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

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Research Article

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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 synthesis bias with downregulation of enzymes of ACSL, ELOVL and FADS families, observed both in filaggrin insufficient cells 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.

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\textsuperscript{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\textsuperscript{5–9}; this is reflected in abnormal functional properties of filaggrin-insufficient cells\textsuperscript{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\textsuperscript{10–12} as a part of innate defence. Filaggrin also modulates dendritic cell capacity to present antigens\textsuperscript{13,14} and T cell responses induced by activated keratinocytes\textsuperscript{15}, it also directly inhibits CD1a lipid neoantigen generation by phospholipase A2 (PLA2)\textsuperscript{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\textsuperscript{16,17}.

Small extracellular vesicles (sEVs), enriched in exosomes, are secreted organelles falling within the 30-150nm size range, released by all nucleated cells, including keratinocytes\textsuperscript{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 (IFNγ) 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 GO terms related to 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.

While primary keratinocytes growing in a monolayer are rather inefficient sEV producers and obtaining enough of those from keratinocytes isolated from atopic skin punch biopsy or 3D organotypic model is not feasible, the epidermis comprises of 10-15 layers of live keratinocytes, which altogether produce a lot of sEVs. Hence, the potential impact of filaggrin insufficiency would likely be substantial in the skin. Here, to overcome the issue of low sEV production from primary cell monolayer and still be able to conclude on the mechanisms relevant to the AD, a filaggrin knock-down keratinocyte line which we previously established by shRNA interference was the model of choice. 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. 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\textsuperscript{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\textsuperscript{26} and Reactome Knowledgebase\textsuperscript{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.

Our 2D monolayer model overcomes the limitation of insufficient exosomal/sEV yields in comparison to the 3D systems or skin samples, but the drawback is the potential lack the stratification-specific effects. Hence, 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.\textsuperscript{9} (Fig. 2, A to F and table S3) as well as the transcriptome dataset obtained from the skin samples of AD patients\textsuperscript{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.

As for the GO terms related to antigen presentation, only the analysis of the \textit{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\(_{sEV}\)).

*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 IFN\(\gamma\) 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 IFN\(\gamma\) 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. We also showed that mast cell-derived exosomes/sEVs may contain active PLA2 enzyme and supply it to induce neoantigen-specific T cell responses. 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 shFLGₜEV and shCₑEV, confirming that the exosomal/sEV lipid content was biased towards phospholipids, as expected. In terms of the changes in the relative content between shFLGₜEV and shCₑEV 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. 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. We saw increased contribution of saturated or monounsaturated long-chain fatty acids (LCFA)-containing PCs 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\textsubscript{SEV} vs shC\textsubscript{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 unsaturated fatty acids (UFAs; 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). This suggests that the UFAs were more likely to impact CD1a-mediated responses. When lipid sources were compared, it was clear that FAs identified as more abundant in shC\textsubscript{SEV} represented much greater variety and were also more suitable for CD1a presentation than those in shFLG\textsubscript{SEV} (Fig. 3J). Strikingly, when assessing the saturation of the FA chains, we found no single UFA to be more abundant in the shFLG\textsubscript{SEV} (Fig. 3J); at the same time, we detected three times more PUFA over MUFA species in shC\textsubscript{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\textsubscript{SEV} (Fig. 3L). Lastly, the number of double bonds in the FA chains also differed greatly, with no single FA more abundant in shFLG\textsubscript{SEV} containing those (Fig. 3M).

Altogether, our results imply that phospholipids in exosomes/sEVs secreted on the filaggrin insufficiency background do not contain FAs of sufficient variety and characteristics to constitute a diverse repertoire of CD1a ligands promoting T cell response.

\textit{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 IFN\textsubscript{γ} 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\textsuperscript{14,31,33–37}. While we noted some reactivity with certain donors manifesting a level of IFN\textsubscript{γ} 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 the 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\textsubscript{SEV}, together with PLA2, resulted in the induction in CD1a-specific IFN\textsubscript{γ} 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<sub>SEV</sub> 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).

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<sub>SEV</sub> and shC<sub>SEV</sub> undergo complete digestion by the enzyme and disappear from both shC<sub>SEV</sub> and shFLG<sub>SEV</sub> 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<sub>SEV</sub> in comparison to the digested control shC<sub>SEV</sub> (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<sub>SEV</sub>; shFLG<sub>SEV</sub> 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<sub>SEVs</sub> 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. Specifically, we included 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<sub>SEV</sub> vs shFLG<sub>SEV</sub> 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)<sup>38</sup>. 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<sup>38</sup>, i.e., SMd42:1, SMd42:2 and SMd42:3 (fig. S5D) which exhibit blocking potential on T cell activation.

