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CD1a presentation of endogenous antigens by group 2 innate lymphoid cells

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Abstract

Group 2 innate lymphoid cells (ILC2) are potent effectors of barrier immunity, with critical roles in infection, wound healing and allergy. In addition to rapid production of cytokines, a proportion of ILC2 express MHC-II 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 on ILC2 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 new role for ILC2 in lipid surveillance, and identify shared pathways of CD1a- and PLA2G4A-dependent ILC2 inflammation amenable to therapeutic intervention.

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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 have been primarily identified at mucosal and skin barrier sites where they have been shown to 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 the transcription factor RORα for development (8), and more broadly the ILC family is thought to differentiate from the Common Lymphoid Progenitor and have been shown to require signaling via IL-2R common γ (γc) chain receptor, inhibitor of DNA binding 2 (Id2), nuclear factor interleukin-3 (Nfil3), T cell factor 1 (TCF1), GATA-binding protein 3 (GATA3), promyelocytic leukemia zinc finger (PLZF) and Notch (9). In humans, ILC2 have been identified in the blood, skin, nasal, gut and lung tissue (10), where they are identified by a lack of cell surface markers of known lineages and are positively defined by IL-7Rα and CRTH2 expression (11). CRTH2 is the receptor for the lipid mediator and ILC2 activating factor PGD2, which is released from activated mast cells and other cells during infection and allergy (12). ILC2 are also characterized by expression of the cell surface receptors for the alarmin cytokines IL-25, IL-33 and TSLP (13). These cytokines are released predominantly by epithelial cells following infection and damage. 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 previously showed that ILC2 are enriched and activated 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 has been established between antigen-specific $CD4^+$ T cells and a population of $MHCII^+$ ILC2. ILC2 presentation of peptide antigen to T cells induces IL-2 production from the T cells, which in turn promotes ILC2 proliferation and IL-13 production. ILC2-derived IL-13 induces *Nippostrongylus brasiliensis* expulsion which 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 a wide variety of ligands to CD1a-reactive T cells, including both self-lipids and ligands derived from foreign sources (20, 21). Until recently it was believed that T cell receptor signaling was induced following ligand binding, with the lipid acyl chain buried in the hydrophobic antigen-binding groove, from where the polar head group protrudes to interact with the TCR of CD1a-responsive T cells. Such CD1a ligands include sphingolipids and phospholipids, glycolipids such as sulfatide and the mycobacterial lipopeptide didehydroxymycobactin

(22). Within the last few years however, our understanding of the diversity of ligands that can be presented by CD1a has extended. Smaller headless oily antigens derived, for example, from the sebum of the skin were shown to bind CD1a and were capable of stimulating T cells, without direct interaction of the TCR with the ligand. Interestingly in this case the TCR interacts only with the CD1a molecule itself (23).

Expression in the skin positions CD1a proteins 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 via regulation of CD1a-mediated T cell activation (25, 26). CD1a-reactive T cells have been found to circulate at relatively high frequencies and reside in healthy skin (27) and to secrete a variety of 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 group 2 phospholipase (PLA2) activity has been found in house dust mite (HDM) allergen and wasp and bee venom to generate CD1a ligands by acting on membrane phospholipids and releasing neolipids that have the potential to activate T cell inflammatory cytokine production (25, 29). In a recent study from our group, endogenous human phospholipase, PLA2G4D, released from mast cells in exosomes, was shown to generate ligands that activate CD1a-reactive T cells in psoriasis lesions (28).

In contrast, the PLA2G4A member of the PLA2 enzyme family is known to be elevated in atopic and allergic environments (30–36). PLA2G4A is a cytosolic PLA which generates arachidonic acid pathway derivatives, but has not been examined for its potential role in contributing to CD1a-reactive T cell responses. PLA2G4A has been documented to be 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 CD1-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

ILC2 have been shown to regulate cutaneous barrier protection, through orchestrating re-epithelialization during cutaneous wound healing (16). To analyze the phenotype of ILC2 in

the skin upon allergic challenge, skin suction blisters were formed after intradermal injection of HDM allergen and the ILC2 were isolated by fluorescence guided cell sorting and gene expression analyzed by RNA sequencing (Fig. 1A, fig. S1A and 1B). While technically challenging to obtain sufficient cell numbers, the advantage of the skin suction blister system is that cells can be isolated directly from the skin without the need for physical or enzymatic processing which is required for conventional skin biopsies with the attendant risks of modulation of gene expression. In addition, ILC2 from the blood were isolated and analyzed in parallel, to aid identification of skin specific surface markers or other selective gene expression. These analyses showed that the gene expression of the group 1 lipid presentation molecule CD1a was highly and specifically upregulated in skin blister-derived ILC2, in comparison to blood derived ILC2 or to T cells. Contrary to the recent report of murine ILC3 expression of CD1d (41), we did not detect significant CD1d expression on human blister-infiltrating ILC2 (Fig. 1B).

ILC2 are thought to be tissue resident and are reportedly present in unchallenged healthy skin (7). To validate the RNA sequencing skin suction blister result in multiple donors and under steady state conditions, samples of healthy unchallenged human skin were 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 Langerhans cells which express high levels of CD1a (Fig. 1D and fig. S1B), that ILC2 from the epidermis might also be enriched for CD1a expression. Indeed, CD1a was detected on approximately 20% of ILC2 within the epidermis, at significantly higher levels than in the whole skin ILC2 population (Fig. 1E, 1F and fig. S1C). Langerhans cells showed a similar expression level (mean fluorescence intensity) of CD1a as epidermal $CD1a^+$ ILC2, although CD1a was present on a greater proportion of the Langerhans cells than ILC2 (Fig. 1D - F). As expected from the RNA sequencing data, 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. Although RNA levels of these cytokines are known to be elevated in atopic dermatitis lesions (7), there are few data which have examined the cytokine protein concentrations in skin. 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). We could not reliably identify IL-25 in the skin, and levels of IL-33 did not significantly change ($p = 0.9289$) with HDM challenge (Fig. 2E). We therefore investigated the effects of TSLP on CD1a expression by ILC2, and showed TSLP induced an increase in CD1a expression. Notably removal of human serum from culture media, and replacement with fetal calf serum (FCS), has been previously shown to release the inhibition of CD1a expression by cultured DCs (42). The authors showed that cardiolipin and lysophosphatidic acid within human serum modulates

CD1a expression. Therefore we cultured blood-derived ILC2 in FCS-containing media for 72 hours, without human serum, which resulted in a significant increase in CD1a expression by the blood derived ILC2 which was further enhanced by TSLP (Fig. 2F). In the presence of FCS, TSLP showed the greatest CD1a induction compared to IL-25, IL-33, PGD2 and LTE4 at concentrations previously shown 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 certain locations to limit 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 performed ELISpot assays to analyze the ability of ILC2 to present lipid antigens to polyclonal T cells. ILC2 were co-cultured with autologous *ex vivo* blood derived polyclonal T cells for 24 hours. Peptide-MHC-TCR interactions were blocked by addition of anti-MHC I/II antibodies to allow detection of CD1a dependent responses. Specificity of the responses to CD1a was analyzed by blocking with anti-CD1a antibody (OKT6) in control co-cultures. IFNγ and IL-22 production following co-culture was assessed by ELISpot analysis and showed that ILC2 could mediate a CD1a-dependent T cell response (Fig. 3A and 3B).

