HUMAN IMMUNOLOGY

CD1a presentation of endogenous antigens by group 2 innate lymphoid cells

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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 (major histocompatibility complex 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 components. 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 up-regulated by TSLP (thymic stromal lymphopoietin) 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 Toll-like receptor–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 interleukin-13 (IL-13) and IL-5, as well as IL-6, IL-9, IL-4, granulocyte-macrophage colony-stimulating factor (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 (retinoic acid–related orphan receptor α) 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–IL-3–regulated, 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 the lack of cell surface markers of known lineages and by the expression of IL-7Rα and CRTH2 (*11*). CRTH2 is the receptor for the lipid mediator prostaglandin D_2 (PGD2), which is released from mast cells and other cells during infection and allergy (*12*). ILC2 are also characterized by the expression of receptors for alarmin cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (*13*). Such characteristics thus position ILC2 as rapid effectors and sentinels capable of mediating responses to cutaneous and mucosal barrier breach.

In addition to 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*). Abrogation of these ILC2 responses impaired efficient wound closure. Murine and human ILC2 have been shown to express functional major histocompatibility complex II (MHCII) (*17*, *18*), and a dialog 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 *Nippostrongylus brasiliensis* expulsion is dependent on ILC2 expression of MHCII.

CD1a is predominantly expressed in the skin, with constitutively high expression on Langerhans cells (LCs), 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 (TCR) signaling was predominantly induced upon 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 dideoxymycobactin (*22*). Within the past 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 the presentation of endogenous or exogenous lipids. In addition to promoting homeostasis and immunity, $CD1a^+$ antigen-presenting cells (APCs) are enriched in atopic dermatitis lesions (*24*). 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 interferon-γ (IFN-γ), IL-22, IL-13, TNFα (tumor necrosis factor–α), IL-17A, and GM-CSF (*25*, *28*).

Recently, we identified that allergen-derived phospholipase A_2 (PLA_2) within house dust mite (HDM) and wasp and bee venom

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generates 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 PLA_2 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*), DCs, 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 *Staphylococcus 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 a therapeutic benefit.

RESULTS

Human skin ILC2 can express CD1a

To analyze the phenotype of ILC2 in the skin upon allergic challenge, we formed skin suction blisters after intraepidermal injection of HDM allergen, isolated ILC2 by fluorescence-guided cell sorting, and analyzed gene expression by RNA sequencing (Fig. 1, A and B, and fig. S1A). Although it is technically challenging to obtain sufficient cell numbers, the advantage of the skin suction blister system is that skin cells can be isolated without the need for the physical and 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 up-regulated 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, we analyzed healthy unchallenged human skin ex vivo for expression of CD1a by ILC2 using flow cytometry. CD1a was observed in a small proportion of ILC2 analyzed within the whole-skin biopsies, suggesting functional heterogeneity (Fig. 1C). However, we reasoned that, given the specific epidermal sublocation of LCs, which express high levels of CD1a (Fig. 1D and fig. S1B), epidermal ILC2 might also be enriched for CD1a expression. CD1a was detected on about 20% epidermal ILC2, at significantly higher levels than whole-skin ILC2 ($P = 0.0116$; Fig. 1, E and F, and fig. S1C). LCs showed a similar mean fluorescence intensity of CD1a as epidermal $CD1a^+$ILC2, although CD1a was present on a greater proportion of the LCs than ILC2 (Fig. 1, D to 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. 2, A and B, and fig. S1, E and F) and on ILC2 cultured in human serum (Fig. 2C). 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