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<sup>31,32,37–39</sup>. The results of our analysis showed that shFLG<sub>SEV</sub> 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<sub>SEV</sub> 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<sub>SEV</sub>. However, while the structure of this isomer is not known, the SMd42:1 species increased in abundance in shFLG<sub>SEV</sub> has the same composition as the one shown to be non-permissive by Cotton et al.<sup>38</sup>

With these new insights we recognized that the enrichment of lipids with inhibitory function in shFLG<sub>SEV</sub> 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<sub>SEV</sub> significantly inhibited IFNγ secretion from T cells while the addition of digested shC<sub>SEV</sub> 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<sub>SEV</sub> 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<sub>SEV</sub> but not by shC<sub>SEV</sub> 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., PC040:7 and PC036:6) 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. 7F). 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).

We also observed that filaggrin insufficiency significantly alters cellular content of several lipid species which are not PLA2 substrates, 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.

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 \textit{in vitro} models, whereas we could observe downregulation of \textit{ELOVL1}, \textit{ELOVL3}, \textit{ELOVL4} and \textit{ELOVL5} mRNA in AD skin (Figure 8F to I). In addition, we observed upregulation of the \textit{FADS1} mRNA expression in the cells (Figure 8J), likely of a compensatory nature in the 2D model, but downregulation of \textit{FADS1}, \textit{FADS2} and \textit{FADS6} mRNA in the skin of AD patients, suggesting more complex regulation of those enzymes during stratification process (Fig. 8K-M).

**Discussion**

Loss-of-function mutations in \textit{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\(^ {6,48}\). \textit{FLG} null mutations predispose to microbial dysbiosis\(^ {49}\) and reduced ability to control skin infections, resulting in \textit{S. aureus} superinfections\(^ {50}\) and a predisposition to eczema herpeticum\(^ {51}\).

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 \textit{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 \textit{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. 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; the longer the time the more type 1 bias. This “structure–activity relationship” has been proposed to result in a ligand-specific “cytokine fingerprint”. 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 insufficiency on keratinocytes as a whole and their exosomal/sEV compartment specifically. In addition, the loss of control of PLA2 activity in the filaggrin insufficiency scenario may lead to even greater dominance of the inhibitory ligands release from sEVs to 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; given that sEVs enter circulation and can be transferred to distant body organs, such a mechanism could provide some potential 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* which correspond to the lipid abnormalities previously reported in AD skin *in vivo*. 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 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. 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; they also have a preference towards polyunsaturated fatty acids. An increase in saturated fatty acids and a decrease in polyunsaturated fatty acid content has been described in rat hepatocytes in an ACSL3 knockdown model. As for the allergic manifestations, methylation of the ACSL3 5'CGI has been found to correlate with asthma status in children and reported to increase in an allergen-induced airway hyperreactivity model in mice. Furthermore, methylation of the ACSL3 gene has also been determined as a signature predictive of clinical food allergy in children. Interestingly, this enzyme was also found in exosomes/sEVs isolated from colostrum but not from mature breast milk; 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. 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. The upregulation of FADS1, which we believe may be a secondary compensatory mechanism, 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, by the effect on ceramide expression, e.g., silencing of the enzyme results in the accumulation of ceramides and sphingomyelin analogue in Drosophila (phosphoethanolamine ceramide; CerPE). 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.
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-restricted 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 \textit{M. tuberculosis} infection\(^{86}\) and to a range of \textit{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 \textit{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 allergens\(^{14}\) and pathogens may constitute\(^{49,50,89}\) a source of the phospholipase A2 activity, either directly\(^{90-92}\) or indirectly\(^{76,93,94}\) and any additional impact of those external factors may also add another layer of complexity to this system. 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\(^{95}\). Since keratinocyte-derived exosomes/sEVs transfer into the circulation and into peripheral tissues, the impact could potentially extend beyond the local tissue environment, affecting antimicrobial and homeostatic responses in the locations distant from the skin and promoting chronic inflammation and Th2 bias. This could be important given 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; however, the precise role of the sEV-mediated transfer of lipid antigens would have to be experimentally confirmed.

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 in those cells, resulting from aberrant differentiation that is apparent both \textit{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.

**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°C, 5% CO₂. 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\textsuperscript{104} and analysed with LIMMA\textsuperscript{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 measurement 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% CO₂. 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 H₂SO₄ 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; Ca²⁺ concentration was adjusted to 20 mM with CaCl₂. 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 with cysteine alkylation.
by iodoacetamide and proteolytic digestion by trypsin. Obtained digests were desalted by the STAGE Tips\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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.

Declarations

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).

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.

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**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 Appovals**
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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**Figures**
mRNA and protein expression signatures signify alterations in GO terms for proteins enriched in the exosomal/EV compartment in filagrin-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;
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.

Figure 2

Changes in cellular compartments are signified by differential GO term enrichment in the epidermal organotypic models and 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.

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
Figure 4
shFLGsEV 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.

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 Šidák's multiple comparisons test; *, p<0.05; **, p<0.01; PLA2, phospholipase A2; VIP, variable importance in projection; SMd, sphingomyelin; Cerd, ceramide.

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 glycerophosphoethanolamine; SFA, saturated fatty acid; UFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

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.
Figure 8

Supplementary Files

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- Kobielaetal.supplementaryfigures.pdf
- TableS1.xlsx
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- TableS6.xlsx