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Title: CD1a presentation of endogenous antigens by group 2 innate lymphoid cells

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One Sentence Summary: Human skin-derived ILC2 express CD1a and present endogenous PLA2G4A-dependent antigens to T-cells.

Abstract:

Group 2 innate lymphoid cells (ILC2) are effectors of barrier immunity, with roles in infection, wound healing and allergy. A proportion of ILC2 express MHCII and are capable of presenting peptide antigens to T-cells and amplifying the subsequent adaptive immune response. Recent studies have highlighted the importance of CD1a-reactive T-cells in allergy and infection, activated by the presentation of endogenous neolipid antigens and bacterial cell wall components. Here, using a human skin challenge model, we unexpectedly show that human skin-derived ILC2 can express CD1a and are capable of presenting endogenous antigens to T-cells. CD1a expression is upregulated by TSLP at levels observed in the skin of patients with atopic dermatitis, and the response is dependent on PLA2G4A. Furthermore, this pathway is used to sense *Staphylococcus aureus* by promoting TLR-dependent CD1a-reactive T-cell responses to endogenous ligands. These findings define a previously unrecognized role for ILC2 in lipid surveillance, and identify shared pathways of CD1a- and PLA2G4A-dependent ILC2 inflammation amenable to therapeutic intervention.

Introduction

Human group-2 innate lymphoid cells (ILC2) provide a rapid source of type-2 cytokines, producing large amounts of IL-13 and IL-5, as well as IL-6, IL-9, IL-4, GM-CSF and amphiregulin. ILC2 are present at barrier surfaces where they have essential roles in homeostasis and disease, including defense during viral (*1, 2*) and parasitic infections (*3, 4*); with emerging evidence suggesting responses to bacteria (*5*). Dysregulated ILC2 responses contribute to skin allergy and asthma (*6, 7*).

ILC2 rely on RORα for development (*8*), and the ILC family is thought to differentiate from Common Lymphoid Progenitors requiring signals via IL-2R common-γ chain, inhibitor of DNA binding 2, nuclear factor interleukin-3, T-cell factor 1, GATA-binding protein 3, promyelocytic leukemia zinc finger and Notch (*9*). In humans, ILC2 have been identified in blood, skin, nasal, gut and lung tissue (*10*), where they are identified by lack of cell surface markers of known lineages and by expression of IL-7Rα and CRTH2 (*11*). CRTH2 is the receptor for the lipid mediator PGD2, which is released from mast cells and other cells during infection and allergy (*12*). ILC2 are also characterized by expression of receptors for alarmin cytokines IL-25, IL-33 and TSLP (*13*). Such characteristics thus position ILC2 as rapid effectors and sentinels capable of mediating responses to cutaneous and mucosal barrier breach.

As well as being resident in healthy human skin, we and others showed that ILC2 are enriched within atopic dermatitis lesional skin (*7, 14, 15*). Furthermore, analysis of human skin biopsies and murine studies have established that skin trauma induces IL-33-dependent ILC2 proliferation, migration and amphiregulin expression (*7, 14, 16*). Notably, abrogation of these ILC2 responses impaired efficient wound closure. Murine and human ILC2 have been shown to express functional MHCII (*17, 18*) and a dialogue exists between antigen-specific $CD4^+$ T-cells and $MHCII^+$ ILC2. ILC2 presentation of peptide antigen to T-cells induces IL-2 production from T-cells, which in turn promotes ILC2 proliferation and IL-13 production. ILC2-derived IL-13 induced *N.brasiliensis* expulsion is dependent on ILC2 expression of MHCII.

CD1a is predominantly expressed in the skin, with constitutively high expression on Langerhans cells (LC), as well as subsets of dermal dendritic cells (DCs), macrophages and DCs at other sites,

and on thymocytes (*19*). CD1a is capable of presenting lipids to CD1a-reactive T-cells, including both self-lipids and exogenous lipids (*20, 21*). Until recently it was believed that T-cell receptor signaling was predominantly induced following the lipid polar head group interacting with the TCR of CD1a-responsive T-cells. Such CD1a ligands include sphingolipids and phospholipids, sulfatide and the mycobacterial lipopeptide didehydroxymycobactin (*22*). Within the last few years, our understanding of the diversity of CD1a ligands has extended. Smaller headless oily antigens derived, for example, from the sebum of the skin, bind CD1a and are capable of stimulating T-cells, without direct interaction of the TCR with the ligand (*23*).