ILC2 are known to increase in number and cytokine production following murine and human allergen challenge (44, 45). 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 clinical HDM extract overnight at 7 μg /ml, with or without prior incubation of ILC2 with anti-CD1a antibody or isotype control. ILC2 were then washed to remove unbound HDM allergen and co-cultured with polyclonal T cells for 24 hours and ELISpot assay performed. HDM-pulsed ILC2 were observed to activate T cells in a manner partially dependent on CD1a leading to secretion of IFNγ and IL-22 (Fig. 3A and 3B). Anti-CD1a inhibited both the auto-reactive and HDM-induced T cell response. 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 as the result may be confounded by ILC2 derived IL-13. We therefore used flow cytometry to detect intracellular IL-13 production by T cells following co-culture with ILC2. HDM-pulsed ILC2 were capable of eliciting T cell derived IL-13, in a CD1a-dependent manner (Fig. 3C).

Prior activation of FCS-cultured ILC2 with TSLP for 72 hours before co-culture with autologous T cells, amplified the CD1a response. Both the autoreactive response (unpulsed) and the presentation of HDM-induced ligands by ILC2 CD1a were amplified by the stimulatory action of 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 deemed a more physiological concentration, representative of TSLP levels we had 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 allergenic components of HDM, which can also generate neolipid antigens that can be recognized by CD1a. HDM, bee and wasp venom derived sPLA2, and endogenous cPLA2G4D have been identified to activate T cell cytokine production in a CD1a-dependent manner (25, 28, 29). 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 was more highly expressed by blood ILC2 than ILC2 derived from the skin, which is of interest given its preferred substrate of platelet activating factor. Given the enriched skin-specific expression of PLA2G4A, we therefore focused on a potential role for PLA2G4A in the generation of CD1a ligands. Pathogen or allergen-induced ILC2 derived PLA2 activity could be a novel route for generating CD1a ligands, and signaling the presence of infection or damage. Interestingly, Cheung *et al.* showed that cPLA2 (PLA2G4D) could be released from mast cells, and act extracellularly to induce CD1a responses (28). We first showed that TSLP could induce PLA2G4A expression in ILC2, which could amplify the effects of TSLP-induced CD1a expression. TSLP stimulation of ILC2 induced a statistically significant upregulation of PLA2G4A gene expression of approximately 1.5 fold, as measured by real-time PCR. To assess the potential for PLA2G4A to contribute to the activation of T cells, we produced recombinant PLA2G4A. Recombinant PLA2G4A showed biochemical cPLA2 activity which was inhibited in the presence of the irreversible inhibitor of cPLA2, Methyl arachidonyl fluorophosphonate (MAFP) (Fig. 5B). Thus the enzyme was shown to be active and allowed us to test whether it could play a role in ILC2 CD1a responses. PLA2G4A was incubated with FCS-cultured ILC2 overnight, with or without cPLA2 inhibitor (MAFP); the ILC2 were then washed prior to co-culture with T cells. Activation of T cells was induced by interaction with ILC2 treated with exogenous cPLA2G4A, in a manner partially dependent on CD1a. Activated T cells produced IFNγ and IL-22, and the cPLA2 inhibitor 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. Furthermore, the response was amplified by TSLP induced activation of $CD1a^+$ ILC2 (Fig. 5E and 5F). In addition, T cell IL-13 production was analyzed by intracellular flow cytometry and showed that PLA2G4A contributed to ILC2 presentation of endogenous CD1a ligands, and IL-13 production by responding 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

A number of studies have reported that *Staphylococcus aureus* infection promotes PLA2G4A activity (46–49). *Lee et. al.* showed that *S. aureus* infection induces upregulation of PLA2G4A and elicits pro-inflammatory PGE2 and IL-6 responses in the lung, in a TLR2 dependent manner (49). Atopic dermatitis is exacerbated by responses to skin bacteria including *S. aureus* and disease severity is directly associated with bacterial density (50). Thus, we reasoned that *S. aureus* may be sensed by a similar pathway. We therefore investigated whether ILC2 sensing of bacterial products could provide a CD1a-mediated

signal to T cells. 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 indeed activate T cells to secrete IFNγ and IL-22, and this response was partially dependent on CD1a (Fig. 6A and 6B). 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 it is of interest that IL-22 is known to trigger anti-microbial pathways in keratinocytes (51) which may reflect an appropriate *S.aureus*-induced response detected here. These data suggested that CD1a ligands could be present in the preparation of heat killed *S. aureus*, or that *S. aureus* could generate endogenous CD1a ligands. We next incubated ILC2 with heat-killed *S. aureus* and assessed PLA2G4A mRNA expression by real-time PCR, and indeed PLA2G4A gene expression was induced (Fig. 7A). 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. TLR2 and 4 agonists induced an increase in 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 the exosomal 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 and TLR4 ligands, and heat-killed *S. aureus* 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. To further investigate the potential involvement of this pathway in human disease we 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 significantly greater capacity to produce PLA2G4A in response to LPS and PamCSK consistent with disease-relevance of the pathway (Fig. 7D). In order to confirm the direct 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* preparation 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 activity and of TLR2 and TLR4 signaling (Fig. 8A, 8B and 8C respectively). CD1a-dependent activation of T cells was virtually ablated by cPLA2 inhibition, but it remains possible that *S. aureus* also contains lipid ligands or stimuli of other additional pathways that could induce ILC2 presentation of CD1a ligands to T cells. 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 Langerhans cells, 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, 52). The role of Langerhans cells 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 murine model of contact dermatitis and psoriasis (52). With this study 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 than previously considered. We observed that CD1a was highly expressed on a subpopulation of skin ILC2 that was enriched in the epidermis in the absence of inflammation, and expressed at a comparable intensity to skin antigen presenting cells. 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. The CD1a was functional and able to induce a T cell response which was found to be partially dependent on PLA2G4A expression by ILC2, consistent with the known role of PLA2G4A in the arachidonic acid pathway, and the generation of permissive and non-permissive CD1a ligands (27, 28, 53).