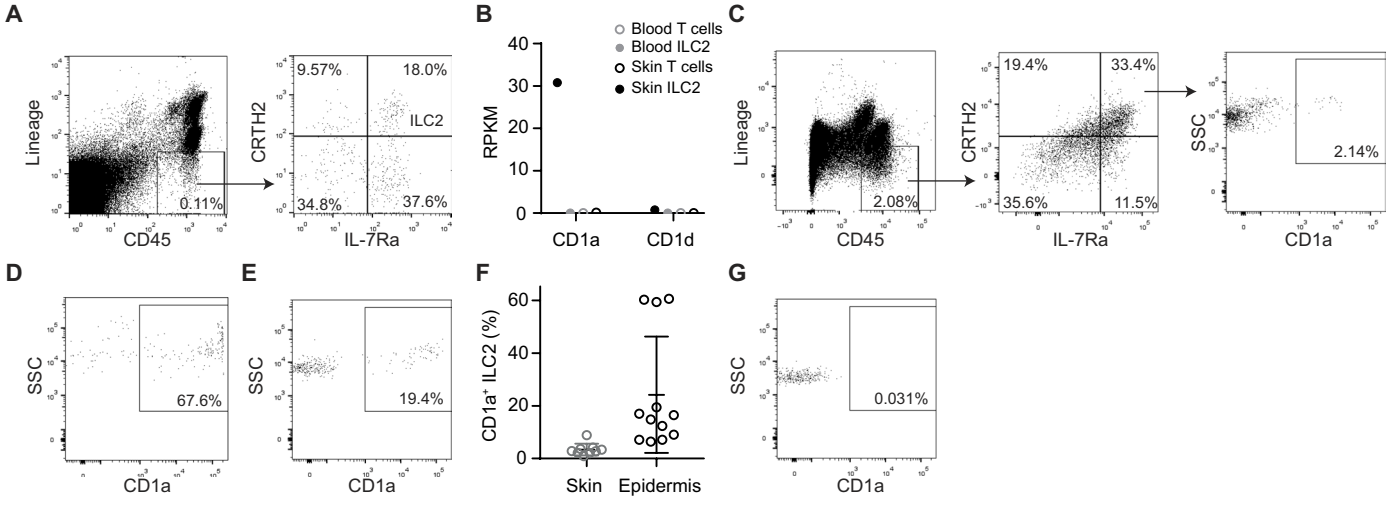

Fig. 1. Human skin ILC2 express CD1a. (**A**) Flow cytometry gating strategy for blister fluid–derived human ILC2. ILC2 are $CD45^+$/$Lineage^-$/$CRTH2^+$/$IL7R\alpha^+$. (**B**) CD1a and CD1d gene expression of skin- and blood-derived ILC2 and T cells determined by RNA sequencing and measured in RPKM. (**C**) Whole-thickness skin samples were homogenized and analyzed by flow cytometry for the presence of ILC2 and expression of CD1a. SSC, side scatter. (**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 are representative of at least 10 independent experiments and $n = 12$ donors.