Skin CD1a is positioned to signal barrier compromise to T-cells through presentation of endogenous or exogenous lipids. As well as promoting homeostasis and immunity, $CD1a^+$ antigen presenting cells (APCs) are enriched in atopic dermatitis lesions (*24*). Indeed the altered lipid milieu of lesional skin has the potential to convey damage (*25, 26*). CD1a-reactive T-cells have been found to circulate at relatively high frequencies and reside in healthy skin (*27*) and to secrete cytokines that contribute to defense against infection, wound healing and skin inflammation including IFNγ, IL-22, IL-13, TNFα, IL-17A and GM-CSF (*25, 28*).

Recently, we identified that allergen-derived phospholipase (PLA2) within house dust mite (HDM) and wasp and bee venom generate CD1a ligands by acting on membrane phospholipids and releasing neolipids that activate T-cell cytokine production (*25, 29*). In a recent study from our group, endogenous human phospholipase, PLA2G4D, released from mast cells in exosomes, generated ligands activating CD1a-reactive T-cells in psoriasis lesions (*28*).

In contrast, the PLA2G4A member of the PLA2 enzyme family is known to be elevated in allergic environments (*30-36*). PLA2G4A is cytosolic and generates arachidonic acid derivatives, but has not been examined for roles in CD1a-reactive T-cell responses. PLA2G4A is expressed by alveolar macrophages, mucosal epithelial cells of the lung, nose and small intestine (*37*), neutrophils (*38*), dendritic cells and mast cells(*39, 40*), and so would be well placed to generate neolipid antigens for presentation by the CD1 protein family at barrier surfaces. Here, we aimed to investigate whether ILC2 could contribute to human CD1a-mediated T-cell responses. Using an *in vivo* human skin challenge model, we observed that human skin ILC2 could express the CD1

group 1 protein CD1a which was regulated by TSLP. Furthermore ILC2 could present endogenous lipid antigens to CD1a-reactive T cells which was explained by their expression of PLA2G4A, and this pathway was used to sense *S. aureus* and promote skin inflammation. These findings link ILC2, PLA2G4A, and CD1a in contributing to human atopic skin inflammation, and raise the possibility that inhibition of this pathway may have therapeutic benefit.

Results

Human skin ILC2 can express CD1a

To analyze the phenotype of ILC2 in the skin upon allergic challenge, skin suction blisters were formed after intra-epidermal injection of HDM allergen and ILC2 were isolated by fluorescence guided cell sorting and gene expression analyzed by RNA sequencing (Fig. 1A, fig. S1A and Fig. 1B). While technically challenging to obtain sufficient cell numbers, the advantage of the skin suction blister system is that skin cells can be isolated without need for the physical/enzymatic processing required for conventional skin biopsies with attendant risks of modulation of gene expression. These analyses showed that the gene expression of CD1a was highly and specifically upregulated in skin blister-derived ILC2, in comparison to blood derived ILC2 or T-cells. Contrary to the recent report of murine ILC3 expression of CD1d (*41*), we did not detect meaningful CD1d expression on human blister-infiltrating ILC2 (Fig. 1B).

To validate the RNA sequencing result in multiple donors and under steady-state conditions, healthy unchallenged human skin was analyzed *ex vivo* for expression of CD1a by ILC2 using flow cytometry. CD1a was observed on a small proportion of the ILC2 analyzed within the whole skin biopsies suggesting functional heterogeneity (Fig. 1C). However, we reasoned that given the specific epidermal sub-location of LC which express high levels of CD1a (Fig. 1D and fig. S1B), epidermal ILC2 might also be enriched for CD1a expression. Indeed, CD1a was detected on approximately 20% of epidermal ILC2, at significantly higher levels than whole skin ILC2 (Fig. 1E, 1F and fig. S1C) ($p = 0.0116$). LC showed a similar mean fluorescence intensity of CD1a as epidermal $CD1a^{+}$ILC2, although CD1a was present on a greater proportion of the LC than ILC2 (Fig. 1D - F). CD1a was not observed on epidermal T-cells (Fig. 1G and fig. S1D). These findings suggested that subsets of skin-derived ILC2 express CD1a and therefore may be capable of presenting antigen to CD1a-reactive T-cells.

