by $shFLG_{sEV}$ but not by $shC_{sEV}$ and a subtle similar trend in IL-17A production but no difference in IL-10 levels (Fig. 5I and fig. S6A and B).

Taken together, we determined that sEVs produced by filaggrin insufficient keratinocytes are enriched in non-permissive ligands which collectively reduce the type 1 IFNγ response from CD1a-restricted T cells and promote a type 2 bias.

*Changes in the lipid composition of exosomes/sEVs secreted by filaggrin insufficient keratinocytes reflect the shift in the cellular lipid landscape*

Finally, to understand the reasons behind the differential enrichment of permissive and non-permissive ligands in $shFLG_{sEV}$ vs. $shC_{sEV}$, we set out to determine if the observed alterations reflected changes in the overall cellular lipid profile resulting from filaggrin insufficiency. Indeed, we observed remodelling of the PC composition in shFLG cells, with significantly lower content of very long-chain PUFAs (Fig. 6, A to C and fig. S7A) in shFLG cells in comparison to shC cells, corresponding to the alterations of exosome/sEV composition. Specifically, the content of the complex ether analogues of PCs, i.e., species containing long chain polyunsaturated fatty acids (e.g., PCO40:7 and PCO36:6) was reduced in the shFLG cells in comparison to the shC cells and shorter saturated or monounsaturated PCOs were dominant in filaggrin-insufficient cells (Fig. 6C). Hence, similarly to the sEVs, FAs identified as more abundant in the shC cells represented much greater variety and closer match to the CD1a-response relevant carbon number and molecular weight benchmarks than those in shFLG cells; this was also true for fatty alcohols (FA-OHs) (Fig. 6, D and E). Interestingly, shC cells contained approximately four times more of the abundant UFA species compared to the shFLG keratinocytes (Fig. 6F). Moreover, among the detected UFAs, a similar number of MUFA and PUFA species was detected in the shC cells, while in the shFLG cells all the very few UFAs were MUFA species; no single PUFA was identified as being more abundant in those cells (Fig. 6, G and H). Finally, filaggrin-insufficient cells had greatly reduced number of double bonds within the identified FAs and FA-OHs (Fig. 6I).

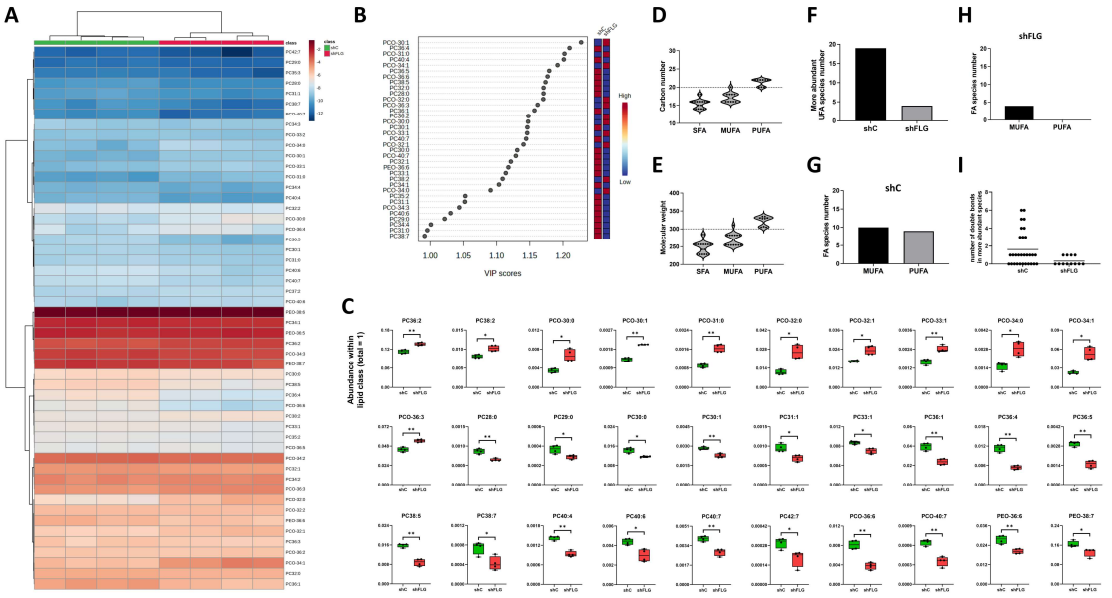


**Figure 6. A filaggrin insufficiency background alters the landscape of the PLA2-digestible lipidome in keratinocytes.** (**A** to **C**) Lipidomic analysis of PLA2-digestible lipid species in shC and shFLG keratinocytes; (A) heatmap of all detected lipid species; (B) lipid species most affected by filaggrin insufficiency accordingly to the PLS-DA analysis; the variance importance for the projection values (VIP) were used to sort lipids accordingly to their contribution to PLS-DA model; (C) boxplots showing lipid species significantly different in abundance; n=4 biological replicates; unpaired t-test; FDR correction; (**D** and **E**) Fatty acid composition of differentially abundant phospholipids in keratinocytes by (D) chain length and (E) molecular weight of fatty acids; dotted line shows the size and mass benchmarks for optimal CD1a-mediated responses; (**F**) Number of the more abundant lipid species in keratinocytes; (**G** and **H**) UFAs represented in (F) found in either shC (G) or shFLG (H) keratinocytes by degree of unsaturation; (**I**) Number of double bonds in the more abundant UFA species in keratinocytes; PLA2, phospholipase A2; PC, diacylglycerophosphocholine; PCO, ether-linked glycerophosphocholine; PEO, ether-linked glycerophosphoethanoloamine; SFA, saturated fatty acid; UFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

We also observed that filaggrin insufficiency significantly alters cellular content of several lipid species which are not PLA2 substates, namely ceramides (Cerds), lactosylceramides (LacCerds) and sphingomyelins (SMds) (Fig. 7, A to C and fig. S7B). As for the differently abundant sphingomyelin species, we found that the shFLG cells were enriched in sphingomyelins with longer chains and higher molecular mass compared to the shC cells (Fig. 7, D and E). Moreover, all the sphingomyelin species abundant in filaggrin-insufficient keratinocytes contained two or three double bonds within their chains; in contrast to the control cells, in which we found only one monosaturated sphingomyelin and all the remaining ones had completely saturated chains (Fig. 7F). Finally, the two sphingomyelin species which contribute to the significant difference in

the CD1a-dependent responses and enriched in shFLG$_{sEV}$, i.e., SMd 42:2 and 42:3, were also highly enriched in the filaggrin knockdown cells.

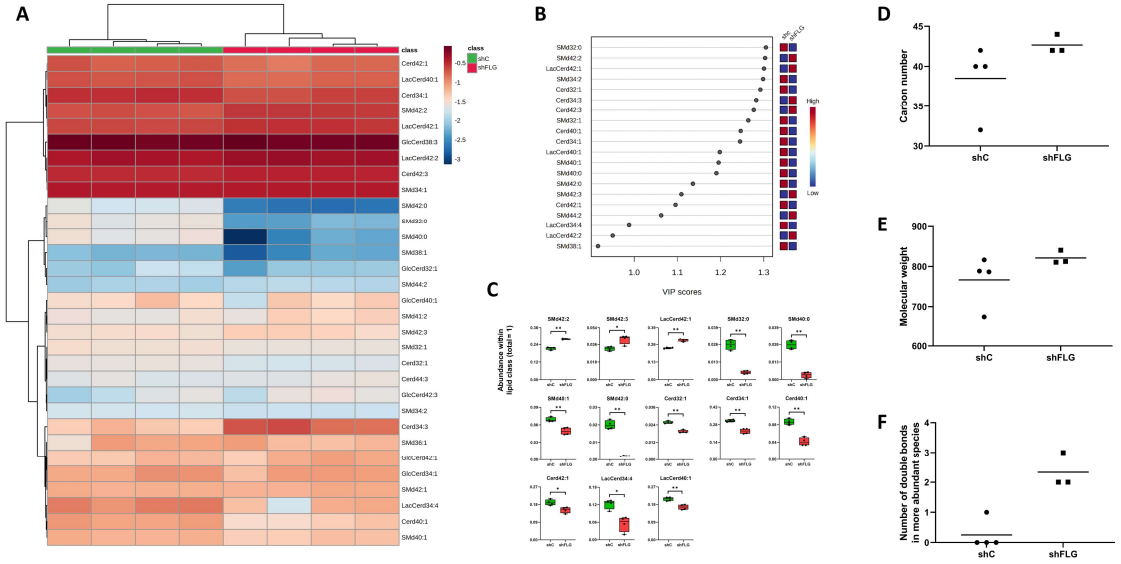


**Figure 7. A filaggrin insufficiency background alters the landscape of non-PLA2-digestible lipidome in keratinocytes.** (**A** to **C**) Lipidomic analysis of PLA2-nondigestible lipid species in keratinocytes; (A) heatmap of all detected lipid species; (B) lipid species most affected by filaggrin insufficiency accordingly to the PLS-DA analysis; the variance importance for the projection values (VIP) were used to sort lipids accordingly to their contribution to PLS-DA model; (C) boxplots showing lipid species significantly different in abundance; data for n=4 biological replicates, unpaired t-test, FDR correction; (**D** to **F**) Differentially abundant sphingomyelin species represented by combined sphingosine and fatty acid chain length; (D), molecular weight (E) and a number of double bonds (F); SMd, sphingomyelin; Cerd, ceramide; LacCerd, lactosylceramide; GlcCerd, glucosylceramide.

*Dysregulation of a long-chain-fatty-acid-CoA ligase capacity on filaggrin insufficiency background*

Alteration in the skin lipid content and dysregulation of the lipid metabolic pathways was previously observed in the AD skin[28 40,41]. Here, given the extent of the changes we detected, affecting multiple lipid classes both in the shFLG cells and their exosomal/sEV compartment, we envisaged that the mechanism contributing to the phenotype would likely involve a pathway(s) with a major role in the lipid metabolism and membrane formation. To this end, we identified that the long-chain-fatty-acid-CoA ligase 3 (ACSL3), implicated in free fatty acid conversion to the activated acyl-CoA esters[42 43], crucial in the membrane phospholipid synthesis process[44], is substantially downregulated in the shFLG cells (Fig. 8A). Interestingly, apart from ACSL3, we found additional isoforms of this enzyme to be also downregulated in AD skin (at mRNA level;

Fig. 8, B to E). In contrast, the enzymes of the elongation of very long (ELOVL) fatty acid family, proposed to be involved in the process of fatty acid extension for CD1a ligands[38], were not differentially expressed in the *in vitro* models, whereas we could observe downregulation of *ELOVL1, ELOVL3, ELOVL4* and *ELOVL5* mRNA in AD skin (Figure 8F to I). In addition, we observed upregulation of the *FADS1* mRNA expression in the cells (Figure 8J), likely of a compensatory nature in the 2D model, but downregulation of *FADS1, FADS2* and *FADS6* mRNA in the skin of AD patients, suggesting more complex regulation of those enzymes during stratification process (Fig. 8K-M).

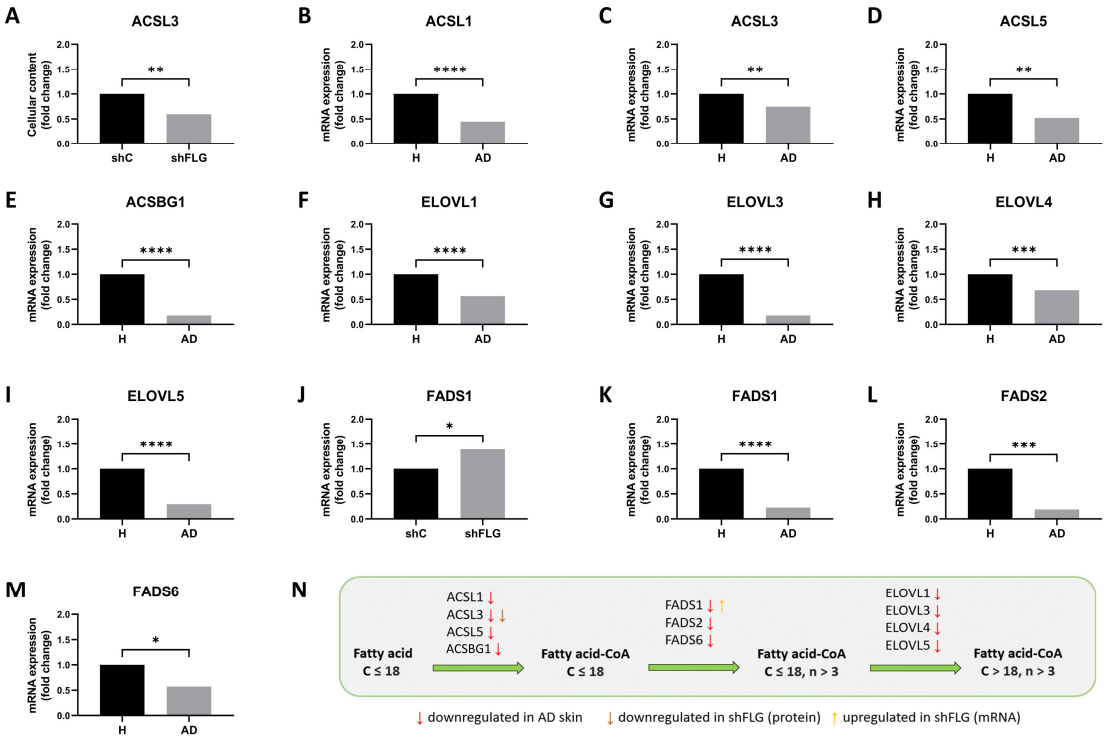


**Figure 8. Filaggrin insufficiency in keratinocytes impacts enzymatic pathways of synthesis of lipids acting as substrates for generation of CD1a-dependent lipid neoantigens.** (**A**) ACSL3 protein expression by cultured keratinocytes; n=4 biological replicates, unpaired t-test, Benjamini-Hochberg FDR; (**B-I**) Analysis of the data from Cole *et al*[28]*.,* showing the expression of (B) *ACSL1*; (C) *ACSL3*; (D) *ACSL5*; (E) *ACSBG*; (F) *ELOVL1*; (G) *ELOVL3*; (H) *ELOVL4*; (I) *ELOVL5* mRNA in AD skin; n=26 AD and n=10 healthy subjects; Benjamini-Hochberg FDR; (**J**) *FADS1* mRNA expression in cultured keratinocytes; n=3 biological replicates, t-test; (**K** to **M**) Analysis of the data from Cole *et al*[28]*.,* showing the expression of (K) *FADS1*; (L) *FADS2*; (M) *FADS6* mRNA in AD skin; n=26 AD and n=10 healthy subjects; all data are normalized to control (shC or H=1); **(N)** Summary of the changes in the lipid metabolic pathways identified in this study; simplified diagram; C, number of carbon atoms; n, number of double bonds; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. *ACSL3*, long-chain-fatty-acid--CoA ligase 3; *ACSL1*, long-chain-fatty-acid--CoA ligase 1; *ACSL5*, long-chain-fatty-acid--CoA ligase 5; *ACSBG1*, long-chain-fatty-acid--CoA ligase *ACSBG1*; *ELOVL1*, *ELOVL* fatty acid elongase 1; *ELOVL3*, *ELOVL* fatty acid elongase 3;

*ELOVL4*, *ELOVL* fatty acid elongase 4; *FADS1*, fatty acid desaturase 1; *FADS2*, fatty acid desaturase 2; *FADS6*, fatty acid desaturase 6.

Taken together, we identified extensive changes in the lipid metabolic pathways, including a pronounced reduction in long-chain-fatty-acid-CoA ligase capacity, in filaggrin-insufficient cells and AD skin as a plausible explanation of the changes observed in keratinocyte lipid composition, including within the exosomal/sEV compartment; the data are summarised in Figure 8N.

## Discussion

Loss-of-function mutations in *FLG*, the gene encoding a late epidermal protein, filaggrin, constitute the most prominent genetic predisposition factor for atopic dermatitis (AD)[3], highlighting the multifaceted role of this protein in supporting epidermal barrier function and controlling the keratinocyte differentiation process. Consequently, reduced filaggrin expression in the skin of AD patients and experimental models impacts numerous processes that are hallmarks of effective epidermal differentiation and cornification[45], e.g., remodelling of the cytoskeleton[46], formation of tight junctions[47], lipid production [46], and changes in enzymatic activity[6,48]. *FLG* null mutations predispose to microbial dysbiosis[49] and reduced ability to control skin infections, resulting in *S. aureus* superinfections[50] and a predisposition to eczema herpeticum[51]. The impact stretches beyond the skin; *FLG* mutations are also linked to other manifestations of the "allergic march", i. e. asthma, rhinitis, food allergy; affecting organs in which filaggrin is not expressed. To date, it is not clear how tissues distant from the skin may be impacted by filaggrin insufficiency, although penetration of allergens through an impaired structural barrier has been proposed as a means by which a systemic immune response is initiated.

In this study, we used a knockdown model to mimic the filaggrin expression downregulation dependent on the isolated inherited factor, which allowed us to dissect out the impact of the AD inflammatory mediators and environmental factors. The use of a stable knockdown line allowed us to overcome the low EV output from primary keratinocytes and the limited size of AD skin samples. Our study, integrating the findings from 2D *in vitro* models with 3D organotypic cultures constructed of primary cells with the AD skin dataset visualised the extent of changes resulting from filaggrin insufficiency and identified the means through which these widespread alterations could promote compartmental remodelling.

The involvement of keratinocyte-derived exosomes/sEVs in antigen-specific presentation was previously only studied by Kotzerke *et al.*, in the context of responses to ovalbumin (OVA) in a murine model which failed to detect any apparent T cell activation of OVA-specific T cells[18]. However, the authors did not investigate filaggrin insufficient mice or from perspective of lipid-specific responses; at the same time, significant differences in the CD1 system between the species (CD1a-c are absent in the rodents), would hamper detection of any such responses, unless a humanised model is used. Recent work, which described *S. aureus* enterotoxin B exosome-mediated transfer from keratinocytes following superantigen exposure described a potential for non-specific T cell activation[52]. Of note, while we did not observe any differential outcome in the class I/class II presentation pathways by simple addition of sEVs during the antigen pulsation, it is still possible that sEVs from keratinocytes insufficient in filaggrin may have additional effects relevant to the peptide presentation, e.g., through their altered ability to transfer peptide antigens or propensity to undergo cellular uptake by antigen presenting cells. To date, it has not been determined whether keratinocyte-derived exosomes/sEVs contribute to lipid-specific T cell responses.

This study, to our knowledge, is the first demonstration that secretory vesicles may constitute an efficient source of ligands for lipid presentation pathways; we showed that exosomes/sEVs are not immunologically inert in this system, but they supply PLA2 substrates to either activate CD1a-specific T cells or lipid ligands of the inhibitory potential with respect to the IFNγ responses and promoting a type 2 bias. Given that sEVs contain a mixture of permissive and non-permissive lipids, such a shift between a type 1 and type 2 response may reflect changes of the overall avidity during CD1a-mediated presentation to T cells. Specifically, it has been shown for both peptides and lipid presentation within the CD1d pathway that changes in ligand affinity (hence the overall interaction avidity) result in differential contact time between the cells and their activation level, leading to differential response[53–56]; the longer the time the more type 1 bias. This "structure–activity relationship" has been proposed to result in a ligand-specific "cytokine fingerprint"[57,58]. Here, increased abundance of the non-permissive ligands, disrupting CD1a-TCR contact zone may reduce the interaction time, resulting in shorter time of cellular interaction and T cell activation more biased towards type 2 responses.

In the context of atopic skin disease, we observed extensive impact of filaggrin knockdown on keratinocytes as a whole and their exosomal/sEV compartment specifically. We subsequently found that, through remodelled sEV composition, the impact is carried through into the long-distance communication stream; in addition, the loss of control of PLA2 activity[14] in the filaggrin insufficiency scenario may lead to even greater dominance of the inhibitory ligands released

from sEVs and compound skin inflammation. We concluded that the sEV-conveyed message determines the involvement of sEVs in CD1a-restricted lipid antigen presentation which links aberrant keratinocyte differentiation with a Th2-biased allergic inflammation and could provide some explanation to the phenomenon of the "allergic march".

Aberrant keratinocyte differentiation resulting from filaggrin insufficiency has previously been shown to contain a broad lipid dysregulation component *in vitro*[9] which correspond to the lipid abnormalities previously reported in AD skin *in vivo*[40,41,59]. Here we determined that the altered exosomal/sEV FA composition in our model of filaggrin-insufficient keratinocytes is a likely consequence of a reduction in expression of the enzymes in the long-chain fatty acyl-CoA ligase family (ACSLs). ACSLs are enzymes upstream of several critical cellular lipid metabolism pathways[43] catalysing the process of fatty acid activation, and formation of fatty acyl-CoA esters which regulate diverse cellular functions, for example providing gene regulation, enzyme inhibition, modulation of ion channel function, and membrane fusion[42]. ACSLs are implicated in membrane phospholipid biosynthesis; their involvement in the process of incorporation of MUFA and PUFA species into membrane phospholipids was previously described for multiple ACSLs[44,60,61]*;* they also have a preference towards polyunsaturated fatty acids[44,60–62]. An increase in saturated fatty acids and a decrease in polyunsaturated fatty acid content has been described in rat hepatocytes in a ACSL3 knockdown model[60]. As for the manifestations of the allergic march, methylation of the *ACSL3* 5'-CGI has been found to correlate with asthma status in children[63] and reported to increase in an allergen-induced airway hyperreactivity model in mice[64]. Furthermore, methylation of the *ACSL3* gene has also been determined as a signature predictive of clinical food allergy in children[65]. Interestingly, this enzyme was also found in exosomes/sEVs isolated from colostrum but not from mature breast milk[66]; in our study, it was not detected in keratinocyte-derived exosomes/sEVs, but it could result from the detection threshold. ACSL downregulation under filaggrin insufficiency background has important immunological consequences; we show that the lipid content in secreted exosomes/sEVs is affected to the extent which abolishes their capacity to provide substrates for generation of the CD1a permissive self-antigens by PLA2; these provide homeostatic T cell activation, contributing to tissue integrity. It has been previously determined that the optimal length of the lipid chain appropriate for accommodation within the CD1a groove is approximately 20 carbon atoms and that unsaturated lipids induce a superior response[33]. Interestingly, when we compared responses obtained from the selected lipids found within the sEVs it was not always the case, i.e., while we could see the highest level of responses to the polyunsaturated long C22:6 DHA,

only some donors responded to this lipid; responses to C14:0 SFA were lower, but more prevalent, while responses to Lyso-PC18:0 were less persistent over time.

It has been suggested that the family of the elongation of very long (ELOVL) fatty acid enzymes, which controls the length of very long fatty acids may be involved in the generation of the long-chain sphingomyelin such as 42:2. While there was no differential expression in our in vitro dataset, we and others have identified decrease of *ELOVL* mRNA in AD skin[59]. The upregulation of FADS1, which we believe may be a secondary compensatory mechanism[67], was the only additional finding relevant to this pathway in the cultured keratinocytes. In contrast, mRNA for several FADS enzymes were downregulated in the AD skin (but not in organotypic model); this may suggest more complex regulation where inflammatory *milieu* may play an important role.

While we did not find any changes in the sphingomyelin synthesis pathway per se, studies focusing on the loss of the ACSL activity provide additional insight. Specifically, ACSL has been shown to regulate composition of fatty acids and membrane lipids in lipid rafts[68], by the effect on ceramide expression, e.g., silencing of the enzyme results in the accumulation of ceramides and sphingomyelin analogue in Drosphila (phosphoethanolamine ceramide; CerPE)[68,69]. Therefore, while the expression of the enzymes in the pathway of sphingolipid synthesis may not be directly affected by filaggrin insufficiency, the increased supply of the substrates channelled into the ceramide/sphingolipid synthesis pathway is a very likely explanation of the accumulation of the non-permissive sphingomyelins[70].