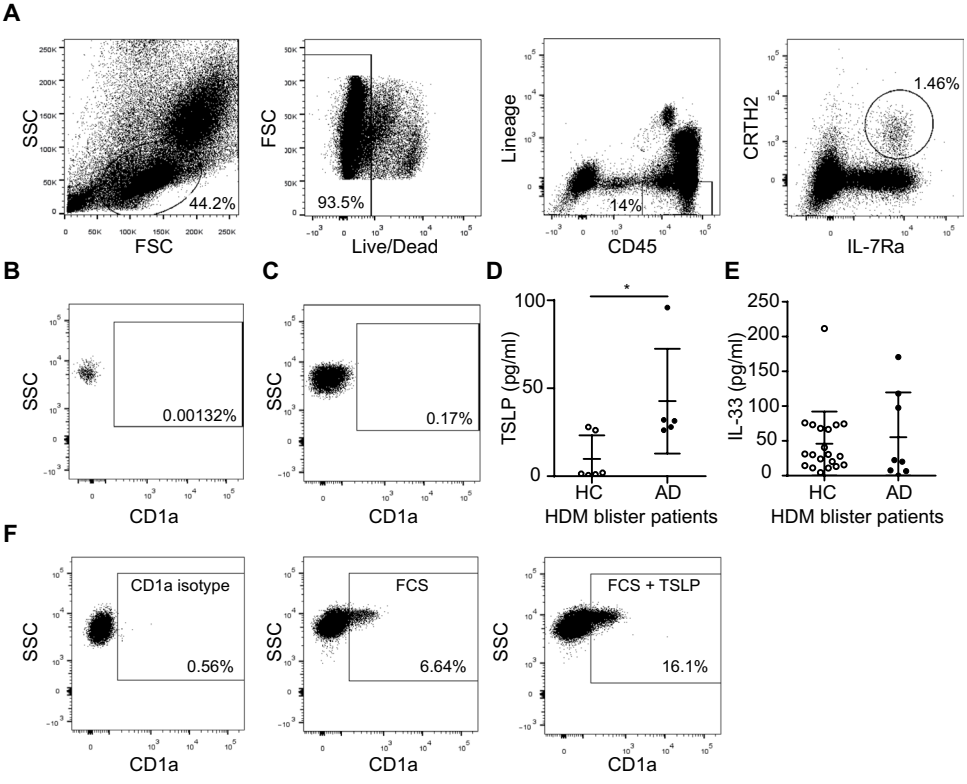

Fig. 2. Human blood ILC2 could be stimulated to express CD1a. (**A**) Flow cytometry gating strategy for blood-derived human ILC2. ILC2 are CD45$^+$/Lineage$^-$/CRTH2$^+$/IL7Rα$^+$. FSC, forward scatter. (**B**) Flow cytometric analysis of CD1a expression on blood ILC2. Flow cytometry data are representative of $n = 8$ donors. (**C**) Flow cytometric analysis of CD1a expression on human serum–cultured blood ILC2 representative of $n = 8$ donors. (**D**) Multiplex bead array analysis of TSLP concentration in HDM-challenged blister fluid of healthy control (HC) and atopic dermatitis (AD) patients ($n = 5$ to 6; $P = 0.0374$, t test). (**E**) Multiplex bead array analysis of IL-33 concentration in HDM-challenged blister fluid of healthy control and atopic dermatitis patients ($n = 8$ to 21, $P = 0.6655$, t test). (**F**) Culture of human blood-derived ILC2 in the absence of human serum. Flow cytometry analysis of anti-CD1a isotype control, of CD1a expression on FCS-cultured blood ILC2, and upon TSLP stimulation (plots from left to right). Data are representative of $n = 3$ donors and three independent experiments. *$P < 0.05$, unpaired Student's t test (mean + SD).

after HDM challenge and was found to be at a higher concentration in the blister fluid isolated from patients with atopic dermatitis ($P = 0.0374$; Fig. 2D). We could not reliably identify IL-25 in the skin, and levels of IL-33 were not significantly different between patient and control groups ($P = 0.6655$; Fig. 2E). We therefore investigated the effects of TSLP on CD1a expression by ILC2 and showed TSLP-induced CD1a expression. 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 medium for 72 hours, which increased CD1a expression by the blood-derived ILC2 ($P = 0.0159$) and was further enhanced by TSLP ($P = 0.0068$; Fig. 2F and fig. S2). In the presence of FCS, CD1a expression by ILC2 was also induced by IL-33, PGD2, and LTE4 (leukotriene E_4) at concentrations known to be functionally relevant for ILC2 (fig. S2) (*12*, *43*). Together, these data suggested that skin-derived ILC2 expression of CD1a is regulated by skin-derived alarmin and by the loss of inhibitory effects of serum. In this way, CD1a expression can be restricted to sublocations 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 the ability of ILC2 to present lipid antigens to polyclonal T cells. ILC2 were cocultured with autologous ex vivo blood-derived polyclonal T cells for 24 hours with or without anti-MHCI/II and anti-CD1a antibodies (OKT6). IFN-γ and IL-22 production after coculture showed that ILC2 could mediate CD1a-dependent T cell responses (Fig. 3, A and B).

We sought to determine whether ILC2, like DCs (*25*), could present HDM-derived lipid ligands to T cells via CD1a. We pulsed CD1a-induced blood-derived FCS-cultured ILC2 with HDM extract (7 μg/ml) overnight, with anti-CD1a or control. ILC2 were washed and cocultured with polyclonal T cells for 24 hours. HDM-pulsed ILC2 activated T cells in a manner partially dependent on CD1a, inducing IFN-γ and IL-22 secretion (Fig. 3, A and B). ILC2 alone did not produce IFN-γ or IL-22, even when stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Fig. 3, A and B). Enzyme-linked immunospot (ELISpot) assays could not be used to analyze IL-13 secretion by T cells because ILC2-derived IL-13 could confound the result. We therefore used flow cytometry and showed HDM-pulsed ILC2 elicited T cell–derived IL-13 in a CD1a-dependent manner ($P < 0.0001$; Fig. 3C).