In contrast to skin ILC2, CD1a expression was not detected on blood derived ILC2 (Fig. 2A, 2B and fig. S1E and S1F), nor ILC2 cultured in human serum (Fig. 2C). In order to investigate whether the CD1a-expressing skin ILC2 represented a distinct lineage, or whether CD1a could be regulated by epidermal-derived cytokines, we measured the levels of IL-33, IL-25 and TSLP in

the skin suction blisters after HDM challenge in patients with atopic dermatitis and controls. TSLP was detected in human skin suction blister fluid following HDM challenge, and was found to be at a higher concentration in the blister fluid isolated from patients with atopic dermatitis (Fig. 2D) ($p = 0.0374$). We could not reliably identify IL-25 in the skin, and levels of IL-33 were not significantly different between subject groups ($p = 0.6655$, Fig. 2E). We therefore investigated the effects of TSLP on CD1a expression by ILC2, and showed TSLP induced CD1a expression. Notably, removal of human serum, and replacement with fetal calf serum (FCS), is known to release the inhibition of CD1a expression by DCs (*42*); cardiolipin and lysophosphatidic acid within human serum modulate CD1a expression. Therefore we cultured blood-derived ILC2 in FCS-containing media for 72 hours which increased CD1a expression by the blood derived ILC2 ($p = 0.0159$); and was further enhanced by TSLP (Fig. 2F and fig. S2) ($p = 0.0068$). In the presence of FCS, CD1a expression by ILC2 was also induced by IL-33, PGD2 and LTE4 at concentrations known to be functionally relevant for ILC2 (*12, 43*) (fig. S2). Taken together, these data suggested that skin-derived ILC2 expression of CD1a is regulated by skin-derived alarmin and by loss of inhibitory effects of serum. In this way, CD1a expression can be restricted to sub-locations limiting potential unconstrained CD1a autoreactivity in the steady state.

ILC2 are capable of activating T-cells via CD1a

To determine the functional significance of ILC2 CD1a expression, we analyzed ILC2 ability to present lipid antigens to polyclonal T-cells. ILC2 were co-cultured with autologous *ex vivo* blood derived polyclonal T-cells for 24 hours with or without anti-MHC I/II and anti-CD1a antibodies (OKT6). IFNγ and IL-22 production following co-culture showed ILC2 could mediate CD1a-dependent T-cell responses (Fig. 3A and 3B).

We sought to determine if ILC2, like dendritic cells (*25*), could present HDM-derived lipid ligands to T-cells via CD1a. We pulsed CD1a-induced blood-derived FCS-cultured ILC2 with HDM extract overnight at 7 μg/ml, with anti-CD1a or control. ILC2 were washed and co-cultured with polyclonal T-cells for 24 hours. HDM-pulsed ILC2 activated T-cells in a manner partially dependent on CD1a, inducing IFNγ and IL-22 secretion (Fig. 3A and 3B). ILC2 alone did not produce IFNγ or IL-22, even when stimulated with PMA and ionomycin (Fig. 3A and 3B).

ELISpot assays could not be used to analyze IL-13 secretion by T-cells because ILC2 derived-IL-13 could confound. We therefore used flow cytometry and showed HDM-pulsed ILC2 elicited T-cell derived IL-13, in a CD1a-dependent manner (Fig. 3C) ($p < 0.0001$).

Both the autoreactive response and presentation of HDM-induced ligands by ILC2 CD1a were amplified by TSLP (Fig. 4A and 4B). ILC2 were stimulated with two different concentrations of TSLP throughout our investigations: 50 ng/ml a typical concentration of TSLP used to stimulate ILC2 *in vitro* and 0.1 ng/ml that was more representative of TSLP levels we found in the skin (Fig. 2D). No statistically significant difference was found between prior treatment of ILC2 with the higher and lower concentrations of TSLP (Fig. 4C and 4D).