Skin is enriched in $CD1a^+$ Langerhans cells abundant in the epidermis[71,72]; in addition, CD1a is also inducibly expressed by dendritic cell populations deeper in the tissue[73,74]. Our findings bear high relevance to the immunological events and tissue integrity[75], since the CD1a-restricted population has been shown to contain many autoreactive T cells, capable of sensing barrier damage and promoting mechanisms engaged in tissue repair[36]. CD1a-resticted responses also contribute to the control of pathogenic skin bacteria[76] and there seems to be an indication of their importance also in the lungs and gut[77,78] where CD1a-expressing cells are also found[79–85]. To this end, CD1a-restricted responses have been shown in the humanised model of *M. tuberculosis* infection[86] and to a range of *M. tuberculosis* lipopeptide (DMM) isomers[87]. Our study determined that neoantigens derived from normal keratinocytes (filaggrin sufficient; replicated by $shC_{sEV}$ in our study) are likely to be CD1a permissive ligands promoting autoreactive responses; their provision may support homeostasis at the skin barrier site or potentially even play an adjuvant-like role in antimicrobial immunity[88]. In contrast, exosomes/sEVs secreted on the filaggrin insufficiency background, containing altered lipid content can inhibit type 1 T cell

responses and promote type 2 bias. Given the preference of the CD1a molecule to bind high affinity inhibitory ligands[38], such as those contained within the sEVs produced by filaggrin insufficient keratinocytes, their presence in the *milieu* would likely affect both the low-level homeostatic and the much more pronounced antimicrobial CD1a-mediated T cell responses.

In contrast, our data indicate that in the absence of PLA2, exosomes/sEVs do not drive marked T cell reactivity, therefore reducing the risk of inflammation in the absence of an external threat. It is important to note that pathogens may constitute [49,50,89] a source of the phospholipase A2 activity, either directly[90–92] or indirectly[76,93,94]. At the same time normal keratinocyte-derived exosomes/sEVs could potentially quench the toxic impact of PLA2 on cellular membranes protecting the body from excess tissue damage during inflammation. Exosomes/sEVs could also shield commensal bacteria which seem to be more susceptible to PLA2 than pathogenic strains[95]. A causative role of dysbiosis[96,97], and chronic inflammation preceding the development or exacerbations in allergic asthma[98,99], intestinal tissue damage [100,101] and food allergy[102], affecting the development of tolerance to the encountered allergens[103] has been previously established. Given that keratinocyte-derived exosomes/sEVs transfer into the circulation and are delivered into peripheral tissues, the impact of filaggrin insufficient keratinocyte-derived sEVs could extend beyond the local tissue environment, affecting responses in the locations distant from the skin and contributing to the development of allergic manifestations in those body sites, by reducing pathogen-directed and regeneration-promoting responses and promoting chronic inflammation and Th2 bias.

In summary, we have shown that small secreted extracellular vesicles constitute a source of antigens for lipid presentation pathways and are active during CD1a-mediated T cell responses. We also established that these responses greatly depend on the filaggrin status of secreting keratinocytes and can be linked to the dysregulation of lipid pathways including reduced ACSL activity in those cells, resulting from aberrant differentiation that is apparent both *in vitro* and in AD skin biopsies. A decrease in provision of the response eliciting CD1a self-antigens and enhanced supply of inhibitory ligands support immune consequences such as persistent allergic inflammation and dysbiosis in the skin; it appears probable that similar mechanisms operate in additional tissue locations to which sEVs can be transferred within the systemic circulation (such as the lungs and gut), contributing to the progression of the “allergic march” at these distant body sites.

## Materials and Methods

### *Samples*

Ethical approval for the study was obtained from the Independent Bioethics Committee for Scientific Research at Medical University of Gdańsk, ethical approval numbers: NKBBN/558/2017-2018 and NKBBN/621-574/2020. Buffy coats were obtained from blood donations from healthy donors the Regional Blood Centre in Gdansk.

### *Cell culture and media*

ShC and shFLG HaCaT keratinocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM-high glucose, Sigma-Aldrich) with 10% FBS (Sigma-Aldrich), 2mM L-Glutamine (Sigma-Aldrich) and 1% Pen/Strep (Sigma-Aldrich). K562-CD1a cells (a kind gift from Prof. Branch Moody) were cultured in RPMI-1640 (Sigma-Aldrich) with addition of 200 µg/ml G418 (Thermo Fisher Scientific), 1% Pen/Strep (Sigma-Aldrich) and 10% heat-inactivated FBS (Sigma-Aldrich) and cultured at $37^0$C, 5% $CO_2$. For EV isolation media contained exosome/sEV depleted FBS; treatments were carried out when the cells reached 80-90% confluence. T cell medium was prepared by supplementation of RPMI-1640 (Sigma-Aldrich) with 5% human male heat-inactivated AB serum (Sigma-Aldrich), 1% Pen/Strep (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 2 mM L-Glutamine (Sigma-Aldrich) 1% non-essential amino acids (Biowest), 50 µM 2-mercaptoethanol (Sigma-Aldrich) and 10 ng/ml IL-2 (PeproTech).

### *Flow cytometry*

Cells were washed, stained with fluorophore-conjugated antibodies for 30 min at 4°C, washed in PBS fixed in 4% formaldehyde (Sigma-Aldrich). Samples were acquired Guava easyCyte (Millipore) and data was analysed with GuavaSoft 3.1.1. Antibodies were from BioLegend: CD14-APC, CD40-FITC, CD80-PE, CD86-PE, CD1a-APC at 1:200 dilution or BD Biosciences: HLA-DR-PE at 1:200 dilution. The catalogue numbers are specified in table S6.

***Western blot***

Cells were lysed in RIPA buffer (Cell Signalling Technologies) supplemented with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche), centrifuged for 15 min at 4°C, 13,000 x g and supernatant was collected. The lysates or EV samples were heated in Bolt™ LDS Sample Buffer (Invitrogen) for 10 min at 80°C and run on Bolt™ 4-12% Bis-Tris Plus Gels (Invitrogen) in the Mini Gel Tank (Life Technologies) connected to the PowerEase™ 300W Power Supply (Life Technologies). The proteins were transferred onto nitrocellulose membranes (iBlot™ 2 Transfer stack; iBlot 2 Dry Blotting System, Invitrogen) and the membranes were blocked in 5% fat-removed milk in PBS. Primary antibody incubations were carried out at 4°C on shaker overnight and secondary antibody IRDye® 800CW or IRDye® 680RD (LI-COR Biosciences, Lincoln, NE, USA) (dilution 1:25,000 in PBS with 0.05% Tween 20) for 30 min at RT. Catalogue numbers of antibodies are specified in table S6. The membranes were scanned and analysed using Odyssey Clx Imaging System (LI-COR Biosciences).

***mRNA microarray***

ShC and shFLG cells were left untreated or subjected to IL-4/IL-13 combination or IFNγ (all cytokines from Peprotech, treatments at 50 ng/ml). After 24h RNA was extracted with RNeasy Plus kit (Qiagen) according to manufacturer's instructions and the microarray was performed by Service XS (Holland) on a HT12 BeadArray platform (Illumina). The data were normalized using lumi[104] and analysed with LIMMA[105.] The data were submitted to Gene Expression Omnibus (GSE203409).

***Monocyte derived dendritic cells (moDCs) generation and exosome/sEV treatment***

CD14+ cells were isolated magnetically from PBMCs using MojoSort™ Human CD14 Selection Kit (BioLegend) according to the manufacturer's protocol. Cells were grown in 24-well plates (Corning) in RPMI-1640 medium (Sigma-Aldrich) supplemented with 1% Pen/Strep (Sigma-Aldrich), 10% heat-inactivated FBS (Sigma-Aldrich) (complete RPMI) and 50 ng/ml GM-CSF and 1,000 U/ml IL-4 (PeproTech). On day 2 and day 4 of the culture, the medium was replaced with fresh complete RPMI and cytokines and the cells were harvested on day 7. For the generation of mature moDCs LPS (Sigma-Aldrich) was added at 1 μg/ml on day 6. moDCs were incubated with 10μg/ml of exosome/sEVs measured by protein concentration on NanoDrop 2000 (Thermo Fisher Scientific) overnight and their marker expression was analysed by flow cytometry.

***EV isolation, purification and characterisation***

Exosome/sEV-free media were used throughout and the protocol followed a scheme depicted in Figure 3A. Briefly, conditioned medium (CM) after 72 hours of culture was harvested and centrifuged at 300 x g (Megafuge 16R TX-400 centrifuge, Thermo Fisher Scientific) for 10 min to remove the cells and cell debris, followed by a spin at 2,000 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific) for 10 min to remove insoluble proteins and apoptotic bodies (AP). The supernatant was ultracentrifuged (OptimaTM L-90K or OptimaTM LE-80K ultracentrifuge, Beckman Coulter) at 10,000 x g (AVG) for 30 min to isolate microvesicles (MVs) and the supernatant was ultracentrifuged at 100,000 x g (AVG) for 16 h to pellet exosome-enriched (100K) fraction. If further purification was needed the exosome-enriched pellet was layered on iodixanol/sucrose discontinuous gradient (iodixanol concentration ranging between 6-18%, increments of 1.2%, 1 ml each fraction). The pellet was top-loaded and ultracentrifuged (OptimaTM L-90K or OptimaTM LE-80K ultracentrifuge, Beckman Coulter) at 198,000 x g for 2.5 h (SW 41 Ti rotor, Beckman Coulter). Fractions were collected separately (1 ml) and pooled when required followed by washing in PBS. The top-loaded sample was pooled with the first fraction and considered as fraction 1 (6%+ added sample). Exosomes/sEVs were stored in PBS at -20°C. Quantification and size measurment of exosomes/sEVs was performed by Nanoparticle Tracking Analysis (NTA) using NanoSight NS300 equipped with a 488 nm laser (Malvern Instruments). 3x30s recordings were taken for every sample. Electron microscopy was carried out as a service by Laboratory of Electron Microscopy on Formvar/Carbon film on Copper 300 mesh (EM Resolutions) and samples were imaged on Tecnai G2 Spirit BioTWIN (FEI Inc.) transmission electron microscope.

***ELISpot and T cell culture***

Human IFN-γ ELISpot BASIC kit (ALP) (Mabtech) was used to assess T cell responses. T cells were magnetically selected using the MojoSort™ Human CD3 T Cell Isolation Kit (BioLegend) according to the manufacturer's protocol and rested in complete RPMI overnight. Immature moDCs were harvested, washed and pulsed with exosomes/sEVs isolated from 1 or 2 mln shC or shFLG cells together with the CEFT Pool (JPT Peptide Technologies) at 1 μg/ml per peptide or 10 μg/ml of CMV pp65 protein (ProSpec-Tany TechnoGene Ltd) overnight. For CD1a-dependent T cell responses, K562-CD1a cells were pulsed with 1 μg/ml PLA2 (Sigma-Aldrich) and exosomes/sEVs isolated from 1 or 2 mln shC or shFLG keratinocytes per 50,000 K562-CD1a cells overnight;

alternatively, K562-CD1a cells were incubated with equivalent amounts of PLA2-digested exosomes/sEVs. For single lipid ELISpot, K562-CD1a cells were pulsed with 10 µM of myristic acid (C14:0; Sigma-Aldrich), docosahexaenoic acid (C22:6; Sigma-Aldrich) or Lyso-PC18:0 (Cayman Chemical Company). Cells were seeded on the pre-coated plate (20,000 immature moDC or 25,000 K562-CD1a per 100,000 T cells) and incubated overnight at 37°C, 5% $CO_2$. After ELISpot cells were harvested after ELISpot and cultured for 13 days in T cell medium with media change every 2-3 days. Then, the cells were rested in complete RPMI and incubated on an ELISpot plate with K562-CD1a cells pulsed with single lipids as described above. For negative control unstimulated T cells were used and 150 ng/ml PMA (Sigma-Aldrich) and 75 ng/ml ionomycin (Sigma-Aldrich) was added to T cells for the positive control. After overnight incubation supernatants were harvested and stored at -80°C for downstream assays. The plate was developed with the AP Conjugate Substrate Kit (Bio-Rad) according to manufacturer's protocol and read using Mabtech IRIS™ reader (Mabtech) or AID reader (Autimmun Diagnostika GmbH).

***ELISA***

IL-10 in cell culture supernatants was measured using the ELISA MAX™ Standard Set Human IL-10 (BioLegend) or Human IL-10 ELISA Set (Diaclone) according to manufacturer's instructions using ELISA Coating Buffer (BioLegend). IL-13 in cell culture supernatants was measured using the Human IL-13 ELISA development kit (HRP) (Mabtech) or Human IL-13 DuoSet ELISA (R&D Systems) according to manufacturer's instructions. For IL-17A measurement Human IL-17A ELISA development kit (HRP) (Mabtech) was used according to manufacturer's instructions. Nunc-Immuno™ MicroWell™ 96 well plates (Sigma-Aldrich) were used. Plates were developed at using the TMB Substrate Set (BioLegend) with $H_2SO_4$ added to stop the reaction. Absorbances were read at 450 nm and 570 nm wavelengths using the Epoch 2 Microplate Spectrophotometer (BioTek) or the Asys UVM340 microplate spectrophotometer (Biochrom). The absorbances at 570 nm were subtracted from those at 450 nm and the concentrations were calculated based on the standard curve equations.

***cPLA activity and PLA2 cell-free digestion***

Calcium-dependent cytosolic phospholipase A2 (cPLA2) content in cell lysates and exosomes/sEVs was assessed by measuring the enzyme's activity towards a synthetic substrate, arachidonoyl thio-PC with the cPLA2 Assay Kit (Cayman Chemical), according to the

manufacturer's instructions. Supernatants obtained after centrifugation (14,000 x g, 10 min., 4°C) of lysed samples were tested in duplicate, and the reaction mixture was incubated for 5 min as well as overnight. Absorbance was measured at 414 nm and 405 nm. For the cell-free digestion cPLA Assay Buffer, a component of the cPLA2 Assay Kit (Cayman Chemical) was used, diluted in PBS according to the manufacturer's protocol; $Ca^{2+}$ concentration was adjusted to 20 mM with $CaCl_2$. Exosomes/sEVs and 1 µg/ml active or heat-inactivated (95°C, 15 min.) PLA2 (Sigma-Aldrich) were added. After 1 h incubation the samples were stored at -20°C.

***Protein mass spectrometry***

Cells and exosomes/sEVs were lysed with 1% SDS; beforehand exosomes/sEVs were purified by gradient to remove contaminating protein aggregates, incl. keratohyalin granules, potentially found in the conditioned media separately to EVs if released from dying cells. Samples were prepared for the mass spectrometry analysis in a Filter Aided Sample Preparation (FASP) procedure[106] with cysteine alkylation by iodoacetamide and proteolytic digestion by trypsin. Obtained digests were desalted by the STAGE Tips[107] procedure on a C18 resin. LC-MS/MS analysis was conducted on a Triple TOF 5600+ mass spectrometer (SCIEX) coupled with an Ekspert MicroLC 200 Plus System (Eksigent). All samples were measured in the data-dependent acquisition mode for the spectral library construction and by the SWATH-MS[108] method in triplicate for relative quantification. Separate spectral libraries for the cell and exosome/sEV samples were built by database search carried out in ProteinPilot 4.5 software (SCIEX) against a SwissProt Homo sapiens database (version from 02.07.2020). SWATH-MS measurements were processed with respective libraries in the PeakView 2.2. software. Resulting protein intensities were normalized by total area sums (TAS) approach and imported into the Perseus software[109], where the technical replicates were median-averaged, and the resulting values were log2-transformed and normalized by z-score. A t-test between the test and control groups was conducted, and the results with FDR-adjusted p-value lower than 0.05 were considered to be statistically significant. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026859[110].

***Lipid mass spectrometry***

Samples were extracted in a cold chloroform/methanol mixture (1/2, v/v), followed by the addition of chloroform and deionized water to separate the aqueous and organic phases. The bottom layer derived from cell extract was used directly in the LC-MS analysis; the exosome/sEV lipid extract was dried and dissolved in methanol. The acquisition was performed on Agilent 1290 LC system coupled to 6540 Q-TOF–MS (Jet Stream Technology, Agilent Technologies). Lipid separation was achieved by reversed-phase column (Poroshell 120 EC-C8; Agilent InfinityLab; Agilent Technologies); the column was maintained at 60°C. The two most abundant peaks obtained were selected for fragmentation. Lipidomic data were processed on the Agilent MassHunter Workstation Profinder 10.0 (Agilent Technologies) using the Molecular Feature Extraction (MFE) algorithm following with the Targeted Molecular Feature Extraction; data alignment and filtration was carried out on Mass Profiler Professional 15.1 software (Agilent Technologies); missing values were exported as missing. Filtration was based on the frequency (the MFs remained in the dataset if they were present in 80% of the samples in at least one specified group) and the QC %RSD. The MFs that were present in the extraction blank with the average peak volume higher than 10% of the average peak volume in the real samples were removed. Further statistical analysis was conducted using MetaboAnalyst5.0 (https://www.metaboanalyst.ca/home.xhtml), reporting adjusted p-value threshold <0.05 (unpaired t-test, unequal variance, Benjamini-Hochberg FDR correction); missing values were replaced with 1/5 of the minimum positive value of each variable if not detected only in one sample group, or by the mean peak area of the compound in a group of samples if not detected in only one of 4 biological replicates or incorrectly integrated by the software. Levels of individual lipid species were normalized to the total amount of the corresponding lipid class. The Euclidean distance algorithm and Ward clustering algorithm were used for the heatmap. The data (relative amounts of lipids within a class) were log-transformed (base10) for a heatmap and log-transformed and autoscaled (mean-centered and divided by the standard deviation of each variable) for PLS-DA analysis. Lipid identification was carried out by a search in custom lipid database of theoretical lipid structures, based on an accurately measured m/z value (Δ5 ppm tolerance), followed by manual interpretation of the obtained MS/MS spectra.

***Functional enrichment and Gene Ontology analysis***

Cellular compartment enrichment analysis of the omics datasets was performed using FunRich 3.1.3 software. The Vesiclepedia[25] database available within the software was used to

investigate the association of proteins/gene products identified in the omics studies with exosomes/sEVs. Gene ontology (GO) and Reactome pathways analysis were carried out via the Gene Ontology tool, available at http://geneontology.org/. Complete GO annotation datasets were chosen. For GO analysis in Figures 1 and 2 top 20 GO terms and Reactome pathways for every dataset were selected based on the lowest FDR values and the number of genes identified within every top 20 term were added together as total for subsequent pie chart analysis. At the time of analysis the latest update for GO database was on 2021-02-01 and Reactome database on 2020-11-17.

***Statistical analysis***

The one-way analysis of variance (ANOVA) tests with indicated correction methods were performed using GraphPad Prism v.7.04 or newer (GraphPad Software). Error bars represent SEM as indicated.

## Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026859. mRNA microarray data were submitted to Gene Expression Omnibus (GSE203409).

## Conflict of Interests

GSO has served on advisory boards or holds consultancies or research grants with Eli Lilly, Novartis, Janssen, BMS and UCB Pharma, Regeneron/Sanofi, Roche, Anaptysbio. GSO has patent filed in the CD1a field. SJB holds or has recently held research grants from the Wellcome Trust, British Skin Foundation, EU/IMI H2020 'BIOMAP', European Lead Factory, Charles Wolfson Charitable Trust, Rosetrees Trust, Stoneygate Trust, Pfizer, and consultancies with Abbvie, Sosei Heptares and Janssen. SG has a patent on B-cell targeting of EVs and is scientific advisor of Anjarium Biosciences.

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## Ethics Approvals

Ethical approval for the study was obtained from the Independent Bioethics Committee for Scientific Research at Medical University of Gdańsk, ethical approval numbers: NKBBN/558/2017-2018 and NKBBN/621-574/2020. Buffy coats were obtained from blood donations from healthy donors the Regional Blood Centre in Gdansk.

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## Author contributions

AK, WHB performed the experiments, analysed the data and contributed to the writing and figure preparation, JEF, LH, AB performed the experiments and analysed the data. NK, RE, AP, ID performed the experiments. SG, SJB, GSO interpreted the data. DGO provided the funding,

planned the study and performed the experiments, analysed the data, wrote the first and subsequent paper drafts. All authors contributed to the article and approved the submitted version.

## Geolocation Information

54.39318849434289, 18.578389264424995

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## Supplementary figures

### siFLG organotypic skin model

| Analysis type | Term | Fold enrichment | FDR |
|---|---|---|---|
| Gene Ontology: Biological Process | Antigen processing and presentation (GO:0019882) | 2.16 | $4.04*10^{-2}$ |
| | Antigen processing and presentation of peptide antigen via MHC class I (GO:0002474) | 2.81 | $4.76*10^{-2}$ |
| Reactome Pathways | Class I MHC mediated antigen processing & presentation (R-HSA-983169) | 2.06 | $2.75*10^{-3}$ |
| | Antigen processing-Cross presentation (R-HSA-1236975) | 3.24 | $3.37*10^{-3}$ |

**Figure S1. Enrichment in the Gene Ontology and Reactome terms associated with antigen presentation identified within the siFLG organotypic skin model.** Proteome dataset analysed by Panther. FDR, false discovery rate.

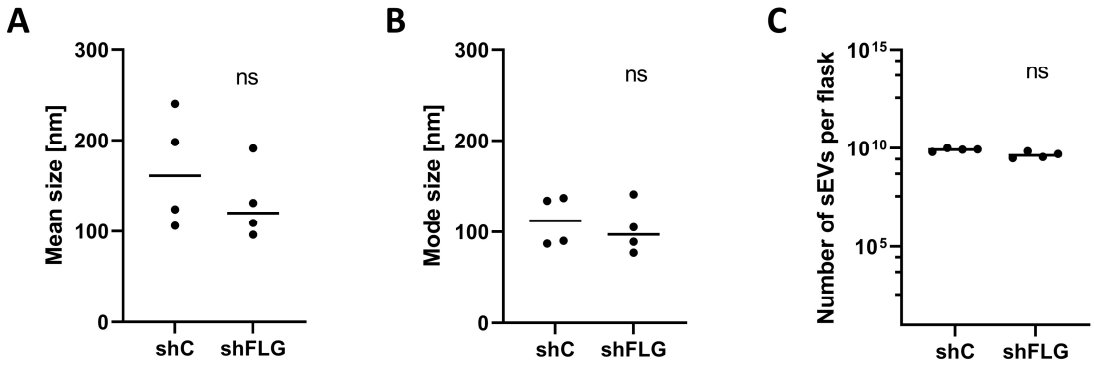


**Figure S2. Filaggrin insufficiency does not affect the size of sEVs or their production by keratinocytes.** (**A** and **B**) Size of keratinocyte-derived sEVs expressed as (A) mean and (B) mode measured by Nanoparticle Tracking Analysis (NTA); (**C**) Number of sEVs produced by keratinocytes per T75 cell culture flask measured by NTA. Combined data for n=4 biological replicates, paired t-test.

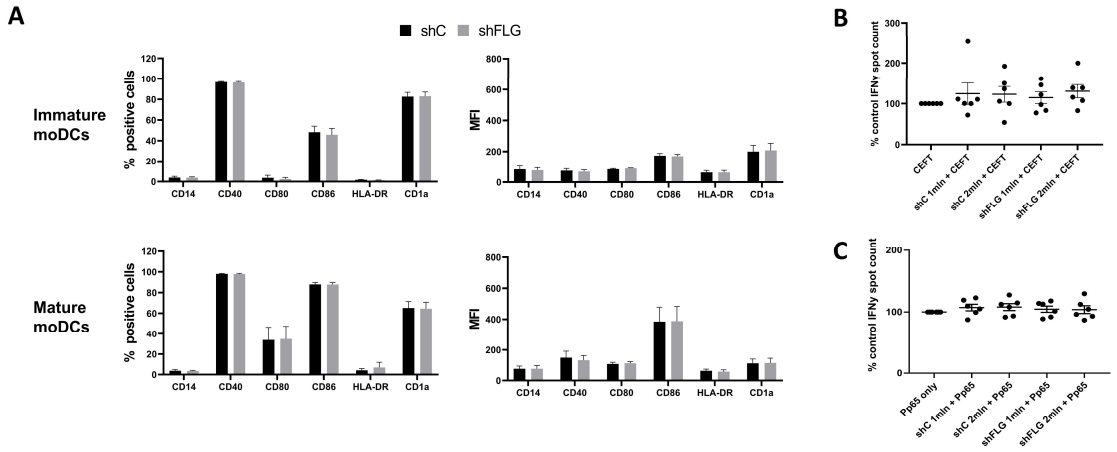


**Figure S3. sEVs secreted by keratinocytes do not impact T cell responses to peptide antigens.** (**A**) Expression of dendritic cell markers by immature and mature moDCs treated with keratinocyte-derived sEVs (means +/- SEM are shown; combined data from n=5 donors; one-way ANOVA with Šídák's multiple comparisons test; (**B** to **C**) IFNy production by T cells stimulated with immature moDCs pulsed with sEVs derived from 1 or 2 million keratinocytes and (B) CEFT peptides at 1 µg/ml per peptide or (C) 10 µg/ml of whole pp65 protein overnight measured by ELISpot assay (means +/- SEM are shown; data are normalized to control; data from n=6 donors; one-way ANOVA with Šídák's multiple comparisons test; moDC, monocyte-derived dendritic cell; MFI, mean fluorescence intensity.