Both the autoreactive response and presentation of HDM-induced ligands by ILC2 CD1a were amplified by TSLP (Fig. 4, A and B). 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 (which was more representative of TSLP levels we found in the skin) (Fig. 2D). No statistically significant difference was found between previous treatment of ILC2 with the higher and lower concentrations of TSLP (Fig. 4, C and D).

ILC2 express PLA2G4A

PLA_2 has been identified as one of the components of HDM, which generates neolipid antigens presented by CD1a (*25*). To determine whether ILC2 could be induced to express host PLA_2 in a HDM response, we analyzed RNA sequencing data from HDM-challenged skin suction blister–derived ILC2 for PLA_2 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 up-regulation of *PLA2G4A* gene expression (1.5-fold), as measured by real time polymerase

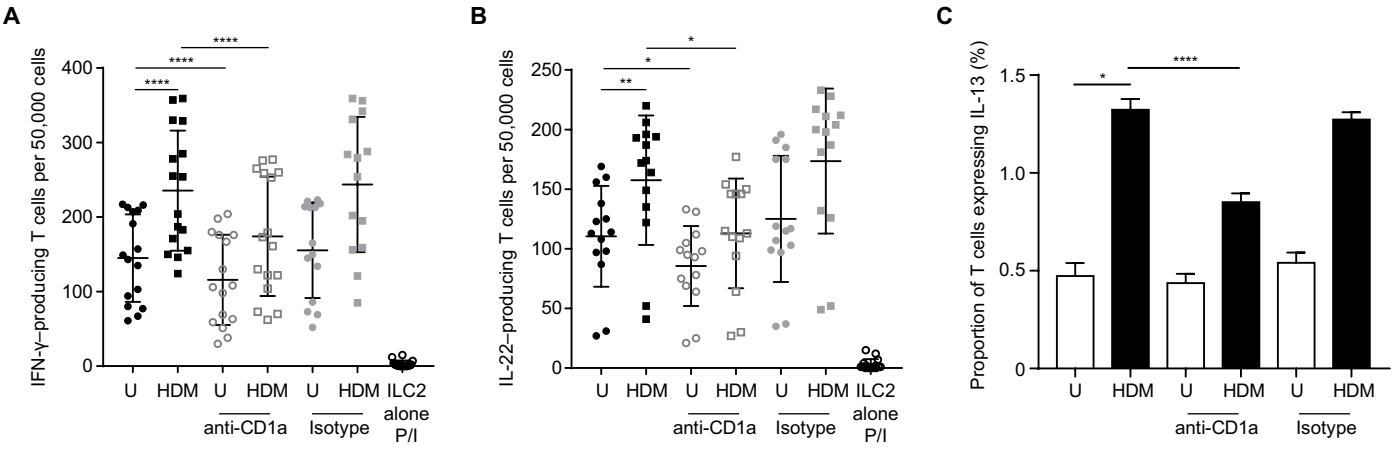

Fig. 3. ILC2 present HDM-derived lipid ligands to CD1a-responsive T cells. Autologous ILC2 and T cells were isolated from donor PBMCs by FACS and CD3 magnetic cell separation (MACS), respectively. (**A** and **B**) Before coculture with autologous T cells, ILC2 were pulsed with HDM extract (7 μg/ml), and IFN-γ (A) and IL-22 (B) production was detected by ELISpot in the presence or absence of anti-CD1a blocking antibody (10 μg/ml) or isotype control (10 μg/ml). In addition, ILC2 alone were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) (P/I; $n = 8$ donors; one-way analysis of variance (ANOVA); data represent at least six independent experiments). U, unpulsed. (**C**) ILC2 were pulsed in the presence or absence of HDM extract (7 μg/ml) and cocultured 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 (10 μg/ml) or isotype control (10 μg/ml) ($n = 3$ donors; one-way ANOVA; data represent three independent experiments). *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$, repeated-measures (RM) one-way ANOVA with Tukey's post hoc test (mean + SD).