ILC2 express PLA2G4A

Phospholipase A2 (PLA2) has been identified as one of the components of HDM which generates neolipid antigens presented by CD1a (*25*). To determine if ILC2 could be induced to express host PLA2 in a HDM response, RNA sequencing data from HDM-challenged skin suction blister-derived ILC2 were analyzed for PLA2 gene expression. *PLA2G4A* and *PLA2G7* were most highly expressed by skin ILC2 (Fig. 5A). PLA2G7 has a preferred substrate of platelet activating factor and was more highly expressed by blood ILC2 than skin ILC2. Given the enriched skin-specific expression of *PLA2G4A*, we focused on its potential role in the generation of CD1a ligands. TSLP stimulation of ILC2 induced a significant upregulation of *PLA2G4A* gene expression (1.5 fold), measured by rtPCR. Cytosolic PLA2 can act extracellularly to generate CD1a ligands (*28*) and so we produced recombinant PLA2G4A and confirmed biochemical cPLA2 activity, which was inhibited by Methyl arachidonyl fluorophosphonate (MAFP) (Fig. 5B). PLA2G4A was incubated with FCS-cultured ILC2 overnight, with/without MAFP; the ILC2 were then washed prior to co-culture with T-cells. Activated T-cells produced IFNγ and IL-22, and MAFP blocked the response (Fig. 5C and 5D). There was no significant effect of MAFP on the unpulsed response suggesting that it is not acting non-specifically (IFNγ $p = 0.1805$, IL-22 $p > 0.9999$). Furthermore, the response was amplified by TSLP-induced $CD1a^{+}$ILC2 (Fig. 5E and 5F), and PLA2G4A

contributed to CD1a^{+}ILC2 presentation to IL-13-producing T-cells (Fig. 5G). These data suggested that the CD1a-mediated activation of T-cells by ILC2 is dependent on PLA2G4A.

Staphylococcus aureus promotes ILC2 presentation of endogenous antigens to CD1a-reactive T-cells

Staphylococcus aureus infection promotes PLA2G4A activity (*44-47*). *Lee et. al.* showed that *S.aureus* infection induces upregulation of PLA2G4A and elicits PGE2 and IL-6 responses in the lung, with TLR2-dependence (*47*). Atopic dermatitis is exacerbated by *S.aureus* and disease severity is directly associated with bacterial density (*48*). Thus, we reasoned that *S.aureus* may be sensed by a similar pathway. FCS-cultured ILC2 were pulsed with heat-killed *S.aureus* and co-incubated with T-cells. We observed that ILC2 exposed to *S.aureus* could activate T-cells to secrete IFNγ and IL-22, which was partially dependent on CD1a (Fig. 6A and 6B) (IFNγ $p < 0.0001$, IL-22 $p = 0.0002$). The IL-22 response was upregulated by prior incubation with TSLP. Interestingly, TSLP did not amplify the *S.aureus*–induced IFNγ response (Fig. 6C and 6D), suggesting distinct cytokine induction mechanisms; however IL-22 is known to trigger anti-microbial pathways in keratinocytes (*49*), potentially reflecting appropriate *S.aureus*-induced responses detected here. These data suggested that CD1a ligands were present in heat-killed *S.aureus*, and/or that *S.aureus* could generate endogenous CD1a ligands. We next incubated ILC2 with heat-killed *S.aureus* and assessed *PLA2G4A* mRNA expression, and observed *PLA2G4A* gene induction (Fig. 7A) ($p = 0.0258$). Heat-killed *S.aureus* can be employed experimentally as a source of TLR2 and TLR4 ligands, and so in order to investigate the underlying mechanisms, we analyzed the effects of Pam3CSK4 (TLR2 agonist) and LPS (TLR4 agonist) on *PLA2G4A* expression. Both agonists induced expression of the *PLA2G4A* gene by ILC2 (Fig. 7B).

These results raised the question of how ILC2-derived PLA2G4A could exert extracellular CD1a dependent effects. As discussed above, PLA2G4A could be produced from ILC2 in a manner similar to release of PLA2G4D from mast cells (*28*). Therefore we assayed ILC2 culture supernatant for cPLA2 activity to determine if cPLA2 was produced by ILC2 *in vitro*. As was observed at the mRNA level, TLR2 ($p = 0.0152$) and TLR4 ligands ($p = 0.0413$), and heat-killed *S.aureus* ($p < 0.0001$) stimulated release of cPLA2 by ILC2 (Fig. 7C). It is of note that the heat-

killed *S.aureus* preparation did not contain cPLA2 or sPLA2 activity. We next compared the capability of ILC2 isolated from the blood of patients with atopic dermatitis (AD) and healthy controls, to express PLA2G4A following stimulation with TLR2 and TLR4 ligands, and heat-killed *S.aureus*. AD patient-derived ILC2 showed a greater capacity to produce PLA2G4A in response to LPS ($p = 0.0329$) and PamCSK ($p < 0.0001$) consistent with disease-relevance of the pathway (Fig. 7D). In order to confirm the roles of TLR2 and TLR4, ILC2 were pre-incubated with cPLA2 inhibitor or anti-TLR2 and TLR4 antibodies, and then pulsed with heat-killed *S.aureus* prior to co-culture with T-cells. The CD1a-dependent activation of T-cell IFNγ, IL-22 and IL-13 production was reduced by inhibition of cPLA2 and of TLR2 and TLR4 signaling (Fig. 8A, 8B, 8C). Taken together, these data showed that the *S. aureus*-derived TLR2 and TLR4 ligands can induce PLA2G4A-dependent presentation of endogenous ligands to CD1a-reactive T-cells.