Recently, we showed that CD1a contributes to the inflammatory response to house dust mite in the skin, and indeed Langerhans cells are enriched within atopic dermatitis skin lesions. House dust mite, an allergen linked to atopic disease, was shown to generate neolipid antigens which could be 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 action. Thus genetic loss of filaggrin inhibition of HDM-sPLA2 may alert CD1a-reactive T cells to barrier compromise, in atopic dermatitis patients. Dysregulated ILC2 responses have also been implicated in allergy, and indeed human ILC2 are enriched in atopic dermatitis lesions and chronic rhinosinusitis nasal polyps (11). Pro-inflammatory ILC2 responses are thought to be activated primarily 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 potently activate ILC2. Many known allergens, such as house dust mite, contain enzymes that cause damage to the epithelium releasing type-2 inducing cytokines (54). This initial damage response then amplifies innate immunity and alerts the adaptive immune system to the barrier compromise, inducing a pro-inflammatory response to the innocuous allergen. Indeed IL-33 induces ILC2 production of cytokines that recruit and activate Th2 cells, eosinophils, mast cells and induce hyper-responsiveness in the epithelium, in response to barrier disruption (7, 13, 45). 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, cytokines involved in the pathogenesis of atopic dermatitis. It is of interest that our results suggest that ILC2 can interact directly with allergens and are not limited to activation by epithelial damage associated cytokine release.

A striking feature of atopic dermatitis lesional skin is the colonization by *S. aureus* (50). 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 the 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 (55). 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 (56) and has been linked to allergic disease in a number of studies (57–60). 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 (56, 61).

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, because CD1a mainly traffics between the cell surface and early endosomes (62). Our study now highlights the potential role of a group 1 CD1 member 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 or 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 $CD1a^+$ skin 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 the investigation of human immunology, despite the need for translational work involving human subjects. Human skin antigenic challenge does offer 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. Despite often being compared to T helper subsets, the interaction of ILC and T cells is still to be fully elucidated. 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.

Materials and Methods

Study design

The study was designed to test the hypothesis that ILC2 present antigen to CD1a-reactive lipid-specific T cells. Atopic dermatitis was diagnosed according to the UK refinements of the Hanifin and Rajka diagnostic criteria, and adult participants were only excluded if on systemic immunosuppression or topical calcineurin inhibitors. Clinic participants were recruited sequentially; blinding and randomization were not required as there was no intervention. Thus variation between the functional responses of different donors is expected as cells were isolated from individuals or different, age, gender, ethnicity and medical history, although broadly defined as healthy controls. Sample size was determined based on previous studies of CD1a-reactive T cell response frequencies in humans (80). All experiments were replicated as presented in the figure legends.

Production of human recombinant PLA2G4A

PLA2G4A was produced in Sf9 insect cells using the baculovirus expression system. Briefly, the human PLA2G4A (Genbank no. BC114340) was inserted into the vector, pOPINE which adds a C-terminal his_6 tag (PMID: 17317681), and a recombinant baculovirus constructed as previously described (PMID: 25502201). Sf9 cells (1.0 L) were infected with the PLA2G4A baculovirus and harvested after 72 hours. Recombinant his-tagged PLA2G4 was purified from the cell lysate by a combination of immobilized metal affinity chromatography and gel filtration.

Antibodies and flow cytometry

For FACS surface staining the cells were labelled with the following anti-human antibodies purchased from BioLegend unless stated otherwise: CD3 (OKT3, Biolegend), CD19 (SJ25C1, BD Biosciences), CD123 (FAB301C, R&D Systems), CD11b (DCIS1/18), CD11c (BU15, Abcam), CD8 (RPA-T8), FcεRI (CRA-1), CD14 (MwP9, BD Biosciences), CD4 (OKT4), CD45 (2D1), CD56 (HCD56), CRTH2 (BM16, Miltenyi Biotec), IL-7Ra (A019D5), Live/Dead violet or Aqua (Invitrogen), CD1a (HI149). Intracellular cytokine staining was completed using the eBioscience FoxP3 Fix/Perm kit as per the manufacturer's instructions with Brefeldin-A (eBioscience) and anti-IL-13 (85BRD, eBioscience) antibody.

Suction blister technique

Suction blister cups were applied to the skin of the forearm of adult patients with atopic dermatitis (AD) and healthy individuals, at a vacuum pressure of 250 mmHg. Blisters were generated over the site of a house dust mite intradermal injection or over unchallenged skin. Blisters were formed within 30 – 90 min of suction application. Blister fluid was aspirated 24 hours later using a 30 - gauge needle. Fluids were then centrifuged at 1500 rpm for 5 min at 4°C and the concentration of TSLP was measured by multiplex array (multiplex bead

array) and in separate studies the cells were stained with cell surface antibodies for flow cytometric isolation of ILC2 and T cells utilized for RNA Sequencing analysis.

RNA Sequencing

Suction blister fluid and blood derived PBMCs were centrifuged at 1500 rpm for 5 minutes at 4°C to pellet the blister-infiltrating cells, which were re-suspended in PBS. Blister and blood cell populations were isolated by flow cytometry and collected directly into TRIzol LS; T cells ($CD3^+$ $CRTH2^+$) and ILC2 (Lin^- $CD45^+$ $CD3^-$ $IL\text{-}7R\alpha^+$ $CRTH2^+$). The manufacturer's protocol was followed for TRIzol LS mRNA extraction as far as "Phase Separation". The RNA containing phase was then processed using Qiagen RNAeasy mini kit and contaminating DNA was removed using Ambion Turbo DNase. The total purified RNA was then processed using a NuGEN Ovation RNA-seq system V2 (Ultralow DR multiplex kit). Samples were sequenced on an Illumina HiSeq 2000. Following QC analysis with the fastQC package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), reads were aligned using STAR (63) against the human genome assembly (NCBI build37 (hg19) UCSC transcripts). Non-uniquely mapped reads and reads that were identified as PCR duplicates using Samtools (64) were discarded. Gene expression levels were quantified as read counts using the featureCounts function (65) from the Subread package (66) with default parameters. RPKM values were generated using the edgeR package (67).

Cell sorting and culture

PBMCs were isolated from healthy adult donors under local ethics approval (National Research Ethics Service Committee South Central, Oxford C, 09/ H0606/71). ILC2 were isolated and cultured as previously described (7). Briefly, the lineage (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, CD123, and $Fc\varepsilon RI)^-$, $CD45^+$, $IL7R\alpha^+$, $CRTH2^+$ ILC2 population was sorted into 96-well plates at 100 cells per well and resuspended in MLR of gamma-irradiated PBMCs from three healthy volunteers ($2x10^6$ cells / ml) coupled with 100 IU / ml IL-2 and PHA. After 4 – 6 weeks the growing cells were tested by flow cytometry staining to ensure a pure population of $lineage^-CRTH2^+IL\text{-}7R\alpha^+$ ILC2 was obtained (fig. S1E and S1F). Autologous T cells were isolated from donor PBMCs prior to isolation of ILC2 using magnetic-activated cell sorting (CD3 Microbeads Miltenyi Biotech.).