chain reaction (rtPCR). Cytosolic PLA_2 ($cPLA_2$) can act extracellularly to generate CD1a ligands (*28*), and so, we produced recombinant PLA2G4A ($cPLA_2\alpha$) and confirmed biochemical $cPLA_2$ activity, which was inhibited by methyl arachidonyl fluorophosphonate (MAFP) (Fig. 5B). PLA2G4A was incubated with FCS-cultured ILC2 overnight, with or without MAFP; ILC2 were then washed before coculture with T cells. Activated T cells produced IFN-γ and IL-22, and MAFP blocked the response (Fig. 5, C and D). There was no significant effect of MAFP on the unpulsed response, suggesting that it is not acting nonspecifically (IFN-γ, $P = 0.1805$; IL-22, $P > 0.9999$). Furthermore, the response was amplified by TSLP-induced $CD1a^+$ILC2 (Fig. 5, E and F), 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.

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

S. aureus infection promotes PLA2G4A activity (*44*–*47*). Lee *et al.* showed that *S. aureus* infection induces up-regulation of PLA2G4A and elicits prostaglandin E_2 and IL-6 responses in the lung, with Toll-like receptor 2 (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 coincubated with T cells. We observed that ILC2 exposed to *S. aureus* could activate T cells to secrete IFN-γ and IL-22, which was partially dependent on CD1a (IFN-γ, $P < 0.0001$; IL-22, $P = 0.0002$; Fig. 6, A and B). The IL-22 response was up-regulated by previous incubation with TSLP. TSLP did not amplify the *S. aureus*–induced IFN-γ response (Fig. 6, C and D), suggesting distinct cytokine induction mechanisms; however, IL-22 is known to trigger antimicrobial 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 ($P = 0.0258$; Fig. 7A). Heat-killed *S. aureus* can be used experimentally as a source of TLR2 and TLR4 ligands, and so, to investigate the underlying mechanisms, we analyzed the effects of Pam_3CSK_4 (PamCSK) (TLR2 agonist) and lipopolysaccharide (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 the release of PLA2G4D from mast cells (*28*). Therefore, we assayed ILC2 culture supernatant for $cPLA_2$ activity to determine whether $cPLA_2$ was produced by ILC2 in vitro. As was observed at the mRNA level, TLR2 ($P = 0.0152$) and TLR4 ($P = 0.0413$) ligands and heat-killed *S. aureus* ($P < 0.0001$) stimulated the release of $cPLA_2$ by ILC2 (Fig. 7C). The heat-killed *S. aureus* preparation did not contain $cPLA_2$ or secreted PLA_2 ($sPLA_2$) activity. We next compared the capability of ILC2 isolated from the blood of patients with atopic dermatitis and healthy controls to express PLA2G4A after stimulation with TLR2 and TLR4 ligands and heat-killed *S. aureus*. Atopic dermatitis 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). To confirm the roles of TLR2 and TLR4, we preincubated ILC2 with $cPLA_2$ inhibitor or anti-TLR2 and anti-TLR4 antibodies and then pulsed with heat-killed *S. aureus* before coculture with T cells. The CD1a-dependent activation of T cell IFN-γ, IL-22, and IL-13 production was reduced by inhibition of $cPLA_2$ and of TLR2 and TLR4 signaling (Fig. 8, A to C). Together, these data showed