Discussion

CD1a is expressed at constitutively high levels by LC, which instigate innate and adaptive immune responses within the skin, presenting both host and foreign lipid ligands to effector T-cells at the site of damage or infection (*25, 29, 50*). The role of LC in presentation of CD1a ligands is well established and has been demonstrated in both human studies (*25, 28, 29*) and *in vivo* using a CD1a transgenic model of contact dermatitis and psoriasis (*50*). We now show a previously unrecognized role for ILC2 as CD1a-expressing barrier sentinels with lipid antigen presentation capacity, and suggest that CD1a antigen presentation occurs through a more diverse population of cells. We observed that CD1a was highly expressed on a subpopulation of skin ILC2 enriched in healthy epidermis, and expressed at a comparable intensity to skin APC. CD1a expression was induced by the epidermal alarmin TSLP and by loss of inhibitory effects of serum. Thus $CD1a^+$ ILC2 may represent an antigen-presenting subpopulation of skin resident ILC2 which can be further regulated under certain stimuli, for example the cytokine milieu associated with skin damage or infection. ILC2-expressing CD1a induced a T-cell response which was found to be partially dependent on PLA2G4A, consistent with the generation of permissive CD1a ligands (*27, 28, 51*).

Recently, we showed that CD1a contributes to the inflammatory response to HDM in the skin, and indeed LC are enriched within atopic dermatitis lesions. HDM generated neolipid antigens presented by CD1a to T-cells in the blood and skin of affected individuals. HDM-derived secretory PLA2 was proposed to cleave membrane phospholipids generating CD1a ligands. This response was controlled by filaggrin inhibition of PLA2. Thus genetic loss of filaggrin inhibition of HDM-sPLA2 may alert CD1a-reactive T-cells to barrier compromise. Dysregulated ILC2 responses have been implicated in allergy, and indeed human ILC2 are enriched in atopic dermatitis lesions and rhinosinusitis nasal polyps (*11*). ILC2 responses are thought to be activated by epithelium-derived cytokines, specifically IL-25, IL-33 and TSLP. These cytokines are rapidly released from damaged epithelial cells in response to stress such as infection, injury and inflammation, and activate ILC2. Many known allergens, such as HDM, contain enzymes that cause damage to the epithelium releasing type-2-inducing cytokines (*52*). This initial damage response then amplifies innate immunity and alerts the adaptive immune system. Indeed IL-33 induces ILC2 production of cytokines that recruit and activate Th2 cells, eosinophils, mast cells and induce hyper-

responsiveness in the epithelium (*7, 13, 53*). Here we show that in addition to production of effector cytokines, ILC2 can present HDM ligands directly to T-cells via CD1a. HDM-challenged ILC2 activated T-cells to produce IFNγ, IL-22 and IL-13. It is of interest that our results suggest that ILC2 can interact directly with allergens and are not limited to activation by damage-associated cytokine release.

A striking feature of atopic dermatitis lesional skin is the colonization by *S.aureus* (*48*). ILC2 and CD1a proteins would be poised to interact with the bacteria, being enriched in lesional skin. Our results suggest that ILC2 could activate inflammatory T-cells in a CD1a-dependent manner through direct sensing of *S.aureus* and through production of PLA2G4A. Indeed a study of human ILC2 in lesional atopic dermatitis skin found elevated ILC2 numbers and observed that ILC2 are in close contact with T-cells in tissue sections (*54*). PLA2 production by ILC2 has not previously been described, however it is of interest that increased expression of PLA2G4A has been observed in HDM-induced dermatitis skin lesions (*55*) and has been linked to allergic disease in a number of studies (*56-59*). These findings implicate an inflammatory cycle in which ILC2 PLA2G4A promotes a CD1a-dependent T-cell response, as well as the production of arachidonic acid pathway derivatives including PGD2, for which the receptor CRTH2 is expressed by ILC2 (*12*). Successful attempts have been made to therapeutically target PLA2G4A, showing inhibition of inflammation in animal models of both skin and lung allergic disease (*55, 60*).