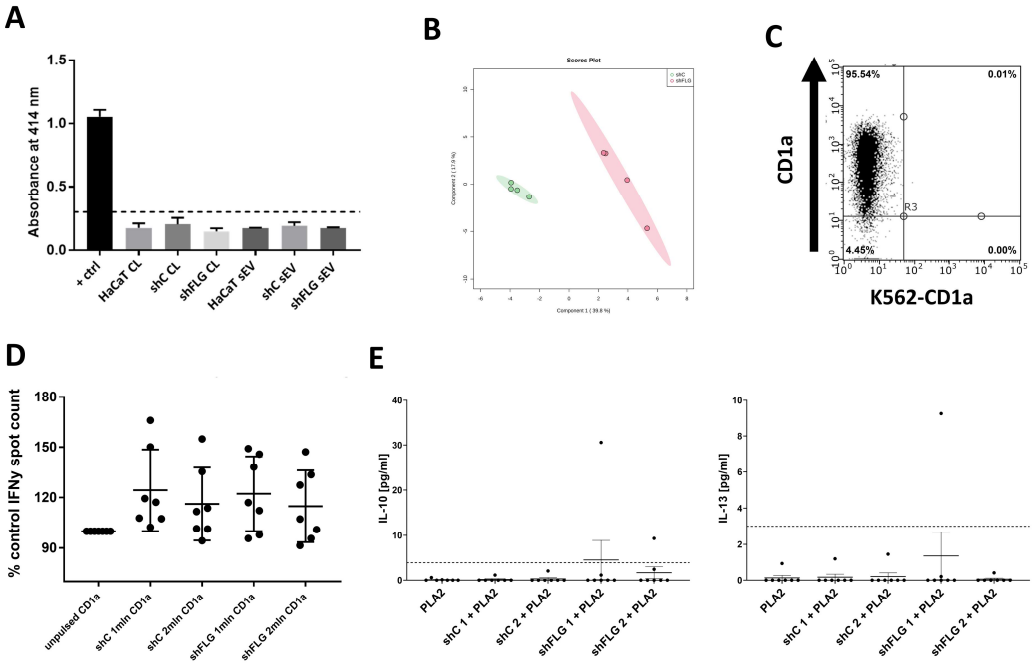


**Figure S4. Keratinocyte-derived sEVs are not a source of readily available CD1a antigens.** (**A**) cPLA2 activity measurement in keratinocytes and sEVs by a colorimetric assay (normalized to control; means from n=3 technical replicates; +/- SEM are shown; dashed line marks the limit of detection; (**B**) PLS-DA analysis of PLA2-digestible lipidome of sEVs; n=4 biological replicates; (**C**) CD1a expression by K562 cells CD1a transfectants; example flow cytometry data shown; (**D**) IFNy

responses from T cells stimulated with K562-CD1a cells pulsed with sEVs derived from 1 or 2 million keratinocytes overnight (means +/- SEM are shown; data are normalized to control=100%; n=7 donors; one-way ANOVA with Šídák's multiple comparisons test; (**E**) Production of IL-10 and IL-13 by T cells stimulated with K562-CD1a cells pulsed with sEVs from 1 or 2 million keratinocytes and 1 µg/ml PLA2 measured by ELISA; means +/- SEM are shown; n=7 donors; one-way ANOVA with Šídák's multiple comparisons test; dotted line marks the limit of detection; CL, cell lysate.

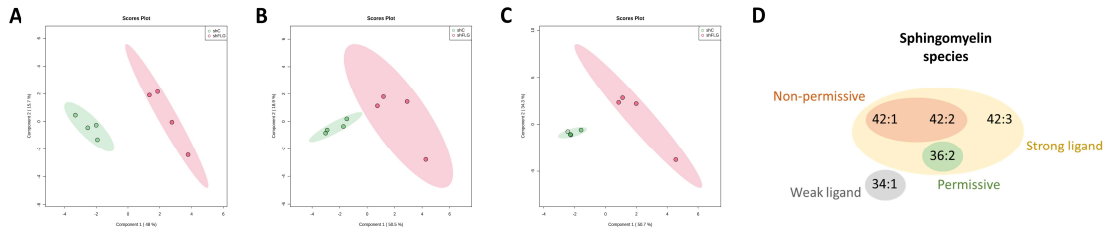


**Figure S5. Filaggrin insufficiency affects both PLA2-digestible and -indigestible lipidome of keratinocyte-derived sEVs.** (**A** and **C**) Score plot between PC1 and PC2 obtained from PLS-DA analysis of (A) glycerophosphocholine-related products of sEV digestion with 1 µg/ml PLA2 for 1h, (B) PLA2-non-digestible lipids in sEVs and (C) PLA2-non-digestible lipids in sEVs digested with 1 µg/ml PLA2 for 1h. (**D**) Characteristics of sphingomyelin species identified as potential CD1a antigens by Cotton *et al.*[38].

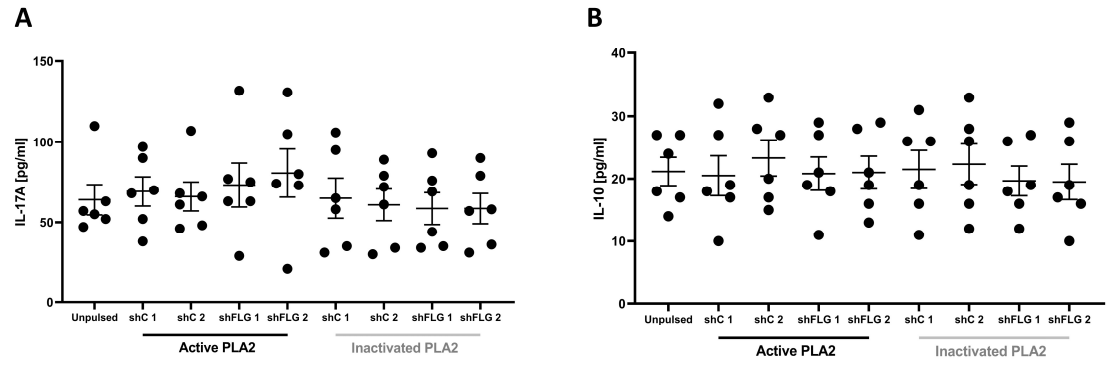


**Figure S6. PLA2-digested sEVs do not alter IL-10 or IL-17A production by T cells regardless of the keratinocyte filaggrin status.** (**A** and **B**) Production of (A) IL-17A and (B) IL-10 by T cells stimulated with K562-CD1a cells pulsed overnight with sEVs from 1 or 2 million keratinocytes digested with 1 µg/ml PLA2 for 1h (means +/- SEM shown, n=6 donors, one-way ANOVA with Šídák's multiple comparisons test).

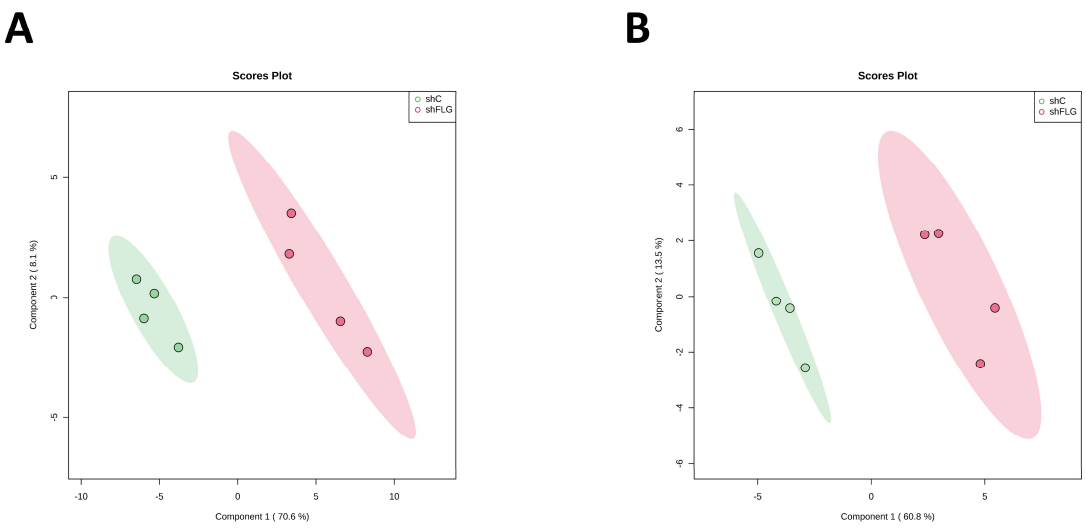


**Fig. S7. Keratinocyte lipid composition is impacted by filaggrin insufficiency.** (**A** and **B**) Score plot between PC1 and PC2 obtained from PLS-DA analysis of keratinocyte lipidome; (A) PLA2-digestible and (B) -non-digestible lipid species.

## Additional supplementary material:

**Table S1.** Gene Ontology and Reactome terms for genes differentially expressed in shFLG keratinocytes with relevance to the proteins identified in the sEV/exosomal compartment by FunRich.

**Table S2.** Gene Ontology and Reactome analysis of proteins differentially expressed in shFLG keratinocytes identified in the sEV/exosomal compartment by FunRich.

**Table S3.** Gene Ontology and Reactome analysis of proteins differentially expressed in siFLG organotypic skin models identified in the sEV/exosomal compartment by FunRich.

**Table S4.** Gene Ontology and Reactome analysis of the genes differentially expressed in AD skin which have relevance to the proteins identified in the sEV/exosomal compartment by FunRich.

**Table S5.** Proteins differentially expressed in shFLG keratinocytes.

**Table S6.** List of antibodies used in the study.

**3.3.** Hovhannisyan L.*, **Kobiela A.***, Bernardino de la Serna J., Bogucka A. E., Deptuła M., Paul A. A., Panek K., Czechowska E., Rychłowski M., Królicka A., Zieliński J., Gabrielsson S., Pikuła M., Trzeciak M., Ogg G. S., Gutowska-Owsiak D. Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion. **In revision in *The Journal of Extracellular Vesicles* (2022).** * joined first authors.

***Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by Staphylococcus aureus in a TLR2-dependent fashion***

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## Abstract

Filaggrin (FLG) protein is indispensable for multiple aspects of the epidermal barrier function but its accumulation in a monomeric filaggrin form may initiate premature keratinocytes death; it is unclear how filaggrin levels are controlled before the formation of storing keratohyalin granules. Here we show that keratinocyte-secreted small extracellular vesicles (sEVs) may contain filaggrin-related cargo providing a route of eliminating excess filaggrin from keratinocytes. Filaggrin-containing sEVs are found in plasma in both healthy individuals and atopic dermatitis patients. *Staphylococcus aureus (S. aureus)* enhances packaging and secretion of filaggrin-relevant products within the sEVs for enhanced export via a TLR2-mediated mechanism which is also linked to the ubiquitination process. This filaggrin removal system, preventing premature keratinocyte death and epidermal barrier dysfunction, is exploited by *S. aureus* which promotes filaggrin elimination from the skin that could help safeguard bacterial growth.

Keywords: extracellular vesicles, exosomes, filaggrin, keratinocyte, atopic dermatitis, *Staphylococcus aureus,* bacteria

## Introduction

A functional epidermal barrier is critical for survival as it protects an organism from the environmental impact. The process of its formation is complex and depends on complete keratinocyte differentiation promoting acquisition of unique cellular functions by these cells and eventual cell death by cornification, leading to *stratum corneum* formation[1]. While multiple mechanisms are involved [2], one remarkable protein, filaggrin (FLG)[3] proved paramount. Specifically, the highest inherited risk for atopic dermatitis (AD), a disease characterised by a pronounced defect in the epidermal barrier function has been shown to be conferred by *FLG* mutation(s)[4]. Several barrier-supporting functions of the protein have been documented, from the mechanical strengthening of the corneocytes through to effect on immunity[5–7] and indirect antimicrobial activity[8]. Filaggrin knockdown models show deep abnormality in keratinocyte differentiation process, including the effect on multiple metabolic pathways[9,10] and enzymatic activity[10] corresponding to the findings from AD skin. The profound role of filaggrin becomes especially apparent upon disintegration of keratohyalin granules (KHGs) which serve as intracellular storage for profilaggrin (PFLG); this results in a sharp increase in the cytosolic content of filaggrin monomer. Accumulation of monomeric filaggrin in the cell is followed by collapse of keratin-based intermediate filaments (IFs), disruption of cellular junctions and initiation of programmed cell death [11]. While those effects are in line with the physiological filaggrin function, given the irreversibility of this process, filaggrin containment and release must be tightly controlled to avoid premature death of keratinocytes and failure of stratification[11]. We have previously described a mechanism involving KHG-associated actin scaffolds controlling filaggrin sequestration and its coordinated release in terminally differentiated keratinocytes[12]; the process requires prior activation of an independent differentiation-linked AKT1-HspB1 pathway[13]. Recently, a complementary mechanism, describing KHGs as biomolecular condensates formed by liquid phase separation has been also proposed[14]. Here we show evidence of low dispersed cytoplasmic level of profilaggrin/filaggrin in keratinocytes before the appearance of KHGs, both in a 2D model and in epidermal sheets. We further demonstrate that filaggrin-related products are loaded as cargo into exosome-enriched keratinocyte-derived small extracellular vesicles (sEVs). This mechanism functions throughout differentiation *in vitro* as well as in the skin *in vivo,* as evidenced by the accumulation of filaggrin-containing sEVs in the peripheral blood, also in AD patients.

*Staphylococcus aureus* (*S. aureus*) is a pathogen frequently colonising the atopic skin and it is linked with clinical deterioration; the bacteria take advantage of the skin barrier defect to promote its growth and entry through the tissue[15]. We show that S. aureus causes dysregulation

of sEV production in keratinocytes and enhanced inclusion of the filaggrin dimer into the cargo for sEV of exosomal characteristics. Using protein interaction modelling and experimental confirmation we determined that the mechanism is TLR2-dependent.

We provide evidence on the existence of a sEV-dependent mechanism regulating levels of cytosolic free filaggrin which escaped KHG sequestration; a mechanism is likely critical to support keratinocyte homeostasis and ensuring full stratification, indispensable for adequate epidermal barrier function. *S. aureus*, growth of which is regulated by the filaggrin breakdown products, seems to exploit this pathway by enhancing profilaggrin processing and product loading into sEVs to accelerate filaggrin removal from the skin.

## Results

*Keratinocytes express low levels of cytoplasmic filaggrin early during differentiation*

Filaggrin expression in keratinocytes increases significantly during differentiation as the result of the positive feedback loop initiated by nuclear signalling of the profilaggrin N-terminal domain [1617]. As such, the expression pattern is expected to follow the model of expression dynamics for proteins undergoing positive autoregulation[18], with synthesis gradually increasing. There is evidence that a substantial local filaggrin concentration threshold must be reached[14] before KHGs may form; before this happens, however, transcriptional bursting[19] would give rise to low levels of RNA expression. Accordingly, *FLG* mRNA transcript can be detected already in basal epidermal keratinocytes (Protein Atlas[20–22]; Figure 1a; Figure S1)[23]. The stochastic nature of transcriptional bursting suggests that this may be possible also in cells other than keratinocytes, e.g., fibroblasts, immune cells and cell lines of a diverse origin; filaggrin transcript can be detected in those too, albeit at low levels (Figure S1). Local protein levels can be much higher in keratinocytes, however, and reach the threshold for KHG formation; we hypothesise that initially, before the threshold is met, unsequestered profilaggrin molecules escape into the cytosol, where they may undergo processing and IF binding.

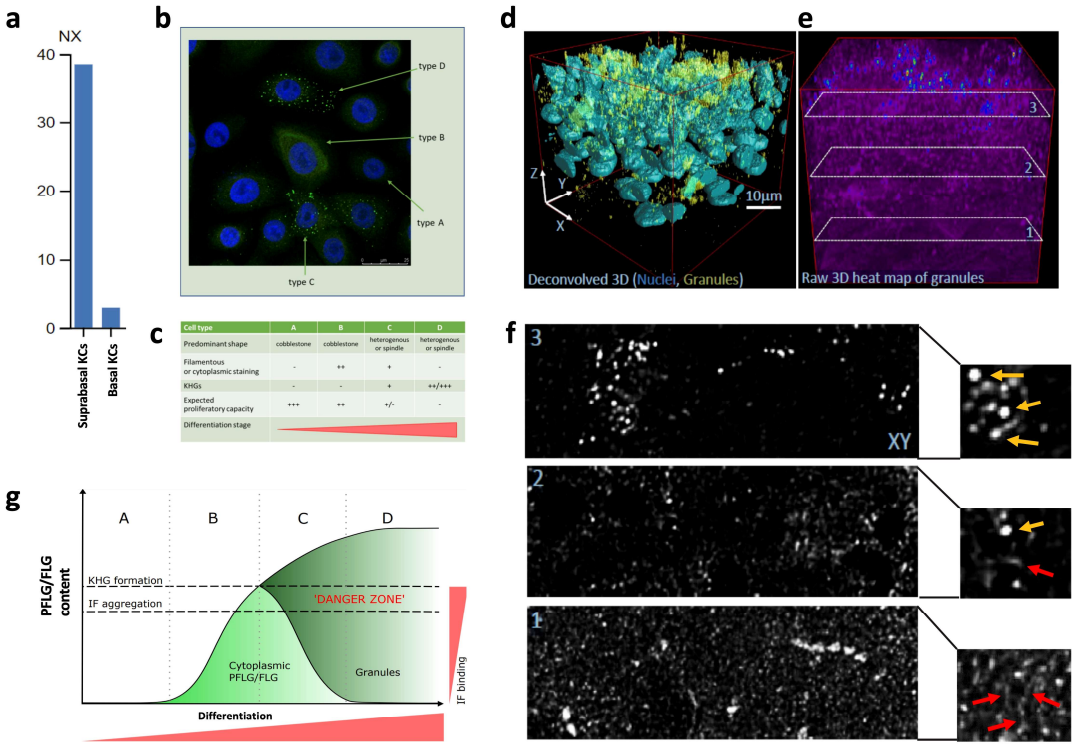


**Figure 1. Low levels of cytoplasmic filaggrin are expressed in keratinocytes during early differentiation, before appearance of keratohyalin granules. a)** filaggrin mRNA expression in primary keratinocytes (KCs), data extracted from ProteinAtlas[20–22]; **b)** example immunofluorescence image of filaggrin protein expression patterns in primary keratinocytes (n=3); nucleus: blue; filaggrin: green **c)** comparison of primary keratinocyte features and filaggrin expression by cells at various differentiation stages; **d)-e)** representative image of whole epidermal sheets (n=5); **d)** deconvolved image, indicating KHG accumulation; **e-f)** representative cross-section of epidermal sheet (n=6) shown in d); **f)** close-up panels indicating filamentous staining suggestive of IFs (red arrows) predominantly in the lower layers and granular pattern of staining indicating KHGs (yellow arrows), enriched in the upper layers; **g)** proposed integrated model of progression of filaggrin expression pattern during keratinocyte differentiation.

To investigate this, we assessed profilaggrin/filaggrin expression in normal human epidermal keratinocytes (NHEKs); depending on the differentiation advancement these cells showed a continuum between cytoplasmic/filamentous and granular filaggrin staining distribution (Figure 1b-c). Specifically, we found proliferatory NHEKs of a cobblestone morphology either negative or showing low/medium intensity of cytoplasmic or filamentous staining and no KHG expression. In contrast, more differentiated keratinocytes showed a degree of “dot positivity” indicative of KHG formation but had either very little or no cytoplasmic/filamentous staining. Finally, we also inspected stratified epidermis and observed low level of cytoplasmic/filamentous staining in the

lower cell layers and complete disappearance of such signal upon the appearance of KHGs (Figure 1d-f, Figure S2).

Based on those results, we formulated a new model of filaggrin expression dynamics integrating the findings (Figure 1g). We propose that the diffusion of the nascent filaggrin into the cytoplasm at early stages of keratinocyte differentiation results in certain tolerated level of protein binding to IFs (which agrees with previously published results by Presland *et al.*[11]) but this ceases upon local concentration reaching the threshold for KHG formation, which results from the positive feedback-driven intensification of filaggrin expression.

*Profilaggrin/filaggrin segregate into small EVs secreted by keratinocytes*

While the proposed model could explain the variable filaggrin expression patterns in NHEKs, it is unclear how the cells withstand free filaggrin during the process of expression escalation and before the KHG formation ("danger zone"; Figure 1g). Hence, we envisaged an active mechanism allowing keratinocytes to efficiently remove excess unsequestered profilaggrin/filaggrin molecules; we hypothesised that keratinocytes may employ small extracellular vesicles (sEVs) such as exosomes for this purpose.

To get some indication if profilaggrin/filaggrin segregate into EVs we first carried out a search in the manually curated EV database (Vesiclepedia; available at http://microvesicles.org/)[24] under terms "filaggrin" and "FLG", complemented with the manual PubMed search (the phrase "filaggrin" or "profilaggrin" and "exosomes" or "microvesicles" or "apoptotic bodies" or "extracellular vesicles"). The results identified several datasets reporting mass spec signal indicative of the presence of profilaggrin/filaggrin-derived peptides in EVs from different sample sources, mostly within the sEV/exosomal fractions. As expected, the protein was mainly reported in samples for which epithelial cells were the most probable source of EVs, e.g., urine, breast milk, nasal lavage, thymic epithelial cell and epithelial cancer cultures (Table 1); however, interestingly signal was also reported in T lymphoblast-derived exosomes, which would be in line with Protein Atlas[20] RNA expression data (Figure S1).

| Source | EV | Isolation methods | Markers | PubMed | FBS info |
|---|---|---|---|---|---|
| **Human colorectal cancer ascites** | MV | Serial centrifugations<br>Sucrose cushion UC (100,000 x g)<br>OptiPrep density gradient UC (200,000 x g) | Alix, CD81, Tsg101, β-actin, ICAM-1, Ezrin, Catenin β-1 | 21630462 | n/a |
| **Breast milk** | EV | Sucrose gradient UC (192,000 x g) | CD9, Annexin A5, Flotillin-1, OLAH and PTHLH - specific to EV derived from milk | 27601599 | n/a |
| **Thymic epithelial culture** | Exo | UC (100,000 x g) | TSG101, HLA-DR, CD9 and CD81, negative for CD45 and close to negative for CD63 | 25776846 | n/a |
| **Human bone marrow mesenchymal stem cells** | MV | UC<br>Ultrafiltration<br>Sucrose cushion UC (100,000 x g)<br>OptiPrep density gradient UC (200,000 x g)<br>UC wash (100,000 x g) | CD63, β-actin, HSP90, galectin-1 | 22148876 | 10% FBS (no information regarding depletion) |
| **Platelet-poor plasma** | MP | Centrifugation (18,890 x g)<br>Centrifugation washes 2, 4, 6, 8, and 10 times | ITGA2B, transthyretin, actin, HSA | 22329422 | n/a |
| **Plasma of coronary artery disease patients** | MP | Centrifugation (19,000 x g)<br>Centrifugation wash | CD31, CD41, CD62E, CD146, CD14 | 23056467 | n/a |
| **Plasma** | Exo | Exo-SpinTM Blood kit<br>Size exclusion chromatography | CD9, CD81, CD5L, LGALS3BP | 26154623 | n/a |
| **Cultured human renal proximal tubule cells** | Exo | UC (200,000 x g)<br>Immunomagnetic isolation with anti-CD63 coupled to magnetic MPs | TSG101 | 24976626 | 10% FBS (no information regarding depletion) |
| **Seminal plasma** | Prostasomes | UC (100,000 x g)<br>XK16/70 Superdex 200 gel column<br>Sucrose gradient UC (85,000 x g)<br>UC wash (100,000 x g) | Flotillin-1, Flotillin-2, Clathrin, Caveolin-1, Caveolin-2 | 26272980 | n/a |
| **Donor primary lymphoblast cells** | EV | UC (100,000 x g) | ERM, filamin, α-actinin, nucleolin, β-actin, Rac1/2, EF-1α | 23463506 | 10% FBS (depleted by overnight centrifugation at 100,000 x g) |
| **Urine** | Exo | UC (200,000 x g)<br>Sucrose (17,000 x g)<br>UC (200,000 x g) | - | 22106071 | n/a |
| **Urine** | Exo | Differential centrifugation (200,000 x g) | - | 22641613 | n/a |
| **Human amnion epithelial cells** | Exo | Differential UC (100,000 x g) | CD9, CD63, CD81, HSC70 | 27333275 | 10% Exo-free FBS (System Biosciences, Mountain View, CA) |
| **Nasal lavage fluid** | Exo | Differential centrifugation (120,000 x g) | CD63, CD9 and TSG101 | 27320496 | n/a |
| **Human Amnion Epithelial Cells** | EV | ExosomeSerial centrifugation<br>UC (100,000 x g) | CD9, CD63, CD81 and HSC70 | 27333275 | n/a |
| **Human adipose-derived mesenchymal stem cells** | Exo | Differential centrifugation<br>UC 100,000 x g | CD9 and CD63 | 33933157 | n/a |
| **Human sweat** | Exo | Differential centrifugation<br>UC 100,000 x g | ALIX, CD63, Hsp70 | 28899687 | n/a |

**Table 1. Studies reporting the presence of profilaggrin/filaggrin-related peptides in extracellular vesicle fractions, as reported in Vesiclepedia, complemented with manual search in Pubmed.** MV, microvesicles; Exo, exosomes; MP, microparticles.