Cytokines and TLR agonists

For stimulation studies ILC2 were incubated in culture with 50 ng / ml of TSLP (Peprotech), or 10 μg / ml PamCSK (Invivogen), 1 μg / ml LPS (Invivogen), 10^8 cells / ml HKSA (Invivogen) for 6 - 72 hours at 37°C as noted in the figure legend. Cells were then centrifuged at 1500 rpm for 5 minutes at 4°C and supernatant saved for enzyme activity and cells used for RT-PCR analysis.

Quantitative RT-PCR

mRNA extraction was performed using a TurboCapture 96 mRNA kit (Qiagen, 72251) following the manufacturer's instructions. cDNA was prepared from the mRNA using M-MLV reverse transcriptase (Invitrogen). Taqman probes for GAPDH (Hs02786624_g1),

PLA2G4A (Hs00996912_m1), PLA2G4C (Hs01003754_m1) were used to analyze gene expression of ILC2 cultures on a QuantStudio7 Flex real time PCR machine.

Analysis of immune cells within human skin biopsies

To analyze $CD1a^+$ populations in the skin, samples of human skin were processed as follows. Samples were taken under GCP guidance with ethical approval of the NRES Committee South Central. Subcutaneous fat was removed using scissors and residual fat cells were scraped from the underside of the skin and hairs from the upper side using a sharp scalpel. To analyze the cells of whole thickness skin samples, the explants were cut in to < 0.5mm pieces using scalpel and scissors in petri dishes and incubated in collagenase P (1 mg / ml Roche) containing media overnight at 37°C in the petri dish. After overnight digestion, the remaining tissue was homogenized with a Pasteur pipette and endonuclease deoxyribonuclease I (DNase I) 200 Kunitz unit/ml (Roche 10104159001) was added for 15 minutes at room temperature then passed through a 70 μm strainer (VWR) and washed with cold 10 mM EDTA solution. After centrifugation, the pellet was resuspended in cold RPMI and passed through a 40 μm strainer ready for further analyses.

To isolate the epidermis 1 cm^2 sections of skin were placed epidermis down in a petri dish containing 5 U / ml dispase at 4°C overnight. The epidermal layer was then peeled from the dermis with forceps and chopped up and placed in an Eppendorf of 0.5 % Trypsin 0.02 % EDTA at 37°C for 15 minutes. The samples were homogenized with a Pasteur pipette and strained through a 40 μM filter and diluted and washed in FCS containing media. The single cell suspension was then stained with cell surface marker antibodies for flow cytometry.

ELISpot functional assays

CD1a reactivity was assessed by IFN-γ and IL-22 ELISpot (Mabtech AB). ELISpot plates (Millipore Corp., MA) were coated with anti-cytokine capture antibody overnight at 4°C (Mabtech AB). ILC2 were pulsed with HDM (7 μg / ml), PLA2G4A (1 μg / ml) (with or without 1 μM inhibitor MAFP (methyl arachidonyl fluorophosphonate)) or heat-killed *S. aureus* (10^8 cells / ml) overnight, and were then washed and resuspended in FCS ILC2 media (RPMI supplemented with 2 mM L-glutamine, 100I U / mL penicillin, and 100 μg / mL streptomycin, Sodium Pyruvate, non-essential amino acids, BME, hepes, plus 10% FCS). The plates were washed six times with RPMI-hepes and blocked for 1 h with ILC2 FCS media. A total of 50 000 T cells were plated per well to which 50 000 ILC2 were added for co-culture with 10 μg / ml anti-HLA A,B,C (W6/32 eBioscience) and HLA-DR (L243, eBioscience) antibodies to inhibit ILC2-MHC-TCR interaction.

In some wells 10 μg / mL anti-CD1a blocking antibody (OKT-6), 10 μg / mL IgG1 isotype control, cPLA2 inhibitor MAFP (10 μM) or anti-TLR2 (pab-hstlr2, Invivogen) and anti-TLR4 (pab-hstlr4, Invivogen) antibodies (10 μg / ml) were added to ILC2 before addition of T cells. Wells were set-up in duplicate or triplicate. Phorbol myristate acetate 10 ng / mL and Ionomycin 500 ng / mL stimulation was included as a positive controls for T cells and ILC2, and T cells alone in the absence of ILC2 and ILC2 alone were included as a negative controls. After overnight incubation at 37°C and 5% CO_2, culture supernatants were recovered, and plates were washed x 6 in PBS-Tween 0.05% and incubated with 1 μg / mL

of biotin-linked anti-IFN-γ or anti-IL22 monoclonal antibody (Mabtech AB, Sweden) for 2 h. After washing 6 times in PBS-Tween 0.05%, the plates were incubated for 1 h with streptavidin-alkaline phosphatase (Mabtech AB). Spots were visualized using an alkaline phosphatase conjugate substrate kit (Biorad, Hercules) and enumerated using an automated ELISpot reader (Autimmun Diagnostika gmbh ELISpot Reader Classic). Number of IFNγ or IL-22 producing T cells per 50 000 cells presented on result graphs was calculated as the total number of spots enumerated minus the number of IFNγ or IL-22 spots in the T cell alone negative control wells.

To assess the functional role of CD1a expressed by ILC2 we relied on the use of CD1a blocking antibody clone OKT6 and calculating the CD1a dependent response by analysing the differential signal of unblocked versus anti-CD1a-mAb-blocked conditions. The variability of human data and of the efficacy/completeness of neutralising antibody experiments was accounted for by careful replication and analysis of a number of donors with experiments completed on different occasions. Concentration of OKT6 used for blocking has been previously optimized (53). Prior incubation of ILC2 with anti-CD1a preceding antigen pulsing was undertaken to aid CD1a blocking.

PLA2 biochemical activity assays

Cytosolic and secreted PLA_2 activity of recombinant PLA2G4A, heat-killed *S. aureus* preparation and ILC2 culture supernatant were measured using either a cytosolic PLA_2 kit (Cayman Chemicals) or a secretory PLA_2 kit (Cayman Chemicals) according to the manufacturer's protocols. Arachidonoyl thio-PC is the substrate for $cPLA_2$ and hydrolysis of the arachidonoyl thioester bond at the sn-2 position by $cPLA_2$ releases a free thiol which can be detected by DTNB (5,5′-dithiobis[2-nitrobenzoic acid]) producing a colored precipitate. For the $sPLA_2$ kit in the presence of PLA2, cleavage of the substrate (diheptanoyl thio-PC) at the sn-2 position results in release of the thiol group. In both assays absorbance was measured with a spectrophotometer (Clariostar) (wavelength 414 nm) to give a measure of PLA2 activity.