Given the known PLA2G4A inducing capacity of *S.aureus*, we reasoned that this CD1a pathway may contribute to the *S.aureus* associated skin inflammation. We confirmed *S.aureus* induced PLA2G4A and showed that this could be mediated by TLR2 or TLR4 stimulation. The PLA2G4A-induction associated with enhanced capacity of ILC2 presentation of endogenous neolipid antigens to CD1a-reactive T-cells, suggesting that *S.aureus* may be sensed through this CD1a pathway to promote an inflammatory response. Inhibition of cPLA2 activity and TLR2 and 4 signaling demonstrated that PLA played a dominant role in CD1a sensing of *S.aureus*. However we cannot rule out the hypothesis that heat-killed *S.aureus* also contained ligands which could be directly presented by CD1a on ILC2, even though only a minor PLA-independent effect was measured. It will be important to investigate the nature of such potential ligands. CD1a can capture and display extracellular lipids without the use of more complex intracellular processing

pathways, trafficking between the cell surface and early endosomes (*61*). Our study now highlights the potential role of CD1a in ILC2 lipid antigen presentation in humans with dependence on PLA2G4A; indeed we show that ILC2 derived from atopic dermatitis patients displayed greater capacity to express PLA2G4A.

While the skin suction blister technique offered us access to human skin fluid and cells directly *ex vivo* without the need for further processing, it does add a potential limitation of the study. Skin suction blisters inevitably introduce physical trauma to the skin and so comparisons with/ without antigen become important. In addition when studying ILC2, multiple suction blisters are required, thus participant numbers become limiting. The scarcity of ILC2 in the skin and suction blisters lead to modelling of the $CD1a^{+}$ILC2 subpopulation in cultured blood ILC2 to generate sufficient numbers of cells for functional analyses. This added complexity to our study and is something we considered when interpreting the data and in the use of experimental controls and neutralizing antibodies. It can be difficult to prove causality in human immunology, despite the need for translational work involving human subjects. Human skin antigenic challenge offers temporal associations with clinical and immunological findings, lending support of causality, but CD1a transgenic models and human skin grafts in immunodeficient models may offer further evidence in the future.

The identification of skin-derived ILC2 as cells with CD1a antigen presentation capacity furthers our understanding of the cross-talk between ILC2 and T-cells. The presence of $CD1a^{+}$ILC2 resident in the epidermis facilitates rapid sensing of immunological stress and defense against infection and wound healing. Therapeutic strategies to regulate $CD1a^{+}$ ILC2 and PLA2G4A activity may provide novel treatment opportunities for inflammatory skin disease.

Supplementary materials

Fig. S1. Gating strategy for analysis of ILC2.

Fig. S2. Stimulation of CD1a expression by blood derived ILC2

Supplementary Materials and Methods

Table S1. PDF file of Source data

Table S2. Table of R.P.K.M expression values for RNA sequencing data.

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Figure legends

Fig. 1: Human skin ILC2 express CD1a.
A. Flow cytometry gating strategy for blister fluid derived human ILC2. ILC2 are $CD45^+$/ $Lineage^-$ /$CRTH2^+$/$IL7R\alpha^+$. **B.** CD1a and CD1d gene expression of skin and blood derived ILC2 and T-cells determined by RNA Sequencing and measured in Reads Per Kilobase of transcript per Million mapped reads (RPKM). **C.** Whole thickness skin samples were homogenized and analyzed by flow cytometry for presence of ILC2 and expression of CD1a. **D.** CD1a expression by epidermal $CD11c^+$ cells was analyzed by flow cytometry. **E.** CD1a expression by epidermal ILC2 was analyzed by flow cytometry. **F.** Summary of CD1a expression on ILC2 as a proportion of total ILC2 derived from whole-thickness skin and epidermis. **G.** CD1a expression by epidermal T-cells was analyzed by flow cytometry. Flow cytometry data representative of at least 10 independent experiments and n = 12 donors.