Next, we set out to confirm if profilaggrin/filaggrin can segregate as cargo in sEV secreted by keratinocytes. We cultured NHEKs and induced their differentiation using a standardised calcium switch assay. Small EV fractions (sEV), which contain both exosomes and smaller microvesicles (MVs)[25] were then isolated from conditioned media by sequential

centrifugation/ultracentrifugation method (exosome-enriched 100K pellet, Figure 2a). We confirmed the characteristic “cup-shaped” morphology by electron microscopy as well as the expected size distribution by Nanoparticle Tracking Analysis (NTA) (Figure S3a-b and e-f). Since conditioned keratinocyte media could potentially contain KHGs released upon cell death and contaminate our 100K pellets, we next followed with purification and fractionation of those preparations by iodixanol/sucrose gradient (Figure 2a). Once again, electron microscopy and NTA analysis confirmed the expected morphology and size distribution of the density gradient-purified sEVs (called “sEV/exosomes” from now on) (Figure 2b-c). Western blot showed that top fractions 1-5 contained vesicles positive for exosomal markers (Figure S3c-d and g-h); we noted that syntenin expression was not well pronounced for some donors. Filaggrin-related bands (incl. products of profilaggrin processing) were observed in both the sEV/exosomal fractions and in fractions containing small MVs (Figure S3d and h). Bands were also observed in the last iodixanol/sucrose gradient fraction (fraction 11), either due to the presence of KHGs or aggregated material released from ruptured sEV/exosomes; we noted that the signal was not detectable in some of the donors. Intriguingly, we did not observe any substantial differences in the filaggrin-related cargo as far as the calcium level was concerned which is unexpected given the large increase in filaggrin expression upon NHEK differentiation. To improve the sensitivity of detection we next pooled fractions 1-5 (Figure 2d) adjusting the samples against cell counts (as in [26–28]), which revealed the band of ~75 kDa in size (possibly filaggrin dimer, Figure 2d) to be the most prominent; the 37 kDa filaggrin monomer band could be also sometimes detected at lower levels in the vesicles harvested from differentiated NHEKs. Unexpectedly, the differences between sEV/exosomes obtained from the two calcium conditions were relatively small. Taken together, we determined that filaggrin and profilaggrin-processing products are sorted into keratinocyte-derived sEV/exosomes *in vitro*, also in cells capable of proliferation, in which KHGs are not yet present.

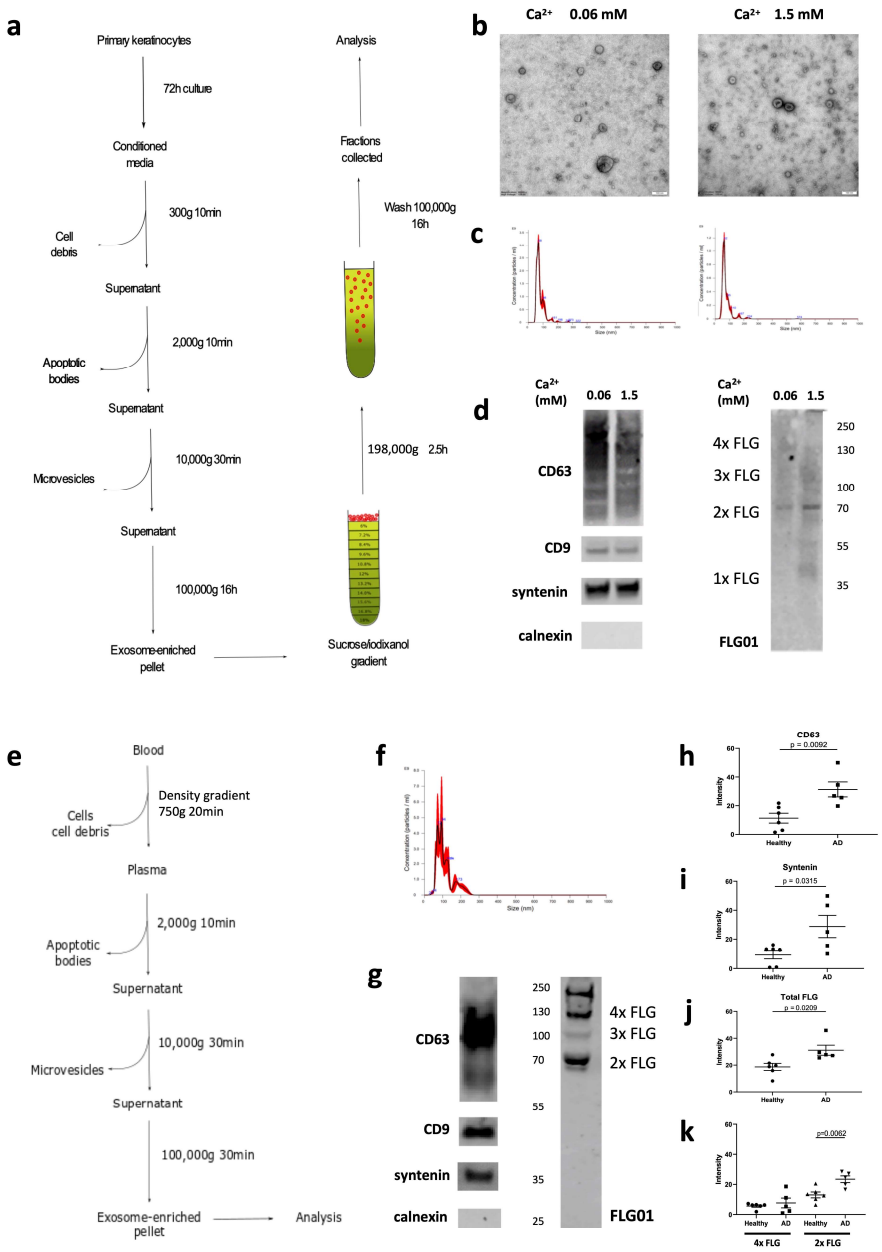


**Figure 2. Profilaggrin/filaggrin is present in small extracellular vesicles secreted by keratinocytes and the blood of healthy individuals and atopic dermatitis patients. a)** protocol for sEV isolation and purification from conditioned cell culture media; **b)-c)** confirmation of the typical sEV **b)** morphology by electron microscopy and **c)** size distribution by Nanoparticle Tracking Analysis of purified primary keratinocyte-derived sEV/exosomes; **d)** detection of exosomal markers and profilaggrin/filaggrin products in purified sEV/exosomes secreted by primary keratinocytes by western blot, example pictures shown (pooled fractions; n=2); profilaggrin sequence features 10-12 filaggrin monomer repeats; the dimer consists of two such repeats joined by a linker; **e)** protocol for sEV isolation (100K pellet) from human blood plasma; **f)** size distribution of human blood plasma sEVs (100K pellet) by Nanoparticle Tracking Analysis; n=3; **g)** Identification of exosomal markers and profilaggrin/filaggrin products in sEVs (100K pellet) isolated from human blood plasma, example pictures of n=5 donors shown; **h)-k)** quantification of exosome marker and profilaggrin/filaggrin signal in western blot analysis of

sEVs (100K pellet; sucrose/iodixanol-purified) isolated from human blood plasma of healthy individuals and AD patients; data from n=6 healthy donors and n=5 AD patients,: **h)** CD63, **i)** syntenin, **j)** total filaggrin signal, **k)** 2x filaggrin and 4x filaggrin; means +/- SEM are shown, unpaired t-test.

*Profilaggrin/filaggrin cargo-containing sEVs are found in the circulation*

The size and flexibility of smaller EVs, especially exosomes, allow them to be carried into the circulation *in vivo*. Hence, we envisaged that we may be able to detect profilaggrin/filaggrin-signal in blood derived samples; interestingly, filaggrin signal could be found in the total plasma as reported by Protein Atlas (Fig S4a; Peptide Atlas). To this end, since it is unlikely that our samples could be contaminated with KHGs, we used a simplified protocol (Figure 2e) to obtain exosome-enriched sEV fractions from plasma of healthy donor samples; we identified a signal corresponding to the unique filaggrin-relevant peptide present within repeat 1, 2, 6 and 10 by mass spec analysis (Figure S4b-d), and verified that the vesicles of expected size (Figure 2f) contained profilaggrin/filaggrin-relevant bands (Figure 2g). The signal was very strong and distinct bands corresponding to profilaggrin-processing products could be easily identified but we also noticed that the 37kDa band was not easily detectable in this case, suggesting potential degradation of the filaggrin monomer in the circulating sEVs. Subsequent gradient fractionation confirmed that filaggrin was present in the sEVs expressing exosomal markers (Figure S5a-b). In contrast to the culture supernatants, the signals of the tetraspanins CD9 and CD63 was much lower in the fractions 6-10 and the signal for profilaggrin/filaggrin was not seen in those fractions, suggesting that small MVs bearing some of this cargo (as based on the results above) were not detected in the plasma 100K pellets.

*Blood of AD patients contains exosomal filaggrin cargo*

Decreased filaggrin expression resulting from an *FLG* mutation and/or downregulating effect of inflammatory mediators[2930–32] is a hallmark of AD skin and AD tissue sections demonstrate low KHG abundance. Given our formulated model of filaggrin expression (Figure 1g) we hypothesized that such low filaggrin production unable to reach the KHG formation threshold may support continuous cytoplasmic diffusion and redirection of unsequestered protein into the exosomal compartment to maintain cell-tolerated level of IF binding. To investigate this, we first compared the exosome-enriched sEV fractions isolated from equal plasma volumes obtained from healthy donors and AD individuals by assessment of WB markers and determined that AD

patients had an increase in the overall fraction of circulating sEVs/exosomes (Figure 2h-i and S5c). Previously, a study reported an increase in the number of blood plasma sEVs/exosomes (100K pellet) in AD patients presenting with moderate-to-severe illness compared to healthy controls, but the difference was not statistically significant; however, the authors found the sEVs/exosomes of the AD patients to be slightly smaller[33]. Next, we compared the size of the filaggrin-related cargo; we noticed an overall increase of the combined 2x filaggrin and 4x filaggrin signal in AD plasma exosome-enriched sEVs in comparison to those from the healthy controls (Figure 2j and S5d). The previously noted band at ca. 70kDa was also more prominent in the patients, suggesting increased accumulation of the cleaved profilaggrin-processing product corresponding to the filaggrin dimer (Figure 2k). Given the increased accumulation of sEV/exosomes in AD plasma, we also calculated the relative filaggrin load "per sEV/exosome" and we found a non-significant trend towards reduction in the AD patients (Figure S5e-j).

*S. aureus exerts pronounced effect on the sEV compartment*

*S. aureus* is a common pathogen colonizing AD skin and contributing to the clinical deterioration in the patients. It has been shown that mildly acidic filaggrin breakdown products, i.e. urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA) execute control over the bacteria in the skin [34]; reduced level of UCA and PCA in AD contribute to the elevated skin pH and loss of this antimicrobial control mechanism [35]. Exposure to the growth supernatant from the bacteria did not have any obvious effect on profilaggrin/filaggrin expression by NHEKs; however, we noticed increased processing of the protein, with increased presence of 37kDa monomer band and breakdown products smaller in size (Figure S6a). This aligns with previous report suggesting that *S. aureus* promotes profilaggrin breakdown by increasing activity of endogenous serine proteases, such as kallikrein 5 (KLK5) in keratinocytes [36] . Hence, we next investigated if the exposure of keratinocytes to *S. aureus* may influence the sEV/exosomal protein cargo. To this end, we exposed immortalised primary keratinocytes (N/TERT-1)[37] grown at either low or high calcium level to *S. aureus* culture supernatant. We noticed extensive morphological changes in the treated cells, as expected given the *S. aureus* toxicity[38], including formation of syncytia by the cells at high calcium level (Figure 3a). Strikingly, upon sucrose/iodixanol fractionation, we observed a pronounced dysregulation within the sEV compartment, as evidenced by increase of CD63 and CD9 signal in the fractions of the lower buoyancy in the gradient (fractions 6-11; Figure 3b-e). Interestingly, this was much more noticeable in the vesicles secreted by the calcium-differentiated cells. Regrettably, due to the discontinuation of the previously used anti-syntenin antibody a different reagent was used, which did not detect much signal in the control sEV

fractions. However, very high syntenin presence in the exosomal fractions 1-5 could be still detected upon the *S. aureus* treatment, suggesting potential specificity for differentially modified (e.g., glycosylated) syntenin and suggesting induction of such form upon the treatment. Interestingly, CD63 was more liable for change in comparison to CD9 and we have observed an increase in the CD63/CD9 ratio also in the fractions 1-5 upon addition of the supernatants (Figure 3f).

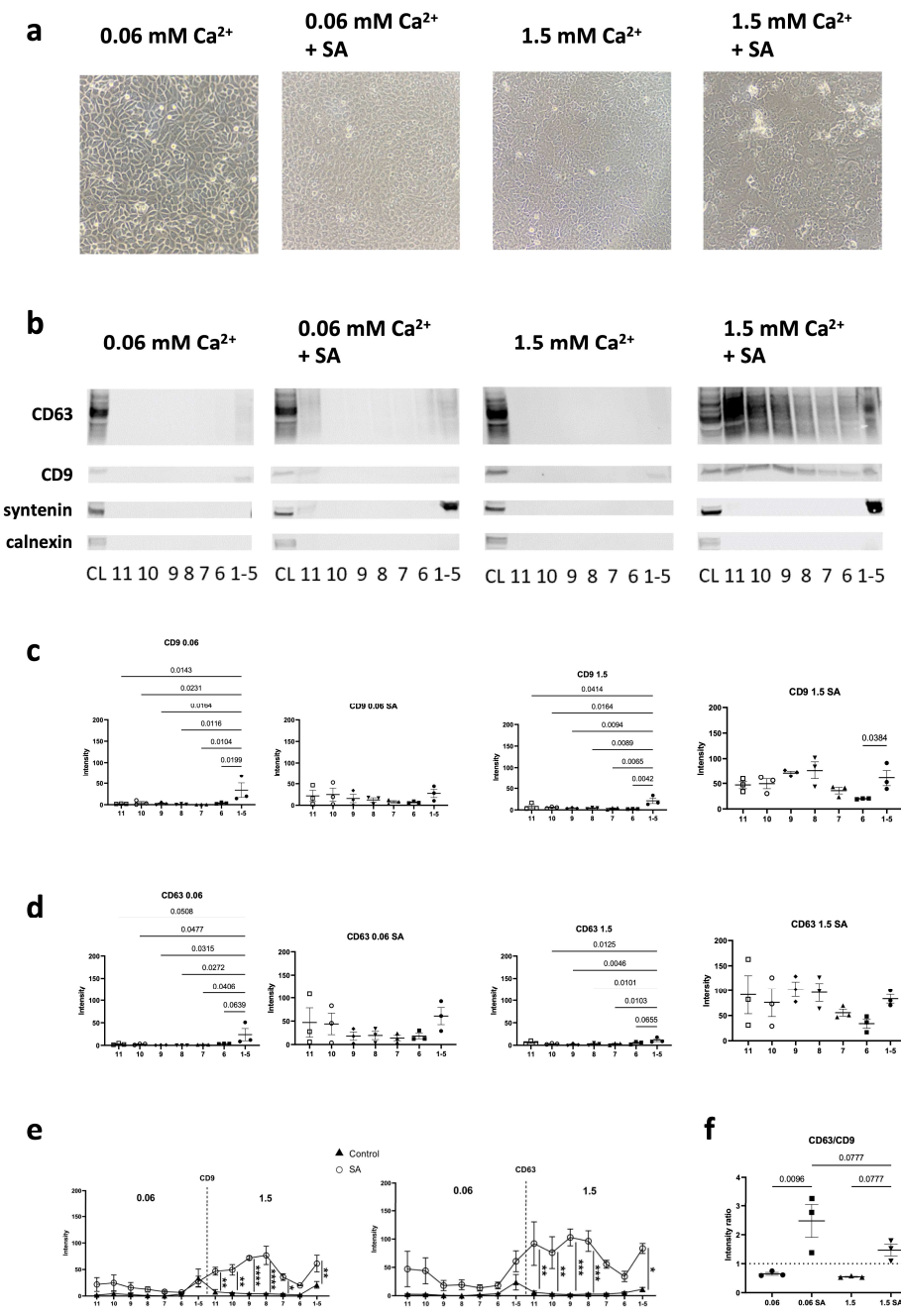


**Figure 3. *S. aureus* dysregulates the small extracellular vesicle compartment in keratinocytes.** **a)** example bright field microscopy pictures of N/TERT-1 cells cultured at low or high calcium level and treated with *S. aureus* growth supernatant; n=3 biological replicates; **b)** detection of exosomal markers in iodixanol/sucrose gradient fractions following purification of sEV/exosomes produced by N/TERT-1 keratinocytes treated with *S. aureus* growth supernatant by western blot, example data of n=3 biological replicates shown; for 0.06 mM $Ca^{2+}$ and 0.06 mM $Ca^{2+}$ + SA cell lysates (CL) from the 0.06 mM $Ca^{2+}$ untreated condition were used while for 1.5 mM $Ca^{2+}$ and 1.5 mM $Ca^{2+}$ + SA CL from the 1.5 mM $Ca^{2+}$ untreated condition were used as controls; **c)-d)** quantification of the **c)** CD9 and **d)** CD63 signal in the post-purification fractions; **e)** comparison of CD9 and CD63 signal intensities measured in *S. aureus* growth supernatant-treated vs. untreated (control) conditions; n=3 biological replicates; **f)** CD63/CD9 signal intensity

ratio comparison between conditions; quantified data are from n=3 biological replicates, means +/- SEM are shown, one-way ANOVA with c)-d) Dunnett's correction, e) Šidák's correction and f) Holm-Šidák's correction; SA, addition of *S. aureus* growth supernatant.

*S. aureus promotes filaggrin inclusion into the sEVs of exosomal characteristics*

N/TERT-1-secreted sEVs did not contain much profilaggrin/filaggrin cargo at baseline, possibly due to the individual characteristics of this particular keratinocyte donor; we noticed this for some donors in the study as mentioned earlier. However, upon the treatment with *S. aureus* substantial content of profilaggrin and profilaggrin breakdown products in sEVs was found. We noticed that this was limited to the exosome-enriched fractions 1-5 at both low and high calcium conditions, with greater effect in the latter (Figure 4a-c; arrows). In addition, treated samples also demonstrated noticeable signal in the fraction 11, most likely due to pulling down protein aggregates that pelleted on the bottom of the gradient; this, again, was not observed in the control conditions. As for the fractions 1-5, preferential inclusion of the filaggrin dimer band was evident again; in contrast, no relevant profilaggrin/filaggrin cargo was found in the remaining sMV fractions (Figure 4a-c). Furthermore, when we analysed the ratio between the intensity of the most prominent filaggrin band in the fractions 1-5 versus the respective CD63 intensity, we also noticed a enhancing effect of both the *S. aureus* supernatant treatment and the calcium level (Figure 4d), which resulted in increased filaggrin loading into vesicles.

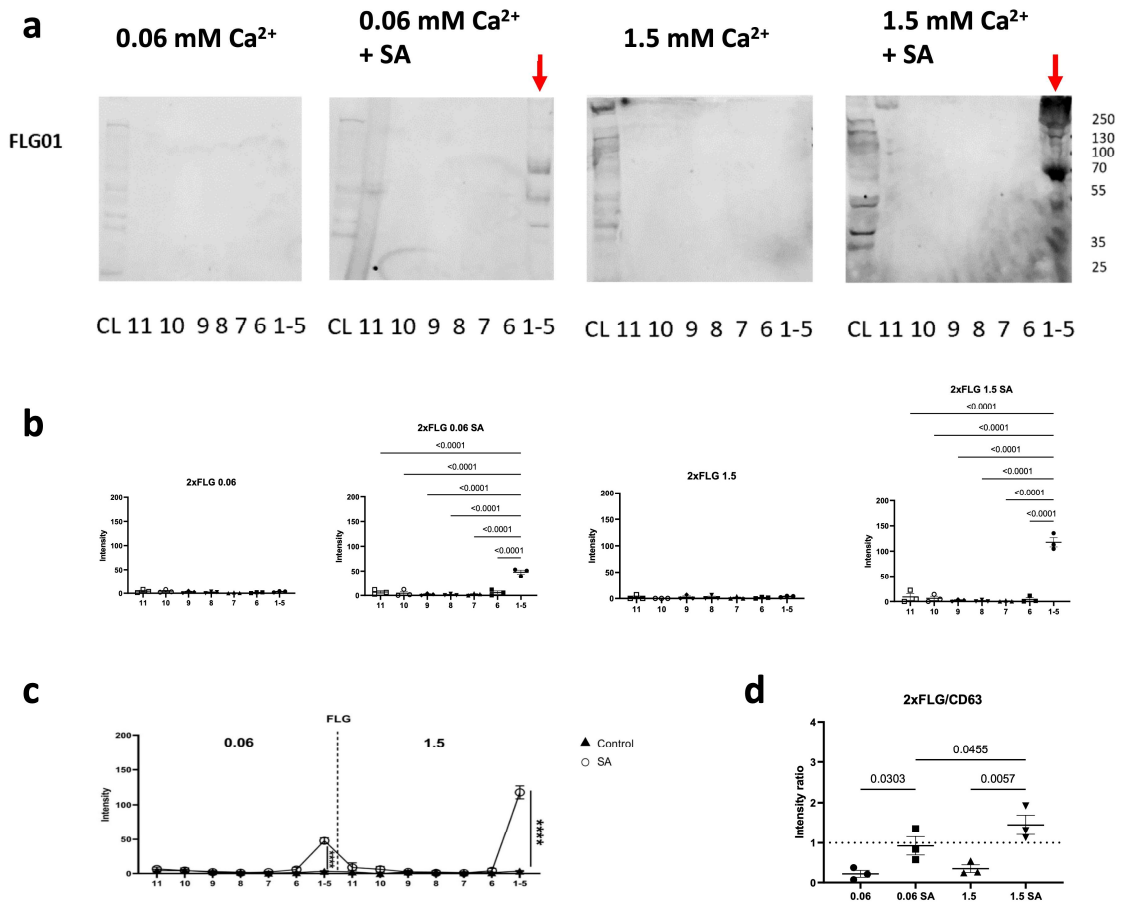


**Figure 4. *S. aureus* promotes loading of profilaggrin/filaggrin into small extracellular vesicles.** **a)** detection of profilaggrin/filaggrin products (red arrows) in iodixanol/sucrose gradient fractions following purification of sEV/exosomes produced by N/TERT-1 keratinocytes treated with *S. aureus* growth supernatant by western blot, example data of n=3 biological replicated shown; for 0.06 mM $Ca^{2+}$ and 0.06 mM $Ca^{2+}$ + SA cell lysates (CL) from the 0.06 mM $Ca^{2+}$ untreated condition were used while for 1.5 mM $Ca^{2+}$ and 1.5 mM $Ca^{2+}$ + SA CL from the 1.5 mM $Ca^{2+}$ untreated condition were used as controls; **b)** quantification of the 2x filaggrin signal in the post-purification fractions; **c)** comparison of the 2x filaggrin signal in *S. aureus* growth supernatant-treated vs. untreated (control) conditions; **d)** 2x filaggrin/CD63 signal intensity ratio compared between conditions; quantified data are from n=3 biological replicates, means +/- SEM are shown, one-way ANOVA with b) Dunnett's correction, c) Šidák's correction and d) Holm-Šidák's correction; SA, addition of *S. aureus* growth supernatant.