Statistical analysis

The ANOVA tests, paired and unpaired T-tests were performed using GraphPad Prism version 6.00 (GraphPad Software). Error bars represent standard deviation of the mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One Sentence Summary

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

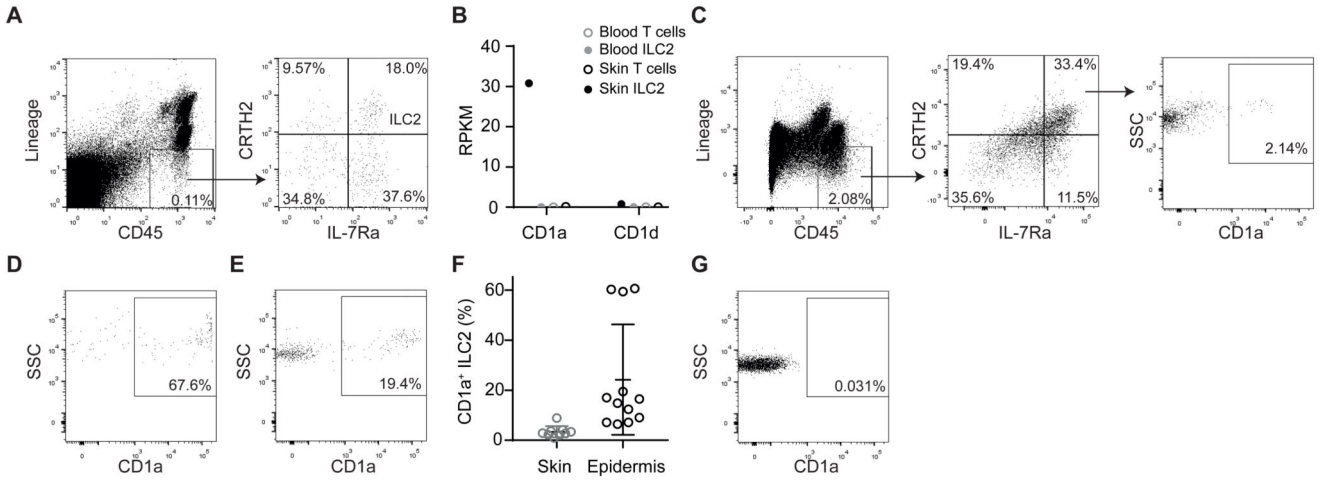

Fig. 1. Human skin ILC2 express CD1a.

A. Flow cytometry gating strategy for blister fluid derived human ILC2. ILC2 are $CD45^+/CD3^-/Lineage^-/CRTH2^+/IL7R\alpha^+$. **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.

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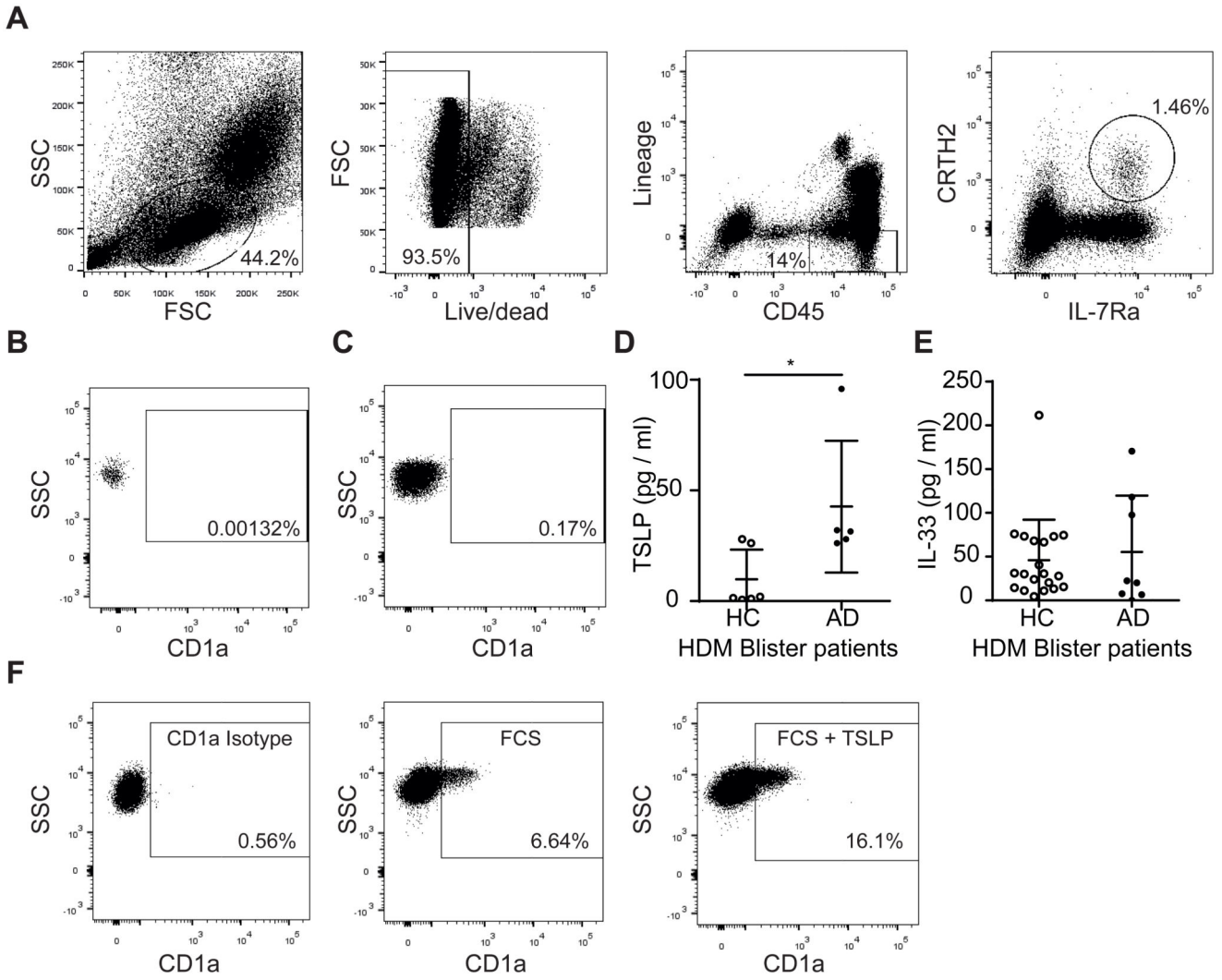

Fig. 2. Human blood ILC2 could be stimulated to express CD1a.