Fig. 2: Human blood ILC2 could be stimulated to express CD1a.
A. Flow cytometry gating strategy for blood derived human ILC2. ILC2 are $CD45^+$/$Lineage^-$ /$CRTH2^+$/$IL7R\alpha^+$. **B.** Flow cytometric analysis of CD1a expression on blood ILC2. Flow cytometry data representative of n = 8 donors. **C.** Flow cytometric analysis of human serum cultured CD1a expression on blood ILC2 representative of n = 8 donors. **D.** Multiplex bead array analysis of TSLP concentration in HDM challenged blister fluid of healthy (HC) and atopic dermatitis (AD) patients ($p = 0.0374$, n = 5 – 6, t-test). **E.** Multiplex bead array analysis of IL-33 concentration in HDM challenged blister fluid of healthy (HC) and atopic dermatitis (AD) patients ($p = 0.6655$, n = 8 – 21, t-test). **F.** Culture of human blood derived ILC2 in the absence of human serum. Flow cytometry analysis of CD1a isotype control, CD1a expression on FCS cultured blood ILC2 and upon TSLP stimulation (plots left to right). Data representative of n = 3 donors and 3 independent experiments. *, $P < 0.05$; unpaired Student's *t* test (mean and SD).

Fig. 3: ILC2 present HDM-derived lipid ligands to CD1a responsive T-cells.
Autologous ILC2 and T-cells were isolated from donor PBMCs by fluorescence activated cell sorting and CD3 MACS microbead separation respectively. **A. and B.** Prior to co-culture with autologous T-cells, ILC2 were pulsed with HDM extract (7 µg / ml), and IFNγ **(A.)** and IL-22 **(B.)** production was detected by ELISpot in the absence or presence of 10 µg / ml anti-CD1a blocking antibody or isotype control. In addition ILC2 alone were stimulated with PMA (10 ng / ml) and Ionomycin (500 ng / ml) (P/I). (n = 8 donors, one-way ANOVA, data represent at least 6 independent experiments). **C.** ILC2 were pulsed in the presence or absence of HDM extract (7 µg / ml) and co-cultured with autologous T-cells. Intracellular staining for flow cytometry was used to assess the proportion of T-cells expressing IL-13 in the presence or absence of anti-CD1a blocking antibody or isotype control, 10 µg / ml. (n = 3 donors, one-way ANOVA, 3 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ RM-one way-ANOVA with Tukey's post-hoc test (mean and SD).

Fig. 4: TSLP further enhances the ability of ILC2 to present CD1a ligands to T-cells.
ILC2 were cultured in 10 % FCS and stimulated in the presence or absence of TSLP for 72 hours prior to pulsing with HDM extract and subsequent ELISpot analysis of capacity to activate T-cells. **A. and B.** Effect of the presence or absence of prior stimulation of ILC2 with TSLP on number of IFNγ **(A.)** or IL-22 **(B.)** producing T-cells induced by co-culture. Fold change was calculated

relative to the unpulsed autoreactive baseline response in the absence TSLP (represented by dotted line). **A.** Graph shows the effect of TSLP on the unpulsed ($p = 0.0221$) and HDM-induced ($p = 0.0111$) IFNγ responses. **B.** Graph shows the effect of TSLP on the unpulsed ($p = 0.0091$) and HDM-induced ($p = 0.0006$) IL-22 responses. (n = 8 donors, t-test, data represent at least 6 independent experiments). **C. and D.** Effect of TSLP concentration upon amplification of CD1a dependent T-cell production of IFNγ **(C.)** or IL-22 **(D.)**. Fold change calculated between cytokine spots produced following T-cell culture with unstimulated and TSLP stimulated ILC2. Graph showing two concentration of TSLP 50 ng / ml and 0.5 ng / ml. (n = 8 donors, t-test, data represents 3 - 6 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ paired Student's *t* test (mean and SD).

Fig. 5: ILC2 express PLA2G4A which generates CD1a ligands.
A. PLA2 gene expression analysis of skin and blood derived ILC2 and T-cells determined by RNA Sequencing and measured in Reads Per Kilobase of transcript per Million mapped reads (RPKM). **B.** Cytosolic PLA2 activity of recombinant PLA2G4A irreversibly inhibited by 1 μM MAFP measured using a biochemical assay kit. **C. and D.** Autologous ILC2 and T-cells were isolated from donor PBMCs by fluorescence activated cell sorting and CD3 MACS bead separation respectively. Prior to co-culture with autologous T-cells, ILC2 were either unpulsed (U) or pulsed with 1 μg / ml PLA2G4A or PLA2G4A inhibited with 1 μM MAFP. IFNγ **(C**.) and IL-22 **(D.)** production was detected by ELISpot in the absence or presence of 10 μg / ml anti-CD1a blocking antibody or isotype control (n = 8 donors, one-way ANOVA, data represent at least 6 independent experiments). **E. and F.** ILC2 were cultured in FCS and stimulated in the presence or absence of TSLP for 72 hours prior to pulsing with PLA2G4A. Effect of the presence or absence of prior stimulation of ILC2 with TSLP upon number of IFNγ **(E.)** ($p = 0.0226$) or IL-22 **(F.)** ($p = 0.0029$) producing T-cells induced by co-culture. Fold change was calculated relative to the unpulsed autoreactive response in the absence TSLP (represented by dotted line). Statistics calculated between the baseline response or as indicated in the figure. (n = 8 donors, t-test data represent at least 6 independent experiments). **G.** Intracellular staining for flow cytometry was used to assess the proportion of T-cells expressing IL-13 in the presence or absence of 10 μg / ml anti-CD1a blocking antibody or isotype control upon co-culture with ILC2 pulsed or unpulsed (U) with PLA2G4A. (n = 3 donors, one-way ANOVA, 3 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ RM-one way-ANOVA with Tukey's post-hoc test (mean and SD).