*Pathways including proteins of innate recognition of S. aureus intertwine with profilaggrin processing and MVB-related vesicle formation*

Multiple pathogen recognition receptors (PRRs) are involved in the innate detection of bacterial pathogens in keratinocytes[39] and the epidermal tissue[40]. Hence, to understand the recognition route resulting in the observed increase in the profilaggrin/filaggrin sorting into exosome-

enriched sEVs, we next searched for recognised links between the innate pathways and profilaggrin-processing enzymes using STRING pathway analysis[41]. This analysis highlighted node connections between two proteins implicated in profilaggrin/filaggrin processing (elastase and HspB1) and TLR2 receptor (Figure 5a). Interestingly, HspB1, which we have previously established as a critical protein implicated in the coordinated release of filaggrin from actin-scaffold caged KHGs[12], seems to constitute a bridge connection within the previously described link TLR2-KLK5 link[42] (via cathepsin D). We next searched for connections between PRRs and the sEV-sorting machinery, to determine if the innate recognition of *S. aureus* may have a potential to affect filaggrin sorting into the sEV/exosomal cargo. To this end, the analysis included a list of 16 proteins identified by Reactome as those related to "cargo recognition and sorting" (Table S1); here, we identified NOD2 as potentially involved, as well as CD14, the known PRR co-receptor for TLR4. Interestingly, those proteins also seem to show direct links to ubiquitin, i.e. UBA52, UBB, UBC and RPS27A (Figure 5b). Lastly, to increase the sensitivity of our search we proceeded with the analysis of the three combined protein networks which identified additional relationship between the clusters, i.e. a link between furin and hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) (Figure 5c). Strikingly, the search identified HspB1 as a central point where the three clusters converged.

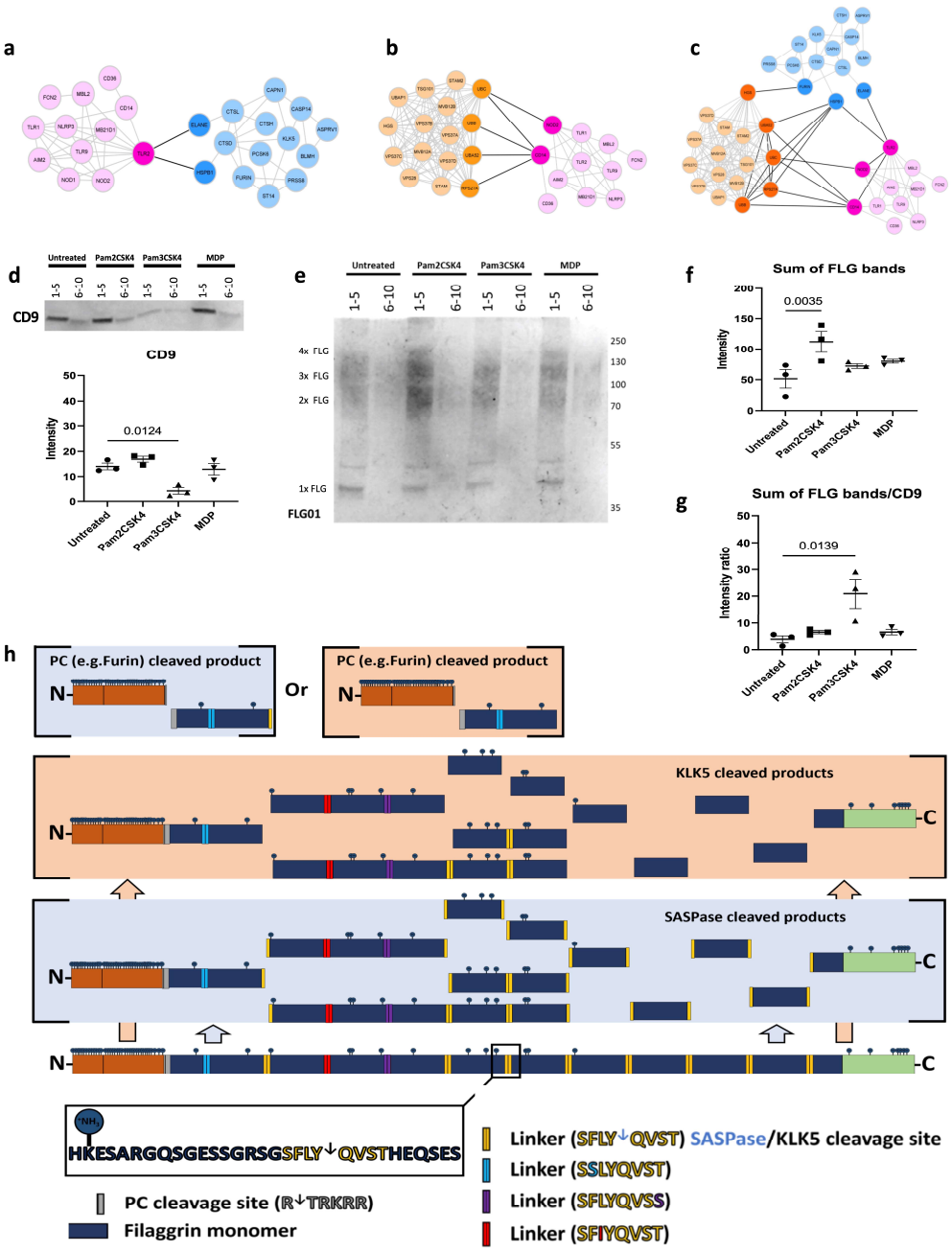


**Figure 5. Loading of filaggrin into small extracellular vesicles is TLR2-dependent. a)-c)** STRING protein-protein interaction map between *S. aureus*-relevant pathogen recognition receptors (PRRs) and **a)** profilaggrin-processing enzymes, **b)** proteins involved in sEV cargo sorting, **c)** combination of the three networks; **d)** presence of CD9 exosomal marker in pooled iodixanol/sucrose gradient fractions following purification of sEV/exosomes derived from N/TERT-1 cells stimulated by TLR2 and NOD2 agonists by western blot and quantification of signals in pooled 1-5 fractions; n=3 biological replicates; **e)** profilaggrin/filaggrin signal in pooled iodixanol/sucrose gradient fractions following purification of sEV/exosomes derived from N/TERT-1 cells stimulated by TLR2 and NOD2 agonists by western blot and **f)** quantification of signals in pooled 1-5 fractions; **g)** ratio between total filaggrin signal/CD9 signal ratio analysis in pooled 1-5 fractions; quantified data are from n=3 biological replicates, means +/- SEM are shown, one-way ANOVA with d) Šidák's correction and e)-f) Dunnett's correction; **h)** cleavage

products resulting from the activity of profilaggrin-processing enzymes with Lys residues indicated as potential ubiquitination targets; Pam2CSK4, TLR2/6 agonist; Pam3CSK4, TLR2/1 agonist; MDP, NOD2 agonist.

*Loading of the profilaggrin/filaggrin-cargo into sEVs is TLR2-dependent*

To experimentally validate the results of our protein network analysis we next investigated changes in the regulation of the sEV compartment as well as the presence of profilaggrin/filaggrin cargo in sEVs upon stimulation of the cells with agonists of identified PRR (TLR2 and NOD2), using immortalized keratinocyte line (N/TERT-1[37,43]). Here we focused on the cells cultured in the presence of 1.5 mM $Ca^{2+}$ as the EV compartment dysregulation and the increase in filaggrin levels in sEVs upon the treatment of cells with *S. aureus* supernatant were much more pronounced in the differentiated cells. Since TLR2 is known to form functional heterodimers with either TLR1 or TLR6, we investigated the effects of the activation of both those heterodimers separately to obtain mechanistic information. With those agonists, we identified CD9 in fractions 1-5 only excluding involvement of those receptors in the previously observed EV compartment dysregulation induced by *S. aureus* (Figure 5d, Figure S6b). However, we found a significant decrease in the intensity of the CD9 signal in fractions 1-5 upon stimulation by a TLR2/1 agonist, Pam3CSK4 (Figure 5d), suggesting a potential reduction of the exosomal output by this stimulation; however, we noticed no difference in the total profilaggrin/filaggrin-relevant cargo (combined 4x filaggrin, 3x filaggrin and 2x filaggrin). Interestingly, however, there was a very pronounced enrichment of those profilaggrin cleavage products in fractions 1-5 signal from the filaggrin bands for N/TERT-1 stimulation with a TLR2/6 agonist Pam2CSK4, (Figure 5e-f). Further calculation of the filaggrin/CD9 ratio (a proxy for cargo inclusion per vesicle) revealed that the engagement of the TLR2/1 heterodimer forced more efficient loading of the protein into the vesicles (Figure 5g). These results suggest that both TLR2/6 and TLR2/1 stimulation contribute to the changes in expulsion of filaggrin from the cells, by either increasing overall amount of the filaggrin cargo export or its loading efficiency under a condition of reduced exosomal output, respectively.

*Product of KLK5-dependent cleavage of profilaggrin are enriched in ubiquitination sites*

Sorting of proteins into the cargo of intraluminal vesicles (ILVs) during the multivesicular body (MVB) formation has been shown to be frequently ubiquitin-dependent[44]. Hence, since the STRING analysis highlighted ubiquitination as a pathway of importance in the *S. aureus*-exposed

keratinocytes we asked if profilaggrin/filaggrin would be prone to such modification. This was especially interesting, since HspB1, which we identified as a central node between the three network clusters has been previously shown to bind ubiquitin and redirect ubiquitinated proteins[45]; HGS, constituting a part of the ESCRT-0 complex[46] has also been shown to recognise ubiquitinated substrates[47]. Indeed, profilaggrin sequence contains multiple Lys residues (canonical ubiquitination marks) which could potentially undergo ubiquitination; interestingly, their spread is uneven within the profilaggrin sequence with the majority of Lys residues accumulated either within the N-terminal domain or clustered at the C-terminal end (Figure 5h, Table S2). In contrast, filaggrin monomer repeats are much less Lys-rich and only the first 8 of those (including the 1$^{o}$ partial repeat) contain at least one such amino acid (Figure 5h, Table S2). Furthermore, when the KLK5[48] and SASPase[49] cleavage-susceptible sites were taken into account it appeared that many of the ~37kDa monomer-sized products generated by the enzyme would contain no ubiquitination sites. In sharp contrast, all the larger products generated during the cleavage would contain several; these modelled cleavage products correspond to the bands observed by western blot. We also noted that the sixth linker region is flanked by five proximal sites of ubiquitin linkage (Figure 5h), potentially masking the cleavage site. We modelled that in a such scenario, specific cleavage through linkers 5 and 7 would result in generation of a stable ~70kDa dimer product containing five ubiquitination sites. The high ratio between the number of ubiquitination sites and weight for this processing product (Table S2) could potentially explain the preferred inclusion of this product within luminal cargo which we observed.

## Discussion

Filaggrin is a protein with a plethora of biological roles supporting the epidermal barrier at both structural and immunological levels. This includes induction of programmed keratinocyte death[11] by free filaggrin monomers to assist *stratum corneum* formation; however, triggering this mechanism prematurely would likely result in a severe barrier defect. Our previous study on the mechanism controlling KHG-dependent filaggrin sequestration[12] determined that low numbers of small profilaggrin-containing granules can be detected already in the lower suprabasal layers of the epidermis[12]. This implied that profilaggrin expression is not confined to the keratinocytes in the granular layer but likely initiated much earlier during the stratification process; however, we did not investigate cells with no visible granules in that study. Formation of KHG requires certain concentration threshold for either local precipitation[12] or phase separation[14] to occur and filaggrin products of small size are not able to form granules, with a tendency to diffuse out into the cytosol[14]. The actin cage-involving, HspB1-dependent

mechanism that we previously defined relies on the profilaggrin accumulation supporting KHG formation and is unlikely to function until high protein production level is reached.

Here we confirm that both the low cytoplasmic filaggrin expression and a degree of IF binding can be observed in less differentiated cultured keratinocytes, as well as in the epidermal tissue, before the appearance of KHGs. There are serious implications to this; a complete lack of control over profilaggrin production and accumulation of (cytotoxic) monomers in the cell early during differentiation would be detrimental and result in cell death and stratification failure. Our proposed model suggests the requirement of an active controlling mechanism to maintain "safe" intracellular filaggrin level that the cell can survive, below a certain "death threshold" as previously showed by regulated filaggrin overexpression experiments carried out by Presland *et al.*[11]. In the current study we show that profilaggrin/filaggrin-derived products are efficiently removed from the keratinocyte cytosol by the means of sEV/exosomal export. We propose that this mechanism enables the cells to maintain safe cytoplasmic levels of filaggrin monomers during the process of profilaggrin synthesis intensification to allow for KHG formation; a novel and basic homeostatic mechanism permitting progression through stratification, essential during the process of the epidermal barrier formation.

Our study also indicates that filaggrin-containing exosomes are transferred into the bloodstream, which may have broader implications systemically, important for the protein of very confined expression pattern, mainly restricted to the epidermis. Interestingly, while the relative abundance of profilaggrin/filaggrin cargo "per a vesicle" remain relatively unchanged, the total detected signal is increased in exosomes isolated from AD plasma, due to the generally increased abundance of sEV/exosomes in the blood of the patients. Consistent with this, recent studies identified increased filaggrin levels in total serum samples from patients with different allergic manifestations[50,51]. We hypothesize that the reduced cellular profilaggrin expression in AD, set at the level insufficient to support KHG formation forces re-direction of the nascent protein into the exosomal secretory pathway and removal from keratinocytes to avoid cell death; these sEV/exosomes can be found in the circulation. While this mechanism could be important at the level of cellular homeostasis, it could further compound barrier dysfunction in the disease due to increased depletion of filaggrin from the epidermis. Currently, we do not know the consequences of the profilaggrin/filaggrin cargo presence in circulation and transfer to peripheral tissues in AD. Given the strong signal observed in exosomes isolated from the blood, sEV/exosome inclusion seem to provide effective protection from proteases in the extracellular space and the bloodstream; this ensures efficient long-distance filaggrin transport into distant tissues. However, it remains to be tested if the increased filaggrin delivery to tissues

in AD may affect ongoing allergic inflammation in the disease, e.g., via the impact on CD1a-mediated T cell responses [52] or contribute to the progression of the allergic march.

Increased level of vesicular secretion has been observed in conditions leading to cell death[53] and this may include enhanced level of blebbing from the plasma membrane and generation of, so called, "apoptotic exosome-like vesicles" (AEVs)[54]; such AEVs have been shown in pathology as a major contributor to inflammation, stimulating expression of proinflammatory mediators and acting as damage-associated patterns (DAMPs)[55]. In the second part of the study, we show that *S. aureus,* a pathogen successfully spreading in the skin of AD patients[56] and contributing to clinical exacerbations may disturb the sEV/exosomal system in keratinocytes, possibly via this mechanism, but with an additional effect on profilaggrin/filaggrin cargo sorting. It is known that *S. aureus* actively targets pathways of keratinocyte differentiation and epidermal barrier formation[57], including expression of filaggrin itself[58] and profilaggrin processing enzymes, i.e calpain-1, caspase-14 and kallikrein-5 [36,58]. *S. aureus* seems to be under control in healthy skin partly thanks to the acidic pH of the skin surface supported by the accumulation of small filaggrin-breakdown products of the natural moisturising factor (NMF), i.e., UCA and PCA, which control bacterial growth[34]. Rapid filaggrin release from keratinocytes dying upon the exposure to bacterial toxins, leading to the intensified NMF generation in the epidermis, would therefore be detrimental to the pathogen. Our study proposes that the bacteria may repurpose the basic homeostatic mechanism contributing to the formation of a protective epidermal barrier that we discovered to reduce the level of available filaggrin in the skin (Figure 6). Specifically, stimulation of profilaggrin cleavage coupled with the cellular redirection and loading into the exosome-enriched sEVs, which are naturally flushed away into the circulation, would result in enhanced filaggrin expulsion from the skin. Such intensified filaggrin removal process could support bacterial growth and colonisation and contribute to the increased amount of filaggrin-related sEV cargo in the circulation of AD patients. Recently, we demonstrated that the exposure of keratinocytes to *Candida albicans* in the context of AD inflammatory *milieu* leads to re-programming of the surface glycosylation pattern on secreted sEVs; sEVs released under these conditions increased their capacity to interact with dendritic cells (DCs) via inhibitory Siglec receptors [59], activation of which has been shown to reduce innate responses to pathogens[53,54]. To our knowledge, no extracellular vesicle-assisted mechanism to reduce host control measures by removal of proteins or compounds with antimicrobial properties from the cells or tissue has been described to date; whether this is a broadly pathogen-utilised mechanism or *S. aureus*-specific phenomenon remains to be clarified. However, filaggrin was previously detected in the unfractionated serum samples from patients with sepsis caused by several different bacteria

species[60]; while the authors interpreted this finding as sample contamination during handling it is plausible that sEVs were the source of the protein.

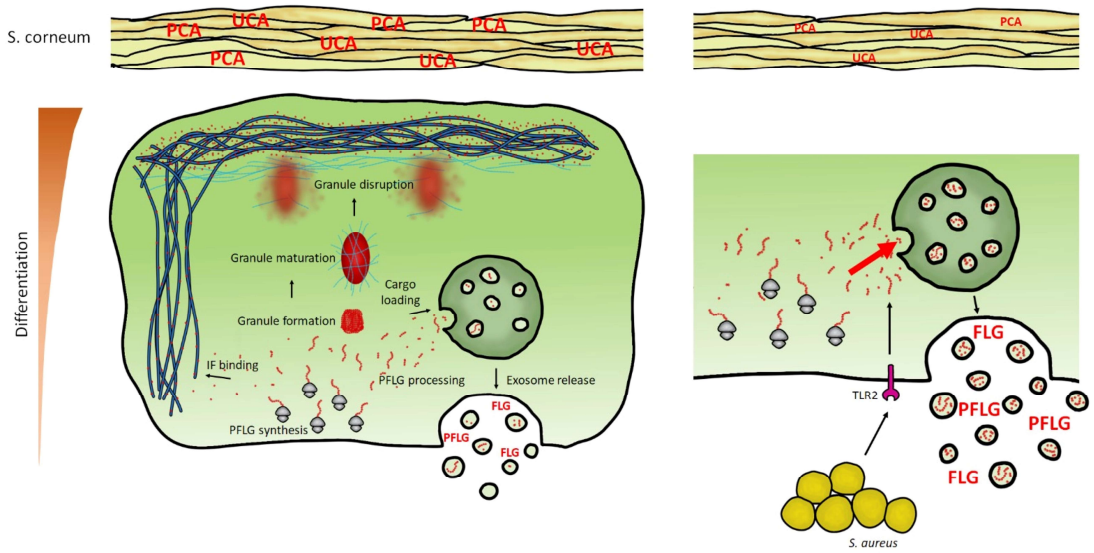


**Figure 6. Proposed model of small extracellular vesicles serving as means of profilaggrin/filaggrin removal from keratinocytes in steady state and during *S. aureus* infection. Left panel:** Low-level profilaggrin expression in the lower suprabasal layers of the epidermis results in the unsequestered protein which can diffuse into the cytosol, where it can bind to keratin-based intermediate filaments (IF) of the cell. A keratinocyte maintains low level of this binding to prevent IF aggregation and cell death, by filaggrin removal via the sEV/exosomal pathway. After the programmed cell death by cornification, a level of antimicrobial filaggrin breakdown compounds (pyrrolidone carboxylic acid; PCA and urocanic acid; UCA) of the natural moisturizing factor (NMF) accumulates in the *s. corneum*. **Right panel**: The mechanism for profilaggrin/filaggrin expulsion via sEV/exosomes is exploited by *S. aureus,* which promotes enhanced profilaggrin/filaggrin removal from the cell via TLR-dependent mechanism. This in turn, reduces filaggrin levels in the skin and limits the exposure of the bacteria to the antimicrobial filaggrin breakdown NMF products that arise following filaggrin release from keratinocytes dying due to the action of toxins released by *S. aureus*.

On the other hand, changes in vesicle secretion upon the exposure to toxins and bacterial cellular constituents could also represent a mechanism utilized by keratinocytes as a part of pathogen defence to initiate immune cell infiltration. Small extracellular vesicles, and especially exosomes, including those secreted by non-immune cells[61], have been shown as important participants in both innate and adaptive immune responses[26,62]. With their small size and high durability, sEVs are particularly suitable for long-distance intercellular communication, including within the immune system. In the skin this could constitute a route allowing for alarming of sentinels, i.e. Langerhans cells (LCs) in the epidermis, or dermal DCs. To this end, secretion of sEVs in the context of infection and cell death could result in enhanced transfer of DAMPs to

those cells. It is not clear how filaggrin transfer within/out of the skin could benefit the host; however, given that the protein has immunoregulatory function, e.g., as an inhibitor of phospholipase A2 (PLA2)[52] in the CD1a-mediated antigen presentation pathway, and as a suppressor of LC activation[6]; hence expulsion of filaggrin away from the site of inflammation would likely promote pathogen clearance.

We determined that the process of enhanced filaggrin loading into sEV/exosomes results from the TLR2 signalling intertwined with the enzymatic processing and MVB sorting networks[3941]. TLR2 is the primary receptor for innate recognition of gram-positive bacteria, including S. aureus[63], with an important role during CD1a-mediated T cell responses[64] ; its polymorphism has been shown in AD previously[65] and linked with the disease severity as well as other allergic manifestations[66]. Our modelling determined that PRR and vesicle networks converge at the level of HspB1 and HSG, which seem to link the exposure to *S. aureus* with the ubiquitination process[45,47]; it is plausible that ubiquitination could mediate MVB trafficking and sorting in this case. Specifically, protein ubiquitination has been shown of importance for cellular localisation beyond redirection of proteins for proteasomal degradation[67]; it also seems to have a role in the transport towards compartments of the endocytic system[44] as well as gene regulation by promoting nuclear localisation[67].

Taken together, we propose that sEV/exosome-aided removal of profilaggrin/filaggrin products exists in keratinocytes to regulate intracellular free filaggrin monomer content and prevent premature cell death during stratification. We believe that this system is of the greatest importance early in epidermal differentiation, before the appearance of KHGs, when it is replaced by the AKT1-HspB1-actin cage-dependent mechanism[12]. While it is conceivable that this mechanism could have additional roles, e.g., constitute a part of keratinocyte pathogen defence system, enhanced filaggrin expulsion from the skin promoted by *S. aureus* inducing profilaggrin processing and MVB sorting into sEVs may benefit the bacteria by reducing the impact of antimicrobial host control and safeguarding its growth. Our study suggests that this pathologic mechanism could be explored as a novel target for targeted therapeutic interventions.

## Methods

### *Skin samples*

Skin samples were collected from healthy donors undergoing surgery on the ethical approvals from UK National Research Ethics Service (14.NW.1153) and the Independent Bioethics Committee for Scientific Research at Medical University of Gdansk (ethical approval numbers: NKBBN/559/2017-2018, NKBBN/621-574/2020 and NKBBN/746/2019-2020) into PBS (Sigma-Aldrich, St. Louis, MO, USA), with 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

### Keratinocyte Isolation and Culture

Skin samples were stored in cold PBS with the addition of 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). For cell isolation subcutaneous adipose tissue was removed before incubation in dispase (12 U/ml, Corning, NY, USA). Epidermal sheets were harvested and digested at 37°C and 0.25% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA). Keratinocytes were seeded on a collagen IV-coated dishes (Corning, NY, USA) and cultured in EpiLife medium (with the addition of EDGS, and antibiotics as above and 10% FBS). The following day, the medium was replaced with a serum-free EpiLife (with EDGS and antibiotics) and cells were cultured at 37°C, 5% $CO_2$. NHEKs were cultured at 37°C, 5% $CO_2$, in animal product-free EpiLife medium (Thermo Fisher Scientific, Waltham, MA, USA) with low calcium (0.06 mM) and EpiLife™ Defined Growth Supplement (EDGS; Thermo Fisher Scientific, Waltham, MA, USA) with addition of 100 U/ml penicillin + 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Pooled NHEK cultures from n=3-4 donors were used when necessary to obtain enough sEVs (harvesting from 2D culture experiments). N/TERT-1 immortalised keratinocyte cell line[37,43], a kind gift from Prof Rheinwald was cultured in keratinocyte serum-free medium (K-SFM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 25 µg/ml bovine pituitary extract (BPE) (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 ng/ml epidermal growth factor (EGF) (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin + 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 0.4 mM $Ca^{2+}$ at 37°C, 5% $CO_2$. For maintenance of the cell line cells were subcultured at no more than 35% confluence. Serum-free EpiLife medium supplemented as described above was used as 'EV-free medium' for N/TERT-1

cells. Cell treatments and EV-free media switches were carried out when the cells reached 80-90% confluence.