A. Flow cytometry gating strategy for blood derived human ILC2. ILC2 are $CD45^{+}/CD3^{-}/Lineage^{-}/CRTH2^{+}/IL7R\alpha^{+}$. **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).

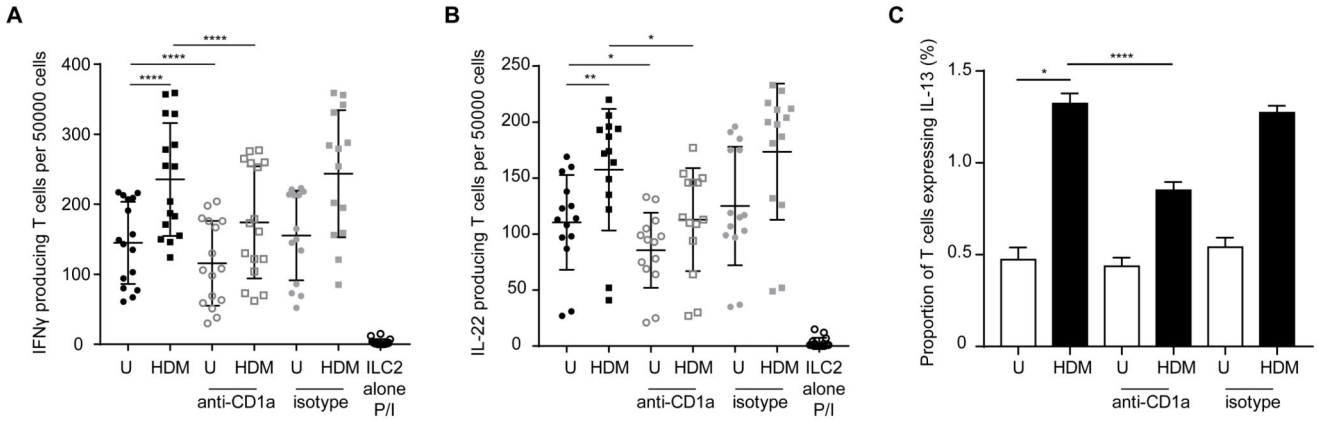

Fig. 3. ILC2 present HDM-derived lipid ligands to CD1a responsive T cells.

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 (15 ng / ml) and Ionomycin (7.5 ng / ml) (P/I). (**A.** $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, **B.** $p = 0.0023$, $p = 0.0158$, $p = 0.0112$, n = 8 donors, one-way ANOVA, and 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. ($p = 0.0124$, $p < 0.0001$, n = 3 donors, one-way ANOVA and 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).

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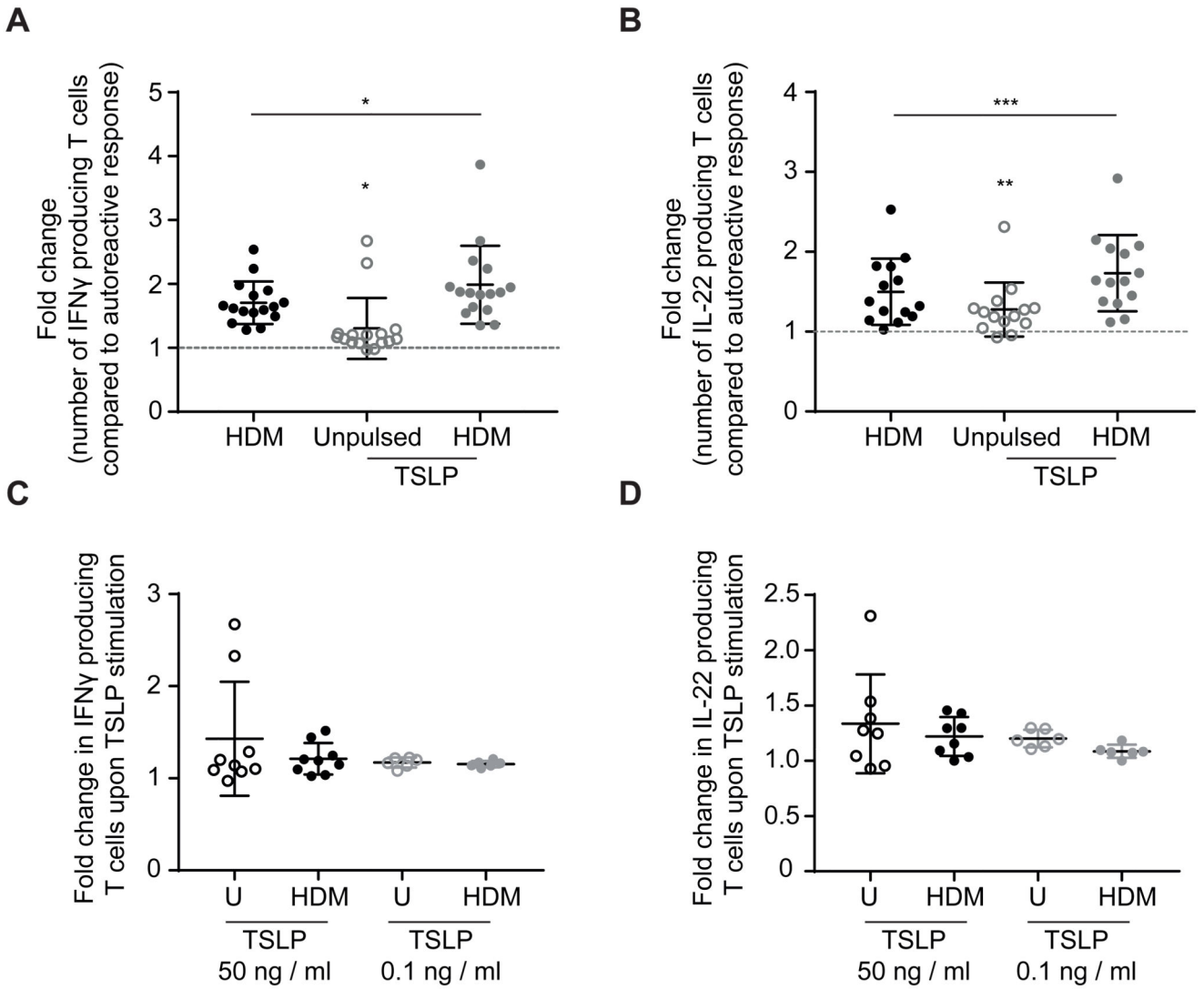

Fig. 4. TSLP further enhances the ability of ILC2 to present CD1a ligands to T cells.