Fig. 6: ILC2 present bacterial lipid ligands derived from *Staphylococcus aureus*
A. and B. Autologous ILC2 and T-cells were isolated from donor PBMCs by flow cytometric sorting and CD3 MACS bead separation respectively. Prior to co-culture with autologous T-cells, ILC2 were pulsed with heat-killed *S. aureus* preparation (HKSA). IFNγ **(A.)** and IL-22 **(B.)** production was detected by ELISpot in the absence or presence of 10 μg / ml anti-CD1a blocking antibody or isotype control. (n = 8 donors, one-way ANOVA, data represent at least 6 independent experiments) **C. and D.** ILC2 were cultured in FCS and stimulated in the presence or absence of TSLP for 72 hours prior to pulsing with HKSA (10^8 cells / ml). Effect of the presence or absence of prior stimulation of ILC2 with TSLP upon number of IFNγ **(C.)** or IL-22 **(D.)** producing T-cells induced by co-culture. Fold change was calculated relative to the unpulsed autoreactive response in the absence TSLP (represented by dotted line). Statistics calculated between the baseline

response or as indicated in the figure. (n = 8 donors, t-test, data represent at least 6 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ RM-one way-ANOVA with Tukey's post-hoc test (mean and SD).

Fig. 7: Bacterial components can stimulate ILC2 to produce cPLA2

A. Real-time PCR analysis of PLA2G4A gene expression by ILC2 following stimulation with heat-killed *S. aureus* preparation (HKSA) ($p = 0.0258$, n = 3, t-test, data representative of 3 independent experiments). **B.** Real-time PCR analysis of PLA2G4A gene expression by ILC2 following stimulation with TLR2 (PamCSK 10 μg / ml) and TLR4 (LPS 1 μg / ml) ligands. (n = 3, t-test, data representative of 3 independent experiments). **C.** Cytosolic PLA2 activity was measured in the supernatant of ILC2 stimulated with TLR2 and TLR4 ligands, or HKSA (10^8 cells / ml) (n = 6, t-test, data representative of 3 independent experiments). **D**. Real-time PCR analysis of PLA2G4A gene expression by healthy or atopic dermatitis ILC2 following stimulation with TLR2 (PamCSK 10 μg / ml) and TLR4 (LPS 1 μg / ml) ligands or heat-killed *S. aureus* (HKSA, 10^8 cells / ml). (n = 5 donors, one-way ANOVA, data representative of 4 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (mean and SD).

Fig. 8: TLR stimulation of ILC2 by *Staphylococcus aureus* induces PLA2G4A and generation of lipid ligands which can be presented to T-cells by CD1a.

Autologous ILC2 and T-cells were isolated from donor PBMCs by flow cytometric sorting and CD3 MACS microbead separation respectively. Prior to co-culture with autologous T-cells, ILC2 were pulsed with HKSA (10^8 cells / ml) (SA) with or without inhibition of cPLA2 (1 μM MAFP) or TLR2 and TLR4 signaling (10 μg / ml anti-TLR2 and anti-TLR4). IFNγ **(A.)** and IL-22 **(B.)** production was detected by ELISpot and IL-13 was detected by flow cytometry **(C.)** in the absence or presence of 10 μg / ml anti-CD1a blocking antibody or isotype control. ELISpot data represent at least 6 independent experiments and n = 8 donors, one-way ANOVA. IL-13 FACS data represent n = 3 donors, one-way ANOVA, 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ RM-one way-ANOVA with Tukey's post-hoc test (mean and SD).