***Epidermal sheet isolation, antibody staining and image analysis***

Epidermal sheets were isolated by incubation in dispase overnight (5 U/ml; Sigma-Aldrich, Gillingham, Dorset, UK) and epidermis was separated from dermal tissues manually with forceps. The sheets were then incubated in 4% formaldehyde (Sigma-Aldrich, Gillingham, Dorset, UK), and 0.1% Triton X-100 (Sigma-Aldrich, Gillingham, Dorset, UK) and then blocked in the buffer (5% FCS, Sigma-Aldrich, Gillingham, Dorset, UK; 2% BSA, Sigma-Aldrich, Gillingham, Dorset, UK in PBS; or 0.4% fish skin gelatin dissolved in TBS and 0.2% Triton X-100). Anti-filaggrin G-20 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was used followed with the secondary anti-goat Alexa 488 (Life Technologies, Waltham, MA, USA). Nuclei were visualized by Hoechst (NucBlue, Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA). The epidermal sheets were mounted on microscope slides and cover-slipped with Mowiol-488 (Sigma-Aldrich, Gillingham, Dorset, UK). Data acquisition was carried out on the Zeiss 780, Zeiss LSM 710 inverted confocal microscope (Zeiss, Jena, Germany) by recording 2D images in different axial (3D) planes. Then, every single confocal plane of a 3D imaging stack was deconvolved using the Huygens Professional software package (Huygens; Scientific Volume Imaging, Hilversum, the Netherlands). Deconvolution allows minimizing image artifacts due to noise and optical aberrations. A theoretical point-spread function (PSF) defined by the software (adjusted as a function of the penetration height/depth) was employed for deconvolution of the 3D images and when 2D planes were used, we employed both our defined PSF (as in[12]) and the theoretical one developed by the software (noting no significant difference). The resulting 3D image was surface rendered (Huygens) to easily observe granules and nuclei without a large contribution from the background and minimizing the effect of in-depth aberration imaging. Raw data was also presented as in (Supplementary data) to show the level of background signal coming from the filaggrin at different depths. Selected planes along the z-axis were shown to highlight the localization and distribution at single planes of the filaggrin.

***EV isolation***

EV isolation was carried out from EV-free media after 72 h of culturing. Briefly, conditioned medium (CM) was centrifuged at 300 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific, Waltham, MA, USA) for 10 min at 4°C to remove the cells and cell debris, followed by a spin at 2,000 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific, Waltham, MA, USA) for 10 min at 4°C to remove soluble proteins and apoptotic bodies (AP). The supernatant was ultracentrifuged (OptimaTM L-90K or OptimaTM LE-80K ultracentrifuge, Beckman Coulter, Brea, CA, USA) at 10,000 x g for 30 min at 4°C to isolate the pellet of microvesicles (MVs) which was washed and stored as above. The supernatant was ultracentrifuged at 100,000 x g for 16 h at 4°C to pellet exosome-enriched small extracellular vesicle fraction (sEV; 100K pellet). The sEV pellet was washed in PBS as above and stored at -80°C for further use. sEV pellet was purified using iodixanol/sucrose discontinuous gradient with iodixanol (OptiPrep™; STEMCELL Technologies, Vancouver, BC, Canada) concentration ranging between 6-18% (increments of 1.2%, 1 ml each fraction). 100K pellet was top loaded on the OptiPrep layers and ultracentrifuged at 198,000 x g for 2.5 h. Fractions were collected separately (1 ml), or pooled where indicated. The top-loaded sample was pooled with the first OptiPrep fraction and together considered the first fraction. After that samples were ultracentrifuged at 100,000 g for 16 h in PBS to wash the sEVs. Blood plasma samples were obtained by gradient centrifugation (Lymphoprep; STEMCELL Technologies, Vancouver, BC, Canada) over 20 minutes at 750 x g (Megafuge 16R TX-400 centrifuge, Thermo Scientific, Waltham, MA, USA) with the centrifuge brake switched off. Collected plasma was diluted in PBS and EV isolation was carried out as above, starting from the 2,000 x g spin.

Clinical samples were obtained from healthy donors and AD patients into vacutainers (containing EDTA, BD, Franklin Lakes, NJ, USA) and plasma was isolated by centrifugation over 10 min at 3,000 x g (Eppendorf, Hamburg, Germany). Collected plasma was kept at -20°C for further use.

***EV Characterisation***

For TEM visualization, the EVs were adsorbed onto formvar/carbon-coated copper grids size 300 mesh (EM Resolutions, Sheffield, UK), then stained with 1.5% uranyl acetate (BD Chemicals Ltd.), and imaged by Tecnai electron microscope (Tecnai Spirit BioTWIN, FEI, Hillsboro, OR, USA). For

the Nanoparticle Tracking Analysis (NTA) the EV samples were diluted 1000x in PBS and run using NS300 NanoSight NTA (Malvern Panalytical, Malvern, UK).

***Western blot***

$1x10^6$ cells were lysed in 100 µl RIPA buffer (Cell Signalling Technology, Danvers, MA, USA) containing protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich, St. Louis, MO, USA) and spun for 15 min at 4°C, 13,000 x g; the supernatant was harvested. 4X Bolt™ LDS Sample Buffer (Thermo Fisher Scientific, Waltham, MA, USA) (10x diluted) was added to the lysates or EV samples and the samples were heated for 10 min at 80°C. Samples were run on Bolt™ 4-12% Bis-Tris Plus Gels (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min using PowerEase™ 300W Power Supply (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were transferred onto nitrocellulose membranes (iBlot™ 2 Transfer Stacks, nitrocellulose, regular size, Thermo Fisher Scientific, Waltham, MA, USA) using iBlot transfer system (iBlot 2 Dry Blotting System, Thermo Fisher Scientific, Waltham, MA, USA) and the membranes were blocked in 5% fat-removed milk in PBS for one hour. Primary antibody incubations (diluted 1:100-1:1,000) were carried out at 4°C on shaker overnight and secondary antibody IRDye® 800CW or IRDye® 680RD (LI-COR Biosciences, Lincoln, NE, USA) (dilution 1:25,000) for 30 min at RT. The membranes were scanned and analysed using Odyssey Clx Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Signal intensity was quantified using ImageJ/Fiji 1.53f51 by the measurement of 'mean grey value' of the bands and background subtraction.

***Cell treatment with S. aureus and PRR agonists***

One ml of overnight culture of *S. aureus* "Newman" strain ($2.4 \times 10^9$ CFU/mL) was spun at 1,700 x g, 5 min. The supernatant was sterile-filtered through a 0.1 µm pore size filter and stored in -20°C. The cell pellet was washed with PBS and spun again at 1,700 x g, 5 min and resuspended in 1 ml PBS. *S. aureus* was incubated at 80°C for 30 min, at 1,000 rpm shaking speed (Thermomixer C, Eppendorf, Hamburg, Germany). The suspension of heat-killed bacteria was cooled on ice and stored in -20°C for up to a month. The NHEKs in P3 or P4 were used to isolate sEVs. Cells growing in 75cm² U-shaped canted neck cell culture flasks (Corning, NY, USA) were treated with 24 µg/ml (total 600 µg in 25 ml cell culture medium) heat killed *S. aureus* (HKSA). N/TERT-1 cells were grown to 80% confluence and treated with the *S. aureus* supernatant at the

time of EV-depleted media switch at 5% v/v of the total media. sEVs were isolated and density gradient-purified as described above. sEVs isolated from conditioned media volume equivalent to 1.5 x T150 cell culture flasks were loaded per gel well in SDS-PAGE.

For the PRR agonist treatment N/TERT-1 cells were stimulated with 50 ng/ml of Pam2CSK4, 1 µg/ml of Pam3CSK4 or 10 µg/ml of MDP (all from InvivoGen, San Diego, CA, USA) at the time of EV-depleted media switch. EVs were isolated and density-gradient purified as described above. sEVs isolated from conditioned media volume equivalent to 3 x T150 cell culture flasks were loaded per gel well in SDS-PAGE.

***Immunofluorescence***

8-well chamber slides (VWR International, Radnor, PA, USA) were coated with coating matrix (Thermo Fisher Scientific, Waltham, MA, USA), then NHEKs were seeded (50,000- 100,000 cells/well) in 500 µl cell culture medium. For staining the cells were fixed with 100 µl/well 4% formaldehyde in PBS for 5 min, then washed and permeabilized with 0.1% Triton X-100, 100 µl per well, for 5 min. For blocking the permeabilized cells were incubated for 1 h with 5% FBS, 2% BSA in PBS on shaker. After blocking 200 µl of the primary Abs were added (1:100 in PBS) and incubated at RT 1 h with gentle shaking. NucBlue (Thermo Fisher Scientific, Waltham, MA, USA) was diluted in PBS (2 drops per ml) and the secondary Abs were diluted in NucBlue/PBS at 1:200. 200 µl of the diluted secondary antibodies were added and incubated 30 min at RT with gentle shaking. Samples were coverslipped with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) and images acquired on the Leica TCS SP8 X microscope (Leica Microsystems, Wetzlar, Germany).

***Mass spectrometry***

Small extracellular vesicles isolated from pooled plasma samples were subjected to lysis with 1% SDS and cysteine residues' reduction by dithiothreitol and processed by the standard Multi-Enzyme Digestion Filter Aided Sample Preparation (MED-FASP) protocol[68] with alkylation of cysteine residues by iodoacetamide and consecutive digestion by LysC, trypsin, and chymotrypsin. Peptide fractions resulting from each digestion step were collected, desalted in a standard STAGE Tips procedure[69] , and analyzed in the data-dependent acquisition mode on a Triple TOF 5600+ mass spectrometer (SCIEX, Farmingham, MA, USA) coupled with an Ekspert MicroLC 200 Plus System (Eksigent Technologies, Redwood City, CA, USA). Resulting files were analyzed in a single database search by PEAKS Studio 10 (Bioinformatics Solutions Inc., Waterloo,

ON, Canada) against the Homo sapiens SwissProt database (version from 23.01.2019) with the corresponding digestion settings for each measurement file. The identification result was adjusted to 5% FDR on the peptide level and 1 unique peptide per protein on a protein level. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD036724[70].

***Data extraction***

Filaggrin expression data was extracted from Human Protein Atlas version: 21.1 (available at www.proteinatlas.org; v21.1.proteinatlas.org), from the record:

https://www.proteinatlas.org/ENSG00000143631-flg and images available at:

https://www.proteinatlas.org/ENSG00000143631-flg/single+cell+type;
https://www.proteinatlas.org/ENSG00000143631-flg/single+cell+type/skin;
https://www.proteinatlas.org/ENSG00000143631-flg/blood+protein.

***STRING and ubiquitination modelling***

Proteins of the query were subjected to the analysis with Protein-protein interaction networks functional enrichment analysis STRING database (version 11.5)[41]. Networks were generated as full STRING networks using confidence mode of display of network edges. Only text mining, experiments and databases served as sources of interactions between proteins with at least medium confidence interaction score (0.4). No expansion of the networks was applied. Generated networks were then graphically adjusted using Cytoscape software platform (version 3.9.0)[71] .

Modelling of ubiquitination was carried out based on the amino acid sequence of human profilaggrin (NP_002007.1, wild type). The sequence was examined for the presence Lys residues (K) as possible ubiquitination sites on individual domains and known enzyme cleavage sites.

***Data analysis***

Statistical analysis of quantitative data was carried out in GraphPad Prism v. 9.3.1 using unpaired t-test, paired t-test or one-way ANOVA with Dunnett's, Šidák's or Holm-Šidák's corrections as

determined by the software based on the data characteristics and spread; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

## Data Availability

Datasets analysed in this study are available in online repositories as indicated below:

https://www.ebi.ac.uk/pride/archive/, PXD036724.

## Conflict of Interests

The authors declare no conflict of interest in relation to this study.

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## Ethics approvals

UK National Research Ethics Service (14.NW.1153) and the Independent Bioethics Committee for Scientific Research at Medical University of Gdansk (ethical approval numbers: NKBBN/559/2017-2018, NKBBN/621-574/2020 and NKBBN/746/2019-2020).

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## Authors contributions

LH, AKo, JF, ABog performed experiments, analysed the data and contributed to the writing and figure preparation. AKr, MD and MR performed the experiments, MT and JZ provided clinical samples. AAP and KP performed data analysis, ECz contributed to the figure preparation. SG, GSO and MP interpreted the data; JBS performed analysis and participated in manuscript writing. DGO provided the funding, planned the study and performed the experiments, analysed the data, wrote the first and subsequent paper drafts. All authors contributed to the article and approved the submitted version.

## Geolocation information

54.39318849434289, 18.578389264424995

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## Supplementary figures and tables

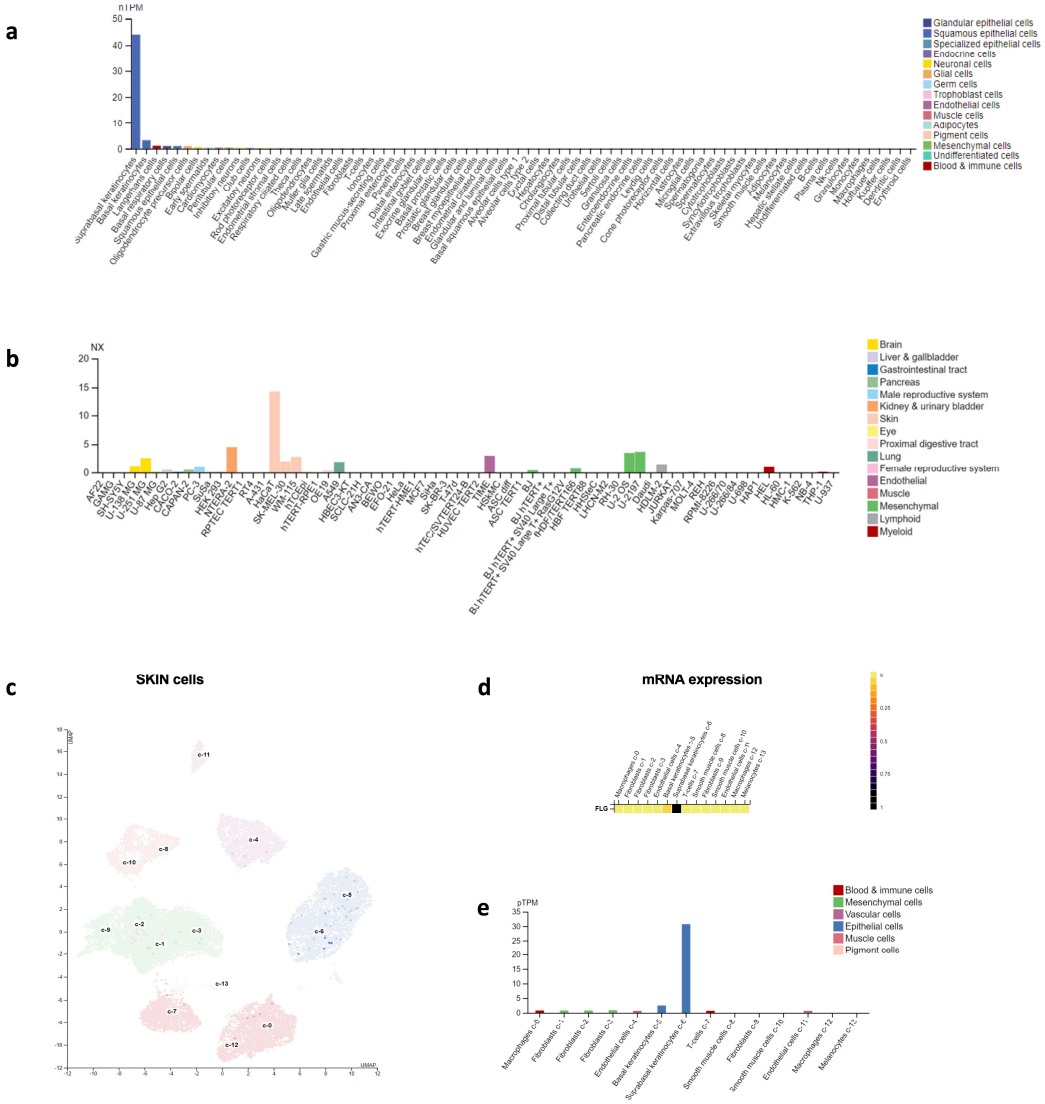


**Figure S1. a)-e)** *FLG* mRNA transcript expression in **a)** primary cells, **b)** cell lines, **c)-e)** skin cells; data extracted from ProteinAtlas[20–22].

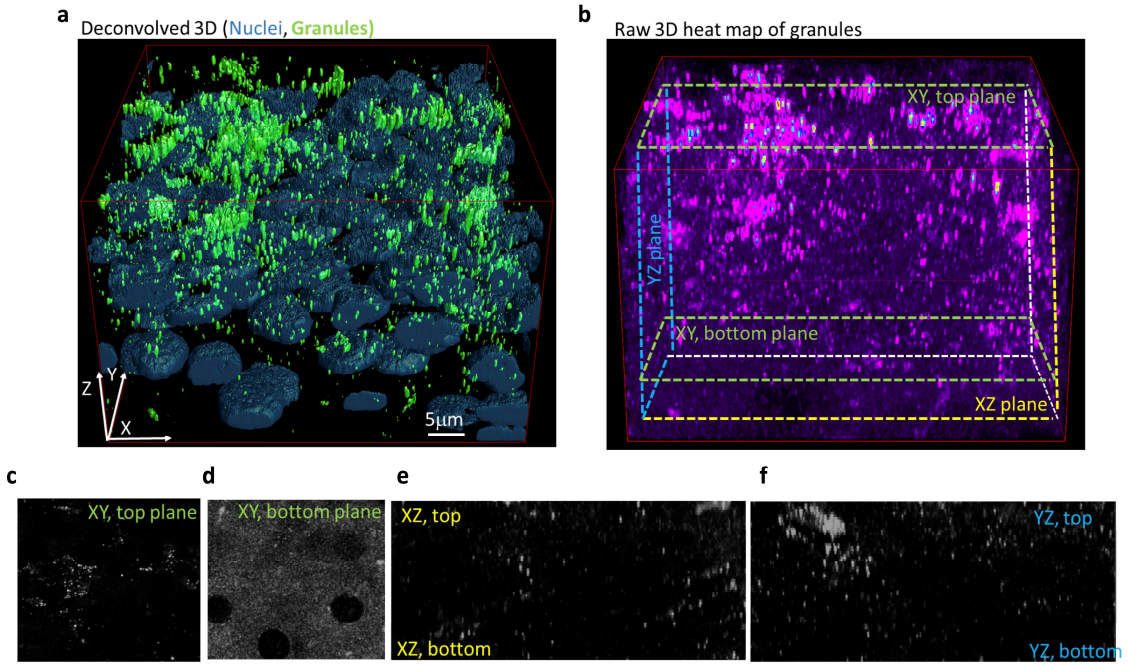


**Figure S2. Representative image of the epidermal sheet. a)** deconvolved image, indicating KHG accumulation; **b)** raw 3D heat map visualizing KHGs representative cross-section of epidermal sheet with indication of the planes; **c)** XY top plane (predominantly granular staining); **d)** XY bottom plane (dispersed/filamentous staining); **e)** XZ cross-section; **f)** YZ cross-section; n=6; scale bar 5 µm.

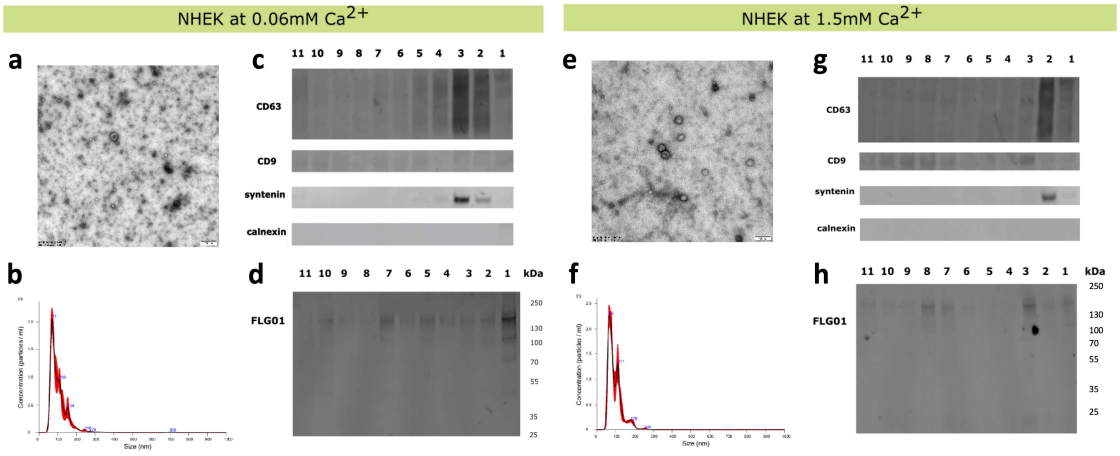


**Figure S3. a)** confirmation of the typical sEV morphology by electron microscopy and **b)** sEV size distribution by Nanoparticle Tracking analysis (NTA) of sEVs (100K pellet) secreted by primary keratinocytes cultured in the presence of 0.06 mM $Ca^{2+}$ ($NHEK_{0.06}$); **c)-d)** detection of **c)** exosomal markers and **d)** profilaggrin/filaggrin products in fractions collected following the iodixanol/sucrose gradient purification of $NHEK_{0.06}$-produced sEV by western blot; **e)** confirmation of the typical sEV morphology by electron microscopy and **f)** sEV size distribution by Nanoparticle Tracking analysis (NTA) of sEVs (100K pellet) secreted by primary keratinocytes

cultured in the presence of 1.5 mM $Ca^{2+}$ ($NHEK_{1.5}$); **g)-h)** detection of **g)** exosomal markers and **h)** profilaggrin/filaggrin products in fractions collected following the iodixanol/sucrose gradient purification of $NHEK_{1.5}$-produced sEV by western blot, example donor with detectable signal in sEVs, n=3 donors.

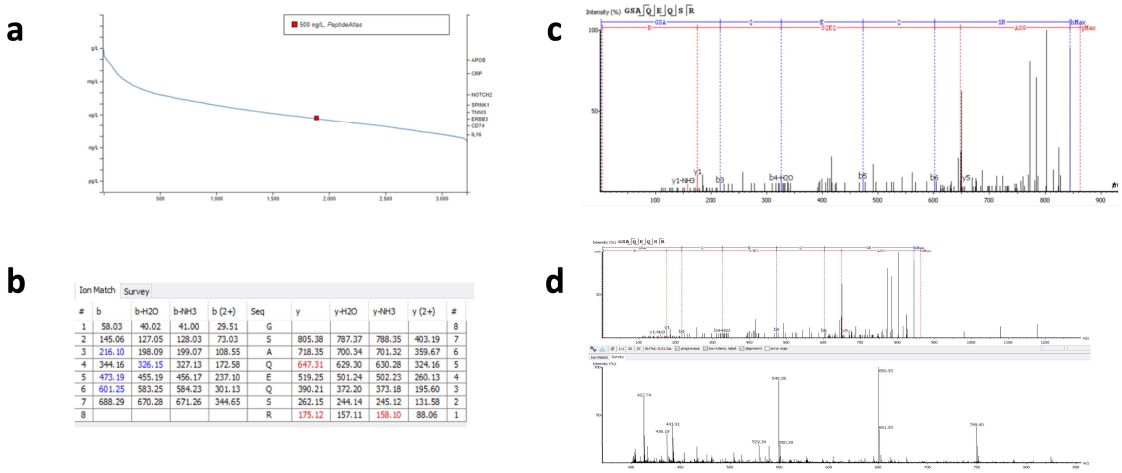


**Figure S4. a)** detection of the filaggrin protein in human blood plasma; data from the Protein Atlas; **b)** intensities and **c)-d)** MS/MS spectra of filaggrin-relevant peptides detected in human blood plasma-derived sEVs (100k pellet); pooled samples from n=3 donors.