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). Statistics calculated between the baseline response or as indicated in the figure. (**A.** p = 0.0221, p = 0.0111, **B.** p = 0.0091, p = 0.0006, 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. (**C.** p = 0.3325, p = 0.4342, **D.** p = 0.484, p = 0.1, n = 8

donors, t-test and data represents 3 - 6 independent experiments). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 paired Student's *t* test (mean and SD).

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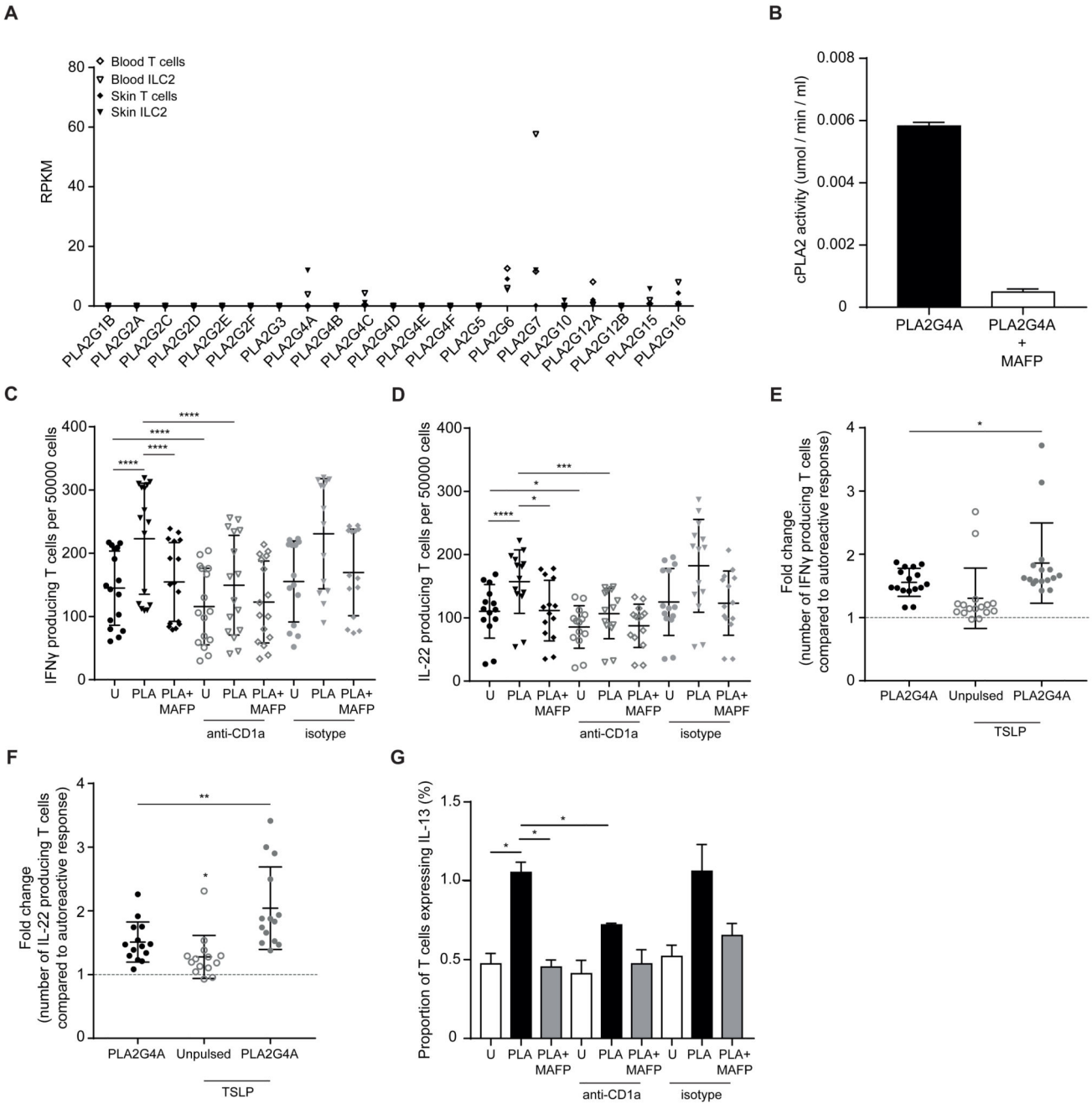

Fig. 5. ILC2 express PLA2G4A which generates CD1a ligands.

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

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MAFP. IFNγ **(C.)** ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$) and IL-22 **(D.)** ($p < 0.0001$, $p = 0.018$, $p = 0.0321$, $p = 0.0007$) 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, and 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 and data represents 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. ($p = 0.041$, $p = 0.0248$, $p = 0.0296$, n = 3 donors, one-way ANOVA, and 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).

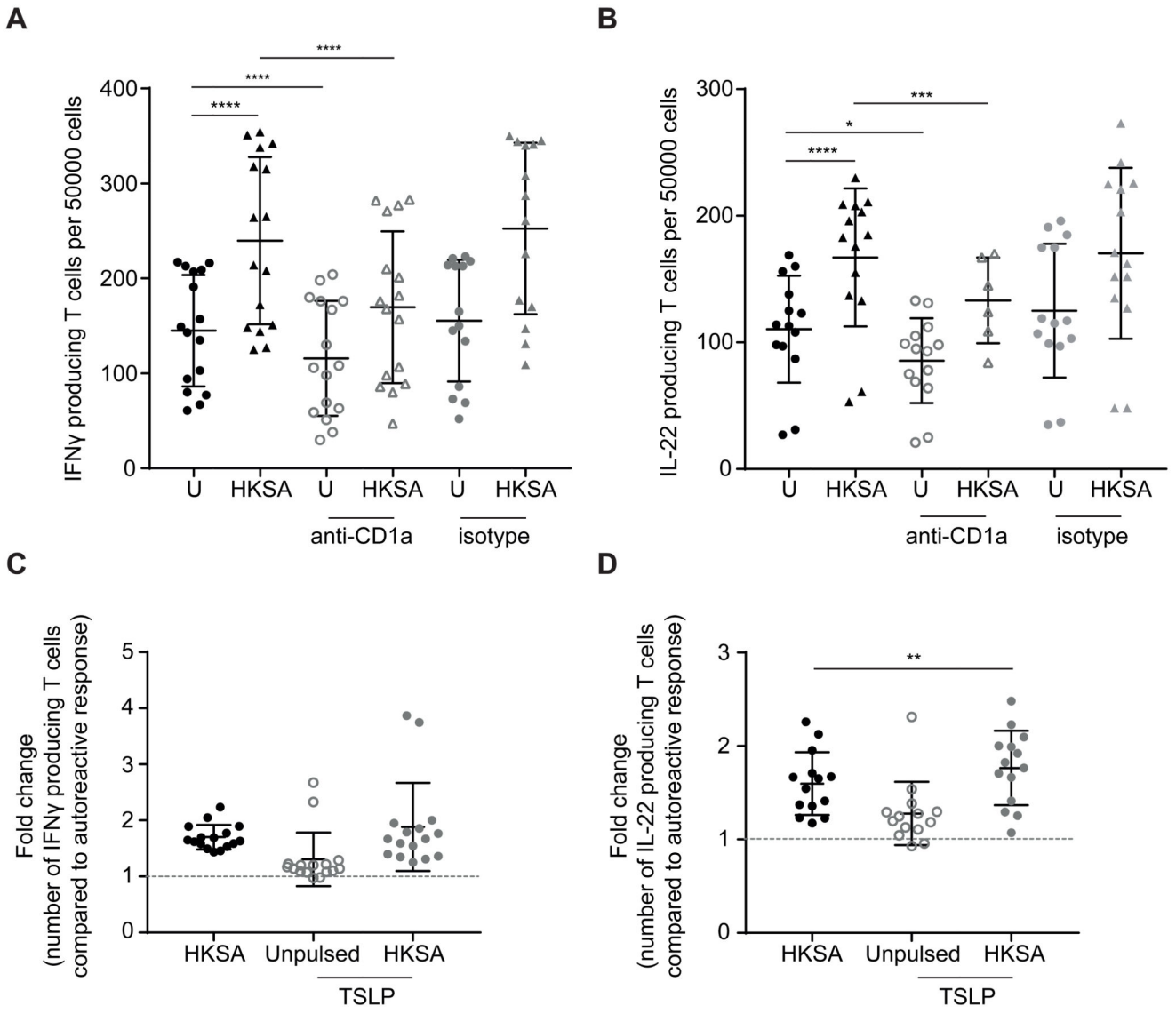

Fig. 6. ILC2 present bacterial lipid ligands derived from *Staphylococcus aureus*

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.)** ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$) and IL-22 **(B.)** ($p < 0.0001$, $p = 0.0158$, $p = 0.0002$) 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, and data represents 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. ($p = 0.0021$, n = 8 donors, t-test, and data represents 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).

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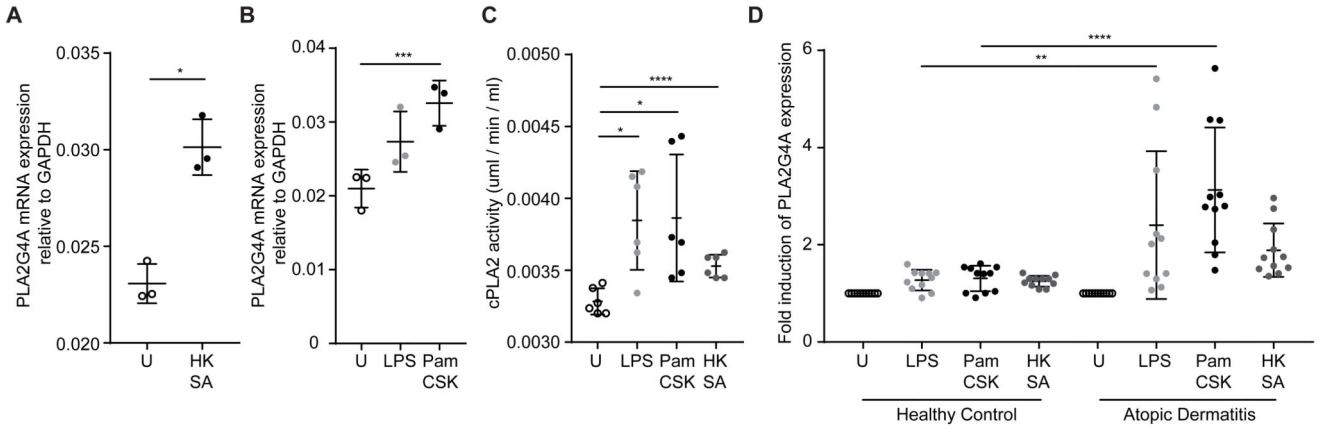

Fig. 7. Bacterial components can stimulate ILC2 to produce cPLA2

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. ($p = 0.0009$, $n = 3$, 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) ($p = 0.0152$, $p = 0.0413$, $p < 0.0001$, $n = 6$, 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). ($p = 0.0329$, $p < 0.0001$, $n = 4$ donors, one-way ANOVA, and data representative of 4 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (mean and SD).

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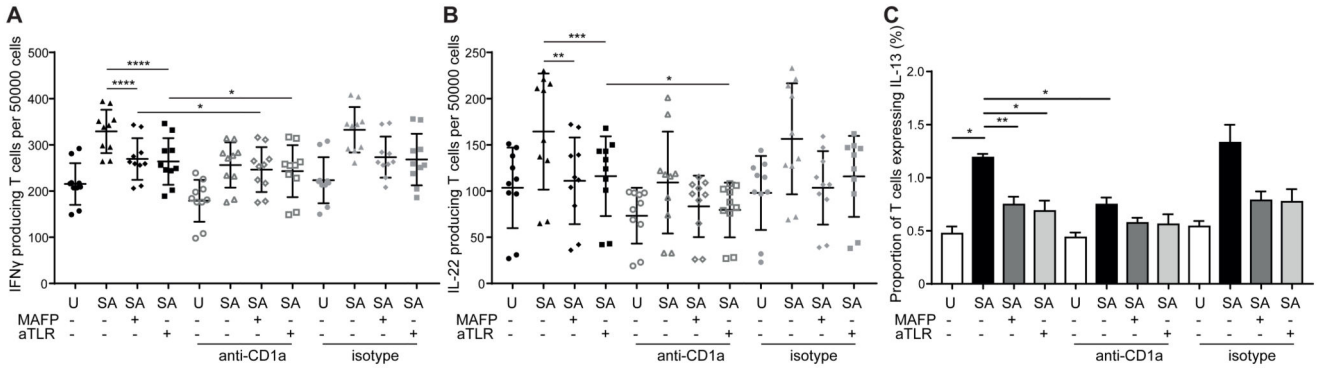

Fig. 8. TLR stimulation of ILC2 by *Staphylococcus aureus* induces PLA2G4A and generation of lipid ligands which can be presented to T cells by CD1a.

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) with or without inhibition of cPLA2 (1 μM MAFP) or TLR2 and TLR4 signaling (10 μg / ml anti-TLR2 and anti-TLR4). IFNγ **(A.)** ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$) and IL-22 **(B.)** ($p = 0.0051$, $p = 0.0007$, $p = 0.0255$) production was detected by ELISpot and IL-13 was detected by flow cytometry **(C.)** ($p = 0.0202$, $p = 0.009$, $p = 0.017$, $p = 0.0119$) 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 and 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).