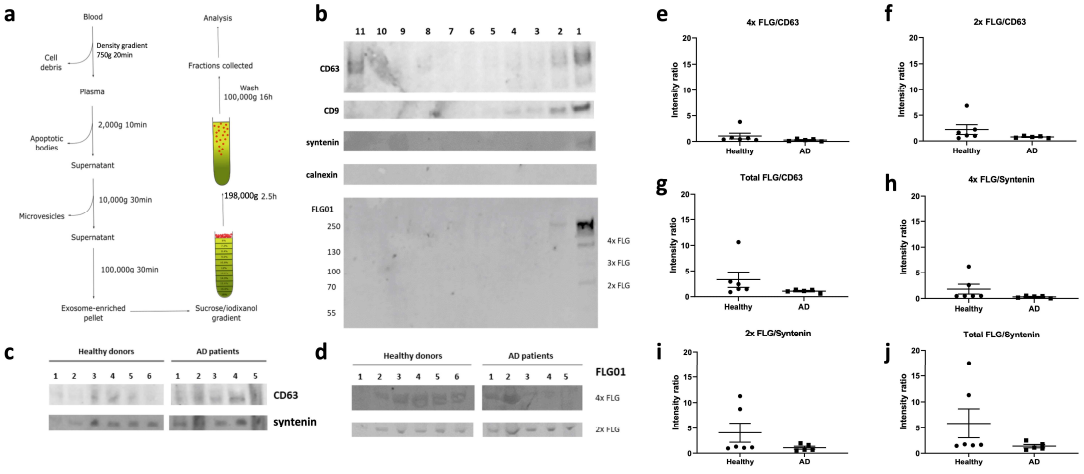


**Figure S5. a)** protocol for isolation and purification of human blood plasma-derived sEVs; **b)** identification of exosomal markers and profilaggrin/filaggrin products in fractions collected after the iodixanol/sucrose gradient purification of human blood plasma-derived sEV/exosomes; **c)-d)** detection of **c)** exosomal markers and **d)** profilaggrin/filaggrin products in sEVs (100K pellet; sucrose/iodixanol-purified) from blood plasma of healthy individuals and AD patients; **e)-j)** analysis of the filaggrin-relevant signal in relation to exosome markers **e)-g)** CD63 and **h-j)** syntenin in sEVs (100K pellet) isolated from blood plasma of healthy individuals and AD patients detected by western blot; combined data from n=6 healthy donors and n=5 AD patients, means

+/- SEM are shown, unpaired t-test, data from one of the assessed AD patient were excluded from the analysis due to high background signal.

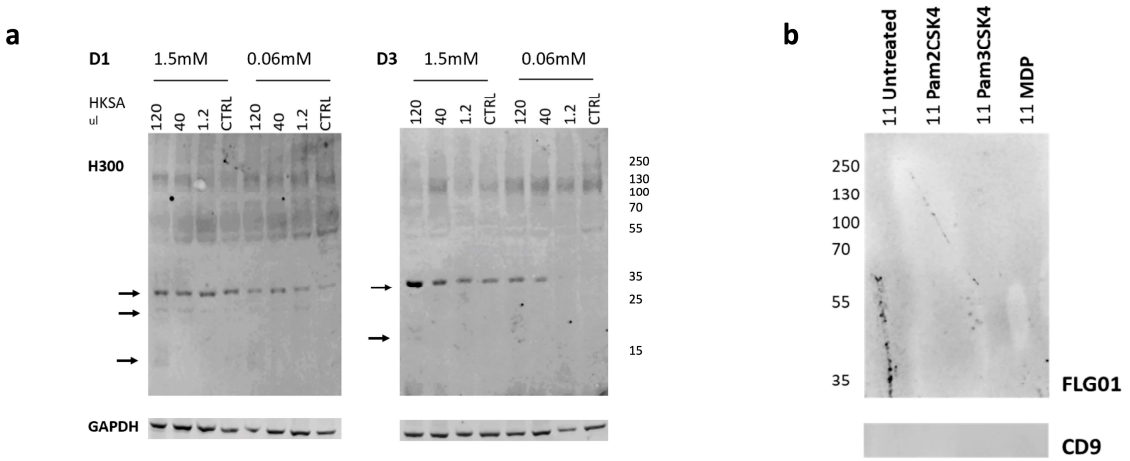


**Figure S6. a)** detection of profilaggrin/filaggrin signal in lysates of heat-killed S. aureus (HKSA)-treated primary keratinocytes on day 1 and day 3 post-treatment, GAPDH was used as a loading control; n=2; **b)** detection of profilaggrin/filaggrin and CD9 signal in fractions '11' collected after iodixanol/sucrose gradient purification of sEV/exosomes produced by N/TERT-1 cells stimulated by TLR2 and NOD2 agonists by western blot, example data from n=3 biological replicates shown; Pam2CSK4, TLR2/6 agonist; Pam3CSK4, TLR2/1 agonist; MDP, NOD2 agonist.

| **S. aureus PR** | STRING aliases | | **PFLG/FLG-processing enzymes** | STRING aliases | | **Cargo recognition and sorting** | | |
|---|---|---|---|---|---|---|---|---|
| TLR1 | TLR1 | | CTSH | CTSH | | UBB | | |
| TLR2 | TLR2 | | CTSL | CTSL | | RPS27A | | |
| TLR9 | TLR9 | | CTSD | CTSD | | UBC | | |
| NOD2 | NOD2 | | CASP14 | CASP14 | | UBA52 | | |
| CD14 | CD14 | | CAPN1 | CAPN1 | | STAM2 | | |
| CD36 | CD36 | | ELANE | ELANE | | STAM | | |
| MBL | MBL2 | | BLMH | BLMH | | HGS | | |
| NLRP3 | NLRP3 | | KLK5 | KLK5 | | MVB12A | | |
| L-ficolin | FCN2 | | HSPB1 | HSPB1 | | MVB12B | | |
| cGAS | MB21D1 | | SASPase | ASPRV1 | | UBAP1 | | |
| AIM2 | AIM2 | | FUR | FURIN | | VPS28 | | |
| | | | PACE4 | PCSK6 | | TSG101 | | |
| | | | PRSS3 | PRSS3 | | VPS37D | | |
| | | | PRSS8 | PRSS8 | | VPS37C | | |
| | | | PRSS14 | ST14 | | VPS37B | | |
| | | | | | | VPS37A | | |

**Table S1.** A list of proteins identified by Reactome as those related to "cargo recognition and sorting" and used for STRING analysis.

| Peptides | | | | | Number of amino acids (w/o terminal linker ) | Predicted molecular weight (kDa) [w/o terminal linker ] | Number of Lysine residues | Ratio of Lysine (Number of Lysine/number of amino acids) |
|---|---|---|---|---|---|---|---|---|
| N terminal | | | C terminal | | | | | |
| Sequence of the enzyme cleavage site (Linker) | Cleaving enzyme | | Sequence of the enzyme cleavage site (Linker) | Cleaving enzyme | | | | |
| N terminal end | | N terminal (S100 fuse type calcium binding domain) | | | 92 | 11.05 | 13 | 0.143 |
| | | N terminal (S100 fuse type calcium binding domain + nuclear localization signal peptide) | R↓TRKRR | PC/furin[1] | 293 | 34.89 | 37 | 0.126 |
| | | N terminal (S100 fuse type calcium binding domain + nuclear localization signal peptide + Truncated filaggrin) | SSLYQVST | ? | 466 | 53.11 | 38 | 0.082 |
| **R↓TRKRR** | PC/furin | Truncated filaggrin | SSLYQVST | ? | 173 | 18.24 | 1 | 0.006 |
| **SSLYQVST** | ? | 1st FLG repeat | SFLY↓QVST | KLK5[2] | 317 | 33.44 | 1 | 0.003 |
| **SFLY↓QVST** | KLK5 | 2nd FLG repeat | SFIYQVST | ? | 316 | 33.69 | 1 | 0.003 |
| **SFIYQVST** | ? | 3rd FLG repeat | SFLYQVSS | ? | 316 | 33.78 | 3 | 0.009 |
| **SFLYQVSS** | ? | 4th FLG repeat | SFLY↓QVST | KLK5[2] | 316 | 33.41 | 1 | 0.003 |
| **SFLY↓QVST** | KLK5 | 5th FLG repeat | SFLY↓QVST | KLK5[2] | 317 | 33.42 | 3 | 0.009 |
| **SFLY↓QVST** | KLK5 | 6th FLG repeat | SFLY↓QVST | KLK5[2] | 316 | 33.04 | 2 | 0.006 |
| **SFLY↓QVST** | KLK5 | 7th FLG repeat | SFLY↓QVST | KLK5[2] | 316 | 33.17 | 1 | 0.003 |
| **SFLY↓QVST** | KLK5 | 8th FLG repeat | SFLY↓QVST | KLK5 [2] | 316 | 33.43 | 0 | 0 |
| **SFLY↓QVST** | KLK5 | 9th FLG repeat | SFLY↓QVST | KLK5 [2] | 316 | 33.84 | 0 | 0 |
| **SFLY↓QVST** | KLK5 | 10th FLG repeat | SFLY↓QVST | KLK5 [2] | 316 | 33.41 | 0 | 0 |
| **SSLYQVST** | ? | 1st + 2nd FLG repeat | SFIYQVST | ? | 633 | 67.11 | 2 | 0.003 |
| **SFLY↓QVST** | KLK5 | 2nd + 3rd FLG repeat | SFLYQVSS | ? | 632 | 67.45 | 4 | 0.006 |
| **SFIYQVST** | ? | 3rd + 4th FLG repeat | SFLY↓QVST | KLK5 [2] | 632 | 67.18 | 4 | 0.006 |
| **SFLYQVSS** | ? | 4th + 5th FLG repeat | SFLY↓QVST | KLK5 [2] | 633 | 66.82 | 4 | 0.006 |
| **SFLY↓QVST** | KLK5 | 5th + 6th FLG repeat | SFLY↓QVST | KLK5 [2] | 633 | 66.44 | 5 | 0.008 |
| **SFLY↓QVST** | KLK5 | 6th + 7th FLG repeat | SFLY↓QVST | KLK5 [2] | 632 | 66.37 | 3 | 0.005 |
| **SFLY↓QVST** | KLK5 | 2th + 3th + 4th FLG repeat | SFLY↓QVST | KLK5 [2] | 948 | 100.85 | 5 | 0.005 |
| **SFIYQVST** | ? | 3rd + 4th + 5th FLG repeat | SFLY↓QVST | KLK5 [2] | 949 | 100.58 | 7 | 0.007 |
| **SFLY↓QVST** | KLK5 | 2nd + 3rd + 4th +5th FLG repeat | SFLY↓QVST | KLK5 [2] | 1265 | 134.25 | 8 | 0.006 |
| **SFLY↓QVST** | KLK5 | C terminal (truncated FLG + C terminal domain) | C terminal end | | 345 | 37.63 | 7 | 0.02 |
| | | C terminal (C terminal domain) | | | 159 | 17.64 | 7 | 0.044 |

**Table S2.** Products of enzymatic cleavage of profilaggrin and their relative Lys content.

# 4. Summary of the research

## 4.1. The effect of pathogens characteristic for atopic dermatitis on the interaction of small extracellular vesicles produced by keratinocytes with dendritic cells (P1)

As keratinocyte differentiation results from greatly altered gene expression, the functionality of the sEVs secreted by those cells may also be changed. Therefore, since sEVs are known to carry antigens and immune mediators there might be differentiation-dependent effects of how $KC_{sEVs}$ influence immune responses to pathogens. Results included in this publication demonstrated that both undifferentiated and differentiated primary keratinocytes stimulated by AD-relevant cytokines (IL-4, IL-13, IL-22 and TSLP) secrete sEVs that interact with DCs; however, the interaction was decreased upon keratinocyte differentiation. This supports the hypothesis that $KC_{sEV}$-mediated signaling or cargo delivery to the immune cells can be affected by keratinocyte differentiation **(Figure 1.** in **P1)**.

To test the effect of AD-relevant environment on the $KC_{sEV}$-DC interaction, keratinocytes were exposed to AD cytokines and, additionally inactivated *S. aureus* or *C. albicans* which often colonize the skin of AD patients **(Figure 3.** in **P1)**. The results demonstrated an enhanced interaction between immature DCs, obtained by differentiation of peripheral blood monocytes, and sEVs produced by undifferentiated keratinocytes simultaneously exposed to cytokines and *C. albicans* compared to sEVs from undifferentiated keratinocytes stimulated just with the cytokines **(Figure 3A** in **P1)**. Similar trend was observed in mature DC-$KC_{sEV}$ interaction but only when keratinocytes were differentiated **(Figure 3B** in **P1)**. Both pathogen-dependent effects were specific to *C. albicans* as exposure of keratinocytes to *S. aureus* did not affect the interaction between DCs and sEVs **(Figure 3A** and **B** in **P1)**.

Cell adhesion of sEVs may depend on the glycosylation pattern of the vesicle surface; protein-protein interaction modeling revealed that some PRRs sensing *C. albicans* but not *S. aureus* were linked to a number of enzymes involved in glycosylation of target molecules **(Figure 3C** and **D** in **P1)**. Transcriptomes of the epidermis of AD patients published in three different studies allowed to narrow down the list of candidate enzymes; ST6 β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) as well as fucosyltransferase 4

(FUT4) were most consistently overexpressed in AD epidermis **(Figure 4A** in **P1)**. Additionally, signaling proteins of the disease-relevant IL-4/IL-13 pathway have been linked to the glycosylation enzymes presented in Figure 3C and D in P1 **(Figure 4C** in **P1)**. This suggests that both AD inflammatory milieu and *C. albicans* may alter keratinocyte glycosylation pathways.

Since *FLG* LoF mutations are the greatest genetic risk factor for AD, the effect of decreased *FLG* expression in keratinocytes on abundance of mRNA related to enzymes involved in glycosylation were investigated. *FLG* gene knockdown keratinocytes (shFLG) and filaggrin-expressing (shC) cells were used for the experiment[265,342]. Among enzymes listed in Figure 3D in P1, ST6GAL1 and core 1 β,3-galactosyltransferase 1 (C1GALT1) were upregulated upon IL-4/IL-13 stimulation of keratinocytes **(Figure 4D** in **P1)**. However, no differences between shC and shFLG cells were found suggesting that the increased expression of glycosylation enzymes in AD is a consequence of inflammation rather than decreased abundance of filaggin.

Next, lectin array was ued to identify changes in sEV surface glycosylation following the exposure of keratinocytes to AD-relevant stimuli. For this experiment sEVs from three conditions were selected based on the degree of their interaction with DCs. Out of 70 tested lectins, 15 were found to bind the strongest to sEVs from the condition that exhibited the highest DC-sEV interaction in Figure 3A and B in P1, i.e., cytokine and *C. albicans*-treated undifferentiated keratinocytes **(Figure 5A** in **P1)**. Upon identification of the carbohydrates that are known to bind those specific lectins, interacting DC receptors were identified through literature search **(Figure 5B** in **P1)**. Among those receptors several candidates were chosen for further experiments to confirm their involvement in the sEV uptake; these included CLRs, i.e., DC-SIGN, MMR or Langerin; sialic acid-binding immunoglobulin-like lectins (Siglecs) Siglec-2, Siglec-7 and Siglec-9 were also selected. Siglecs are a family of transmembrane receptors expressed mostly by the cells of the immune system[343]. These receptors recognize sialic acids often found at the terminal end of cell surface glycoproteins and glycolipids[343]. Pathogens often either synthesize sialic acids or acquire them from the host[343]. Siglecs can either activate or inhibit immune response and the inhibitory kind contain immune receptor tyrosine-based inhibition motifs (ITIMs) in their intracellular region[343]. Siglec-2, Siglec-7 and Siglec-9 all have ITIMs[343].

sEVs from both undifferentiated and differentiated keratinocyte cell line N/TERT-1 were incubated with monocyte cell line-derived DCs in which selected receptors were pre-blocked with receptor-specific antibodies. Results showed that the blocking of Siglec-7 reduced the interaction of undifferentiated keratinocyte-derived sEVs with DCs. At the same time, interaction between DCs and sEVs from both undifferentiated and differentiated keratinocytes was inhibited upon Siglec-9 blocking **(Figure 6A** in **P1)**. In healthy epidermis Siglec-7 and Siglec-9 are expressed almost exclusively in LCs **(Figure 6B** in **P1)**. Increased expression of these two receptors in AD and psoriatic skin; their expression occurs mainly in myeloid cells, which include the APCs of the skin such as DCs, LCs or macrophages even in disease **(Figure 6C** in **P1)**. Taken together, the expression patterns of Siglec-7 and -9 in the skin imply that sialic acids present on the surface of $KC_{sEVs}$ predispose those vesicles to interact specifically with skin APCs.

In summary, the study showed that *C. albicans* modifies the glycosylation pattern of the $KC_{sEV}$ surface by enriching it in sialic acid-containing motifs. This could occur via stimulation of PRRs by the pathogen since the interaction between PRR signaling and glycosylation enzyme network was shown. Such change in glycosylation pattern leads to an increased interaction between sEVs and DCs via Siglec receptors; due to the immune inhibitory nature of these receptors *C. albicans* might exploit this mechanism to diminish immune response of the host and increase its survival. This presents a novel pathway that could be targeted by treatment of *C. albicans* infection in AD and possibly beyond. Moreover, the increased cell-sEV interaction may facilitate delivery of sEV cargo, including antigens to APCs, which could affect the functionality of those cells; therefore, downstream adaptive immune responses to this pathogen may be affected.

## 4.2. The influence of filaggrin insufficiency on the ability of keratinocyte-derived small extracellular vesicles to influence antigen-specific T cell responses (P2)

LoF *FLG* mutations confer a high risk of AD; however, even in the absence of such mutations filaggrin expression is downregulated by the mediators of allergic inflammation. This inflammation is largely a consequence of aberrant T cell activation.

Hence, this study aimed to explore the effect of specifically filaggrin insufficiency on the capacity of $KC_{sEVs}$ to affect peptide and lipid antigen-specific T cell responses.

Four datasets relevant to AD were analysed using bioinformatic tools; this allowed to identify cellular compartments, biological processes and pathways most affected by the disease or its components. First two datasets were generated by mRNA and proteomic profiling of shC and shFLG keratinocytes **(Figure 1.** in **P2).** Additionally, proteomic data from filaggrin-insufficient 3D skin models published by Elias *et al.*[259] and transcriptome of the skin of AD patients by Cole *et al.*[344] were also analyzed **(Figure 2.** in **P2).** Collectively, the datasets showed that the exosomal/sEV compartment is significantly altered following *FLG* knockdown or in the disease **(Figures 1.** and **2.** in **P2)**. Moreover, pathways related to the cell adhesion and immune processes, among others, were identified to be altered in all four datasets **(Figures 1.** and **2.** in **P2)**.

Then, the effect of sEVs from shC ($shC_{sEV}$) and $shFLG_{sEV}$ keratinocytes on expression of the activation markers by DCs differentiated from peripheral blood monocytes was investigated. However, no difference was observed for either immature DCs or mature DCs upon their exposure to $shFLG_{sEV}$ compared to $shC_{sEV}$ **(Figure S3A** in **P2)**.

Next the involvement of $KC_{sEV}$ in peptide antigen-dependent T cell response was explored. Immature DCs were treated (pulsed) with a pool of peptide antigens derived from common pathogens to which the majority of the population had been exposed, either during a natural infection or through vaccination (CEFT peptide pool). $shC_{sEV}$ or $shFLG_{sEV}$ were added to the cells at the time of CEFT pulsation. Following the incubation, the DCs were co-cultured with autologous T cells and the activation of the latter was assessed by their IFNy secretion. However, there was no differential response to CEFT when $shC_{sEV}$ and $shFLG_{sEV}$ conditions were compared with each other or with the CEFT-only control **(Figure S3B** in **P2)**. Similarly, no T cell activation change was observed when whole protein was used as the antigen source instead of CEFT **(Figure S3B** in **P2)**. This suggested no impact of $KC_{sEV}$ on antigen processing pathways through which pathogen-derived proteins go before being presented to T cells.

CD1a presents lipids antigens which can be released from biological membranes by PLA2. Some allergens and pathogens are a source of PLA2. Production of PLA2 in human cells can also be induced in inflammation or infection. Lipidomic profiling was performed on $KC_{sEV}$ to investigate the impact of filaggrin insufficiency on the lipid

composition of these vesicles **(Figure 3E-G** in **P2)**. This revealed that that $shFLG_{sEV}$ are less abundant in long-chain polyunsaturated fatty acids (LC-PUFAs) and are enriched in short-chain saturated fatty acids (SC-SFAs) within their diacyl glycerophosphocholine species (PCs) and their ether analogues (PCOs) which are substrates for PLA2 **(Figure 3H-M** in **P2)**. The analysis of the fatty acids (FAs) within the sEV PCs and PCOs revealed that the mass and chain length of detected LC-PUFAs and are similar to those indicated as optimal for efficient activation of CD1a-dependent T cells[345]. Since we noted differential lipid composition between $shFLG_{sEV}$ and $shC_{sEV,}$ also with regards to the incorporation of potential CD1a ligands, T cell responses mediated by CD1a were examined. For this purpose, CD1a-overexpressing K562 cell line (K562-CD1a) was pulsed with the intact sEVs. K562 transfectant was used as an APC instead of DCs; this was due to the lack of MHC class I or MHC class II expression by the K562 cell line. Hence, using this cell line dissected out any MHC-dependent T cell activation which might be caused by HLA mismatch between keratinocytes and immune cells in the coculture in the allogeneic system; such activation would mask CD1a-specific T cell responses. In this experiment, however, no differences in T cell responses were observed **(Figure S4D** in **P2)**. This suggested that intact sEVs do not constitute a source of a readily-available pool of lipid CD1a ligands. However, when the PLA2 enzyme was added to the K562-CD1a cells during the sEV pulsation, we observed an increase in the IFNγ responses for $shC_{sEV}$ vs. unpulsed cells **(Figure 4A** in **P2)**. In contrast, no differential response was detected upon the exposure to $shFLG_{sEV}$ and PLA2. This indicated that PLA2 aids release of lipids from the sEV membrane allowing them to modulate CD1a-dependent T cell responses.

Apart from PCs, known as classical PLA2 substrates, other lipid species, sphingomyelins and ceramides were also detected in $KC_{sEV}$; sphingomyelin species with long fatty acid chains were enriched in $shFLG_{sEV}$ **(Figure 5A-F** in **P2)**. Some sphingomyelins, often characterized by long fatty acid chains are known be preferentially bound by CD1a over ligands with lower affinity and block the CD1a-TCR interaction. Subsequently, the relative amounts of lipid species known to facilitate (permissive ligands) and block (non-permissive ligands) this interaction were quantified; the latter were enriched in $shFLG_{sEV}$ **(Figure 5G** in **P2)**. Next, T cell activation was measured with sEVs that were first digested prior to K562-CD1a pulsation. This time, inhibition of the IFNy response and increase in IL-13 production was observed for $shFLG_{sEV}$ while $shC_{sEV}$ produced no

difference compared to unpulsed cells **(Figure 5H-I** in **P2).** This suggests that the effect in the experiment shown in Figure 4A in P2 might have been partially masked by the action of PLA2 on the membranes of the cells in the coculture. Overall, the T cell responses indicated that $KC_{sEV}$ can contribute to allergic inflammation when produced by filaggrin-insufficient keratinocytes.

The lipidome alterations in $KC_{sEVs}$ reflected those observed in keratinocytes **(Figures 6** and **7** in **P2)**; accordingly, shFLG keratinocytes were less abundant in PCs and PCOs containing PUFAs compared to shC cells **(Figure 6** in **P2)**. shFLG keratinocytes were also enriched in sphingomyelin species with longer fatty acid chains which contained more unsaturated bonds compared to shC keratinocytes **(Figure 7** in **P2)**.

To determine the reasons behind the altered lipid composition of sEVs produced by filaggrin insufficient keratinocytes, the impact of filaggrin knockdown on the keratinocyte expression of enzymes involved in lipid metabolism was investigated **(Figure 8.** in **P2).** Filaggrin insufficiency affected the lipid metabolic pathways by impacting expression of several enzymes involved in lipid synthesis pathways. Specifically, we found that long-chain-fatty-acid-CoA ligase 3 (ACSL3), an enzyme that preferentially activates long-chain fatty acids for their subsequent incorporation into phospholipids was downregulated in shFLG cells **(Figure 8A** in **P2)**; this could be at least partly responsible for the enrichment of short-chain fatty acid-containing phospholipids in $shFLG_{sEV}$. Enzymes of the ACSL family also have a preference for PUFAs, which could explain the predominance of SFAs in PCs and PCOs of $shFLG_{sEV}$ compared to $shC_{sEV.}$ More ACSL isoforms were downregulated in AD skin at the mRNA level **(Figure 8B-E** in **P2).** Additionally, mRNA of some isoforms of the elongation of very long chain fatty acids enzyme (ELOVL) was downregulated in AD skin **(Figure 8F** to **I** in **P2).** Since ELOVL enzymes elongate fatty acid chains, their downregulation may affect the chain length of fatty acids present in phospholipids of biological membranes in AD.

Taken together, this study has shown that filaggrin insufficiency background alters the $KC_{sEV}$ lipidome which has a profound effect on the pool of lipids that can bind CD1a when released from those vesicles by the activity of PLA2. The decrease in the abundance of permissive CD1a ligands and enrichment in non-permissive lipid species results in a diminished IFNy T cell response in favour of the IL-13 production. This suggests a role for $KC_{sEV}$ produced on a filaggrin insufficiency background in exacerbation of allergic

inflammation in the skin. However, since sEV may be transferred into the circulation and carried into distant tissues, $KC_{sEV}$ may be involved in the pathomechanism of allergy in additional organs, affected by atopic diathesis, i.e., lungs or the intestine.

## 4.3. The effect of *Staphylococcus aureus* on the secretion of small extracellular vesicles by keratinocytes and filaggrin inclusion in these vesicles (P3)

The presence of acidic antimicrobial filaggrin breakdown products, contributing to the lower pH of the *stratum corneum* constitute one of the first line of defenses against colonization of the epidermis by pathogens such as *S. aureus*. However, as beneficial as profilaggrin expression and cleavage is for the protection against harmful microorganisms, given filaggrin's ability to promote cytoskeletonal collapse, its intracellular level must be tightly controlled in order to prevent premature cell death. This study investigated the packaging of profilaggrin/filaggrin into sEVs as means of removal of excess filaggrin. Moreover, the effect of *S. aureus* on this process was explored.

Profilaggrin is highly expressed in *stratum granulosum* where this protein is sequestered in the form of tightly packed keratohyalin granules (KHGs). However, *FLG* mRNA can be detected already in basal keratinocytes **(Figure 1A** in **P3).** Due to the absence of KHGs in basal keratinocytes the expressed filaggrin was expected to interact with intermediate filaments (IF) of the cell; indeed, filamentous filaggrin staining was observed in proliferatory 2D keratinocyte culture but also in the lower layers of stratified epidermis. This pattern disappeared, however, once KHGs were formed **(Figure 1B-F** in **P3).** We hypothesized that profilaggrin expression could reach dangerously high levels before its sequesteration in the granules **(Figure 1G** in **P3).** The possibility of the sEV involvement in the removal of excess filaggrin was explored, given that profilaggrin/filaggrin-derived peptides were identified in sEV/exosomal fractions in previously published proteomic datasets **(Table 1** in **P3)**. Indeed, profilaggrin/filaggrin was identified in sEVs produced by primary keratinocytes; specifically, signal corresponding to two filaggrin repeats joined by a linker (filaggrin dimer) was the most enriched **(Figure 2D** in **P3).** Interestingly, profilaggrin/filaggrin products were also detected in sEVs isolated from the plasma of healthy donors **(Figure 2G** in **P3).** Finally, an increase in both exosomal marker and profilaggrin/filaggrin signal was observed in sEVs from AD serum compared to that

from healthy controls **(Figure 2H-K** in **P3)**. The most prominent profilaggrin/filaggrin signal corresponded to two and four filaggrin monomer repeats (2x FLG and 4x FLG, respectively) **(Figure 2K** in **P3)**. Further analysis revealed a non-significant trend of the reduction in the abundance of the profilaggrin/filaggrin cargo in the plasma sEVs from AD patients **(Figure S5E-J** in **P3)**.

Investigation into the effect of sterile *S. aureus* growth supernatant on the sEV production by keratinocytes revealed an extensive dysregulation of this compartment as evidenced by the increased abundance of CD9 and CD63 markers in sEV samples containing small microvesicles (sMVs) **(Figure 3B-E** in **P3);** this effect was more prominent in the differentiated keratinocytes. Exosome-enriched sEV fractions from sterile *S. aureus* growth supernatant-exposed undifferentiated and differentiated keratinocytes were abundant in profilaggrin and its breakdown product; the filaggrin dimer was, again, the most enriched and more abundant in differentiated compared to undifferentiated keratinocytes **(Figure 4A-C** in **P3)**. Profilaggrin/filaggrin signal was absent from the sMV samples **(Figure 4A-C** in **P3)**. The calculation of the filaggrin dimer/CD63 signal ratio revealed that *S. aureus* enhanced loading of filaggrin into sEVs; the effect was more pronounced in differentiated keratinocytes **(Figure 4D** in **P3)**.

In the search for the mechanism behind the increased filaggrin packaging into the sEVs, protein-protein interaction analysis was conducted **(Figure 5A-C** in **P3)**; *S. aureus*-recognizing PRRs, TLR2 and NOD2 were identified. These PRRs were the most closely linked with proteins involved in profilaggrin/filaggrin processing as well as proteins in the Reactome database 'cargo recognition and sorting' category. To confirm the involvement of these PRRs in filaggrin packaging into sEVs, differentiated keratinocytes were stimulated with TLR2 and NOD2 agonists; since TLR2 forms two functional heterodimers, TLR2/1 and TLR2/6 each dimer was activated separately. Decreased CD9 signal in the sEV fraction upon TLR2/1 stimulation suggested decreased exosome/sEV production while total profilaggrin/filaggrin signal (4x filaggrin, 3x filaggrin and 2x filaggrin combined) was unchanged **(Figure 5D-F** in **P3)**. TLR2/6 stimulation on the other hand resulted in an increase in the total profilaggrin/filaggrin signal despite CD9 remaining unchanged **(Figure 5D-F** in **P3)**. The subsequent analysis of the total profilaggrin/filaggrin in relation to the CD9 signal revealed that TLR2/1 stimulation enhanced loading of the protein into sEVs **(Figure 5D-F** in **P3).**

As ubiquitination has been shown to drive protein loading into ILVs of the MVBs, giving rise to exosomes, profilaggrin and its cleavage products were explored for their potential ubiquitination pattern. Interestingly, this analysis revealed that many of the monomer-sized products would not be ubiquitinated **(Figure 5H** in **P3)**. This is contrary to the larger products which were predicted to undergo ubiquitination; size-wise these correspond to the profilaggrin/filaggrin signal detected in sEVs. Moreover, filaggrin dimers generated by the profilaggrin cleavage also seemed be enriched in the ubiquitination marks compared to the larger processing products when their size is considered. Given these observations, the dimer may be potentially favoured for loading into sEVs at the level of MVB, which would explain the abundance of the filaggrin dimer in the sEVs secreted by the *S. aureus*-exposed keratinocytes.

Overall, the study suggests that profilaggrin/filaggrin produced by keratinocytes can be included as cargo of exosomes/sEVs; this is proposed to serve as a mechanism for a safe removal of unrequested profilaggrin and its processed products from the cells before they can be sequestered within KHGs, the formation of which requires high expression of the protein. The presence of profilaggrin/filaggrin detected in exosomes/sEVs from the plasma of healthy donors and AD patients (higher levels of those vesicles in the latter) could enhance the delivery of profilaggrin/filaggrin to distant organs, leading to local effects in physiology and ongoing allergic inflammation. Since *FLG* expression is almost completely confined to the epidermal keratinocytes, it is highly likely that the circulating profilaggrin/filaggrin-containing exosomes/sEVs are derived from those cells. Such profilaggrin/filaggrin removal from the epidermis may contribute to the reduced abundance of the protein in the epidermis observed in AD skin. *S. aureus* promotes secretion of both exosomes/sEVs and sMVs by keratinocytes, and increases filaggrin loading into the vesicles, which end up removed from the skin into the circulation. This could protect the pathogen from antimicrobial filaggrin breakdown products generated from filaggrin which are released by keratinocytes dying after the exposure to the pathogen.

# 5. Key findings

Research presented in this thesis advances the knowledge on the role of $KC_{sEV}$ in the pathogenesis of AD by providing mechanistic understanding on how the main AD disease components, i.e., allergic inflammation, filaggrin insufficiency and skin pathogens affect the functionality of $KC_{sEVs}$. This provides an insight into previously unknown mechanisms exacerbating the disease which could be exploited as targets for novel treatment approaches.

**Major findings:**

- *C. albicans* and allergic inflammation alter $KC_{sEV}$ surface glycosylation pattern, which increases sEV interaction between with inhibitory Siglec receptors on dendritic cells;
- Filaggrin insufficiency decreases the incorporation of permissive CD1a ligands in $KC_{sEV}$ membranes and enriches them in non-permissive CD1a ligands;
- $KC_{sEV}$ derived from filaggrin-insufficient keratinocytes promote type 2 CD1a-dependent T cell responses;
- Profilaggrin and its processing products are loaded into $KC_{sEVs}$ by undifferentiated and differentiated keratinocytes at steady state and are present in circulating sEVs in both healthy individuals and AD patients;
- sEV expulsion may serve as a homeostatic mechanism in keratinocytes;
- *S. aureus* dysregulates production of sEVs by keratinocytes;
- *S. aureus* enhances loading of profilaggrin/filaggrin into $KC_{sEVs}$ of the exosomal characteristics

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# 5. Other manuscripts/publications

Paul, A. A.*, Szulc. N.*, **Kobiela, A.**, Brown, S. J., Pokrzywa, W., Gutowska-Owsiak, D. *In silico* analysis of the profilaggrin sequence indicates alterations in the stability, degradation route, and intracellular protein fate in filaggrin *null* mutation carriers. **In revision in *Frontiers in Molecular Biosciences* (2022).** * joined first authors.
IF = 6.113

# 6. Conferences

**Oral presentations:**

- Filaggrin insufficiency modifies the impact of keratinocyte-derived small extracellular vesicles on CD1a-mediated T cell responses by altering provision of lipid antigens. **4th Interdisciplinary Foundation for Polish Science Conference, Warsaw (2022)**

- Fibronectin-1 enhances cellular uptake of small extracellular vesicles derived from filaggrin-insufficient keratinocytes. **UK Society for Extracellular Vesicles Virtual Forum, online (2021)**

- Filaggrin insufficiency remodels the exosomal compartment in keratinocytes and impedes uptake of keratinocyte-derived exosomes. **UKEV Early Career Researcher Event, online (2020)**

**Poster presentations:**

- Filaggrin insufficiency enriches keratinocyte-derived small extracellular vesicles in lipid CD1a ligands inhibiting CD1a-dependent T cell responses. **British Society for Immunology Congress, Liverpool, United Kingdom (2022)**

- Fibronectin-1 enhances cellular uptake of exosomes derived from filaggrin-insufficient keratinocytes. **British Society for Immunology Congress, online (2021)**

- Filaggrin-insufficient keratinocytes produce exosomes characterized by reduced capacity to promote CD1a-dependent responses. **XVII Congress of the Polish Society of Experimental and Clinical Immunology, online (2021)**

- Atopic dermatitis conditions promote the ability of allergen-treated keratinocyte-derived exosomes to indirectly induce immune response. **British Society for Immunology Virtual Conference: Connecting immunology in the time of COVID-19, online (2020)**

- Exosomes derived from filaggrin-deficient keratinocytes promote less uptake and alter marker expression in dendritic cells. **British Society for Immunology Congress, Liverpool, United Kingdom (2019)**

# 7. Scholarships and awards

- 1st prize for the best poster at the **XVII Congress of the Polish Society of Experimental and Clinical Immunology (2021)**

- Scholarship within the PROM International scholarship exchange of PhD candidates and academic staff program. Host laboratory and institution: **Translational Research Immunology Group (TRIG), University of Oxford (2020)**

# 8. Co-authorship statements

Gdańsk, 16.03.2023

**Adrian Kobiela**

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## CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

Title: **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors**

Authors: A. Kobiela, J. E. Frackowiak, A. Biernacka, L. Hovhannisyan, A. E. Bogucka, K. Panek, A. A. Paul, J. Lukomska, X. Wang, E. Giannoulatou, A. Krolicka, J. Zielinski, M. Deptula, M. Pikula, S. Gabrielsson, G. S. Ogg, D. Gutowska-Owsiak

Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Performing experiments: sEV isolation, sEV uptake, lectin array; analysis of data from performed experiments and published proteomic/transcriptomic data; protein-protein interaction analysis; statistical analysis; contribution to manuscript writing and figure preparation

Title: **Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation**

Authors: A. Kobiela, W. Hewelt-Belka, J. E. Frąckowiak, N. Kordulewska, L. Hovhannisyan, A. E. Bogucka, R. Etherington, A. Piróg, I. Dapic, S. Gabrielsson, S. J. Brown, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Performing experiments: sEV isolation, flow cytometry, ELISpot, ELISA, T cell line culture,

preparation of samples for lipidomic analysis, Nanoparticle Tracking Analysis; FunRich, Gene Ontology, Reactome analysis; data analysis; statistical analysis; contribution to manuscript writing and figure preparation

and

Title: **Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion**

Authors: L. Hovhannisyan, A. Kobiela, J. Bernardino de La Serna, A. E. Bogucka, M. Deptuła, A. A. Paul, K. Panek, E. Czechowska, M. Rychłowski, A. Królicka, J. Zieliński, S. Gabrielsson, M. Pikuła, M. Trzeciak, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Performing experiments: sEV isolation; western blot *(S. aureus* growth medium treatment and TLR2, NOD2 stimulation experiments); data analysis; statistical analysis; contribution to manuscript writing and figure preparation

Gdańsk, 08th March 2023

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## CO-AUTHOR STATEMENT

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Title: **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors**

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Performing experiments: preparation of heat-killed microorganisms, sEV isolation, sEV uptake, lectin array; data analysis; statistical analysis; figure preparation; contribution to manuscript writing

Title: **Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation**

Authors: A. Kobiela, W. Hewelt-Belka, J. E. Frąckowiak, N. Kordulewska, L. Hovhannisyan, A. E. Bogucka, R. Etherington, A. Piróg, I. Dapic, S. Gabrielsson, S. J. Brown, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Performing experiments: sEV isolation, ELISpot, cPLA2 activity; data analysis; statistical analysis

Joanna Frąckowiak

Gdańsk, 07.03.2023

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# CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

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| Title: | **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors** |
| Authors: | A. Kobiela, J. E. Frackowiak, A. Biernacka, L. Hovhannisyan, A. E. Bogucka, K. Panek, A. A. Paul, J. Lukomska, X. Wang, E. Giannoulatou, A. Krolicka, J. Zielinski, M. Deptula, M. Pikula, S. Gabrielsson, G. S. Ogg, D. Gutowska-Owsiak |
| Published in: | *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530. |

was as follows:

sEV isolation; performing the receptor blocking experiments; nanoparticle tracking analysis of sEVs; data analysis; statistical analysis; contribution to manuscript writing and figure preparation

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## CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

Title: **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors**

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

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Performing experiments: sEV isolation, western blot, nanoparticle tracking analysis of sEV; data analysis; contribution to manuscript writing and figure preparation

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Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Performing experiments: sEV isolation, western blot; data analysis

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Title: **Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion**

Authors: L. Hovhannisyan, A. Kobiela, J. Bernardino de La Serna, A. E. Bogucka, M. Deptuła, A. A. Paul, K. Panek, E. Czechowska, M. Rychłowski, A. Królicka, J. Zieliński, S. Gabrielsson, M. Pikuła, M. Trzeciak, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Performing experiments: sEV isolation, immunocytochemistry, western blot, nanoparticle tracking analysis of sEVs; data compilation by literature search; data analysis; contribution to manuscript writing and figure preparation

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24.02.2023

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# CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

Title: **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors**

Authors: A. Kobiela, J. E. Frackowiak, A. Biernacka, L. Hovhannisyan, A. E. Bogucka, K. Panek, A. A. Paul, J. Lukomska, X. Wang, E. Giannoulatou, A. Krolicka, J. Zielinski, M. Deptula, M. Pikula, S. Gabrielsson, G. S. Ogg, D. Gutowska-Owsiak

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Performing experiments and analyzing data from protein mass spectrometry of cells and small extracellular vesicles.

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Here, I declare that my contribution to the publications:

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Protein-protein interaction modeling; matching lectins with carbohydrate moieties and the latter with recognition receptors (literature search); contribution to figure preparation

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Protein-protein interaction modeling; contribution to figure preparation

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Matching lectins with carbohydrate moieties and the latter with recognition receptors (literature search); protein-protein interaction modeling; contribution to figure preparation

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Profilaggrin ubiquitination modeling; contribution to figure preparation

Warsaw, 08.03.2023

**Joanna Łukomska**

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|---|---|
| Title: | **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors** |
| Authors: | A. Kobiela, J. E. Frackowiak, A. Biernacka, L. Hovhannisyan, A. E. Bogucka, K. Panek, A. A. Paul, J. Lukomska, X. Wang, E. Giannoulatou, A. Krolicka, J. Zielinski, M. Deptula, M. Pikula, S. Gabrielsson, G. S. Ogg, D. Gutowska-Owsiak |
| Published in: | *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530. |

was as follows:

Analysis of the lectin array experiment data

Signature

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Xi'an, 2.24. 2023

Name: Xinwen Wang

Department : Oral medicine

University: The Fourth Military Medical University

Address: 145, Changle West Road, Xi'an, Shaanxi Province, China

## CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

Title: **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors**

Authors: A. Kobiela, J. E. Frackowiak, A. Biernacka, L. Hovhannisyan, A. E. Bogucka, K. Panek, A. A. Paul, J. Lukomska, X. Wang, E. Giannoulatou, A. Krolicka, J. Zielinski, M. Deptula, M. Pikula, S. Gabrielsson, G. S. Ogg, D. Gutowska-Owsiak

Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Keratinocyte treatment and mRNA isolation for microarray analysis; generation of a filaggrin knockdown keratinocyte cell line

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24/2/2023

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Victor Chang Cardiac Research Institute,

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## CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Analysis of keratinocyte microarray data

Gdansk, 24.02.2023

**dr hab. inż. Aleksandra Królicka, prof. UG**
University of Gdansk
Intercollegiate Faculty of Biotechnology UG & MUG
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# CO-AUTHOR STATEMENT

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

and

Title: **Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion**

Authors: L. Hovhannisyan, A. Kobiela, J. Bernardino de La Serna, A. E. Bogucka, M. Deptuła, A. A. Paul, K. Panek, E. Czechowska, M. Rychłowski, A. Królicka, J. Zieliński, S. Gabrielsson, M. Pikuła, M. Trzeciak, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Preparation of microorganisms cell culture

KIEROWNIK
Zakład Badania Związków Biologicznie Czynnych
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Signature

Gdańsk, 03.03.2023

**Prof. dr hab. Jacek Zieliński**

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# CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

Title: **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors**

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and

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Obtaining skin tissue from patients undergoing surgery

Jacek Zieliński

Prof. dr hab. Jacek Zieliński
specjalista chirurgii ogólnej
specjalista chirurgii onkologicznej
Nr ZUS 3476169

Gdansk, 03.03.23

Milena Deptuła

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Here, I declare that my contribution to the publications:

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Isolation of primary keratinocytes from skin samples

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Here, I declare that my contribution to the publications:

Title: **Exposure of keratinocytes to *Candida albicans* in the context of atopic milieu induces changes in the surface glycosylation pattern of small extracellular vesicles to enhance their propensity to interact with inhibitory Siglec receptors**

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

data interpretation and contribution to manuscript writing

and

Title: **Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion**

Authors: L. Hovhannisyan, A. Kobiela, J. Bernardino de La Serna, A. E. Bogucka, M. Deptuła, A. A. Paul, K. Panek, E. Czechowska, M. Rychłowski, A. Królicka, J. Zieliński, S. Gabrielsson, M. Pikuła, M. Trzeciak, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows: data interpretation

Zakład Embriologii
Pracownia Inżynierii Tkankowej
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Profesor

Stockholm,
Feb. 23, 2023

Susanne Gabrielsson

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Here, I declare that my contribution to the publications:

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Experimental design; data interpretation; participation in the manuscript writing

Title: **Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation**

Authors: A. Kobiela, W. Hewelt-Belka, J. E. Frąckowiak, N. Kordulewska, L. Hovhannisyan, A. E. Bogucka, R. Etherington, A. Piróg, I. Dapic, S. Gabrielsson, S. J. Brown, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2023

and

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

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Here, I declare that my contribution to the publications:

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Data interpretation and participation in the manuscript writing

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Data interpretation; provision of funding

24/2/23

Gdańsk, 15.03.2023

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## CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publications:

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Published in: *Frontiers in Immunology*, 2022 Jun 9;13:884530. doi: 10.3389/fimmu.2022.884530.

was as follows:

Provision of funding; experiment planning; data analysis; writing of the first and subsequent paper drafts

Title: **Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation**

Authors: A. Kobiela, W. Hewelt-Belka, J. E. Frąckowiak, N. Kordulewska, L. Hovhannisyan, A. E. Bogucka, R. Etherington, A. Piróg, I. Dapic, S. Gabrielsson, S. J. Brown, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Provision of funding; planning of the study; performing experiments: preparation of keratinocytes for mRNA profiling; data analysis; writing of the first and subsequent paper drafts

and

Title: **Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion**

Authors: L. Hovhannisyan, A. Kobiela, J. Bernardino de La Serna, A. E. Bogucka, M. Deptuła, A. A. Paul, K. Panek, E. Czechowska, M. Rychłowski, A. Królicka, J. Zieliński, S. Gabrielsson, M. Pikuła, M. Trzeciak, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Provision of funding; planning of the study; performing experiments: imaging of epidermis; data analysis; writing of the first and subsequent paper drafts

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## CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publication:

Title: **Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation**

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Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Performing lipidomic analysis on keratinocytes and small extracellular vesicles; analysis of the lipidomic data; figure preparation; participation in manuscript writing

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Olsztyn,
3.03.2023

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Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Performing secreted cytokine quantification by ELISA

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25.02.2023

Dr Rachel Emma Etherington

MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine

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John Radcliffe Hospital

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Here, I declare that my contribution to the publication:

Title: **Filaggrin insufficiency renders keratinocyte-derived small extracellular vesicles capable of affecting CD1a-mediated T cell responses and promoting allergic inflammation**

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Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Scanning of ELISpot plates and analysis of ELISpot data

City, date

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Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Optimization of conditions for lipidomic analysis of keratinocytes and small extracellular vesicles

Signature

Gdańsk. 24/02/2023

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Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Optimization of conditions for lipidomic analysis of keratinocytes and small extracellular vesicles

Edinburgh, Scotland,

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Professor Sara J Brown

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## CO-AUTHOR STATEMENT

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Under review in *The Journal of Extracellular Vesicles*, 2023

was as follows:

Data interpretation; provision of transcriptomic and proteomic data

Sara J Brown

London,
09/03/2023

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National Heart and Lung Institute

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# CO-AUTHOR STATEMENT

Here, I declare that my contribution to the publication:

Title: **Excess filaggrin in keratinocytes is removed by extracellular vesicles to prevent premature death and this mechanism can be hijacked by *Staphylococcus aureus* in a TLR2-dependent fashion**

Authors: L. Hovhannisyan, A. Kobiela, J. Bernardino de La Serna, A. E. Bogucka, M. Deptuła, A. A. Paul, K. Panek, E. Czechowska, M. Rychłowski, A. Królicka, J. Zieliński, S. Gabrielsson, M. Pikuła, M. Trzeciak, G. S. Ogg, Danuta Gutowska-Owsiak

Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Analysis of epidermal sheets microscopy images; contribution to manuscript writing

Jorge Bernardino de la Serna

Gdańsk, 03.03.2023

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Laboratory of Experimental and Translational Immunology

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Contribution to figure preparation

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Gdansk 2023 February 24

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Projects and acquisition of confocal microscopy images of keratinocytes

Signature

Gdańsk, 14.03.2023

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# CO-AUTHOR STATEMENT

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Under review in *The Journal of Extracellular Vesicles*, 2022

was as follows:

Obtaining blood plasma samples and clinical assessment of patients