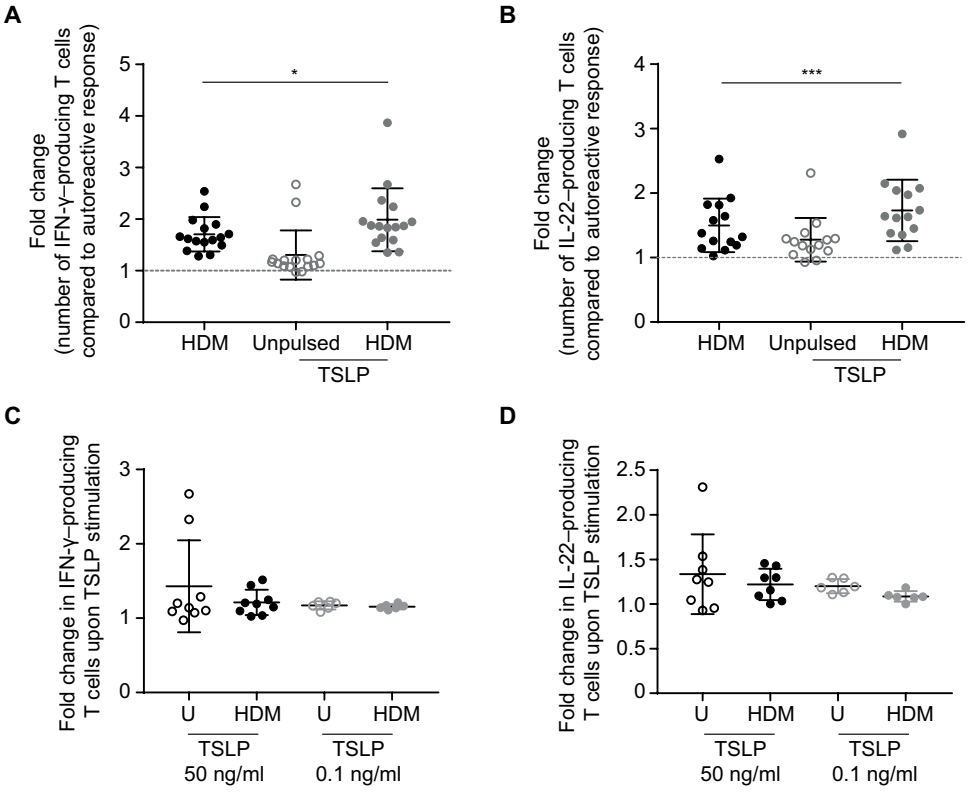

Fig. 4. TSLP further enhances the ability of ILC2 to present CD1a ligands to T cells. ILC2 were cultured in 10% FCS and stimulated in the presence or absence of TSLP for 72 hours before pulsing with HDM extract and subsequent ELISpot analysis of capacity to activate T cells. (**A** and **B**) Effect of the presence or absence of previous stimulation of ILC2 with TSLP on the number of IFN-γ–producing (A) or IL-22–producing (B) T cells induced by coculture. Fold change was calculated relative to the unpulsed autoreactive baseline response in the absence of TSLP (represented by dashed line). Graphs show the effect of TSLP on the unpulsed ($P = 0.0221$) and HDM-induced ($P = 0.0111$) IFN-γ responses (A) and the effect of TSLP on the unpulsed ($P = 0.0091$) and HDM-induced ($P = 0.0006$) IL-22 responses (B) ($n = 8$ donors; *t* test; data represent at least six independent experiments). (**C** and **D**) Effect of TSLP concentration upon amplification of CD1a-dependent T cell production of IFN-γ (C) or IL-22 (D). Fold change was calculated between cytokine spots produced after T cell culture with unstimulated and TSLP-stimulated ILC2. Graphs show the two concentrations of TSLP (50 and 0.1 ng/ml) ($n = 8$ donors; *t* test; data represent three to six independent experiments). $^*P < 0.05$; $^{***}P < 0.001$, paired Student's *t* test (mean ± SD).

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 LCs, 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 LCs in the 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 APCs. CD1a expression was induced by the epidermal alarmin TSLP and by the 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 LCs are known to be 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 $sPLA_2$ was proposed to cleave membrane phospholipids, generating CD1a ligands. This response was controlled by filaggrin inhibition of PLA_2. Thus, genetic loss of filaggrin inhibition of HDM-$sPLA_2$ may alert CD1a-reactive T cells to barrier compromise. Dysregulated ILC2 responses have been implicated in allergy, and human ILC2 are enriched in atopic dermatitis lesions and rhinosinusitis nasal polyps (*11*). ILC2 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. IL-33 induces ILC2 production of cytokines that recruit and activate T helper type 2 cells, eosinophils, and mast cells and induce hyper-responsiveness in the epithelium (*7*, *13*, *53*). Here, we show that in addition to the production of effector cytokines, ILC2 can present HDM ligands directly to T cells via CD1a. HDM-challenged ILC2 activated T cells to produce IFN-γ, 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 notable feature of atopic dermatitis lesional skin is the colonization by *S. aureus* (*48*). ILC2 and CD1a proteins would be poised to interact with 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 the production of PLA2G4A. 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*).

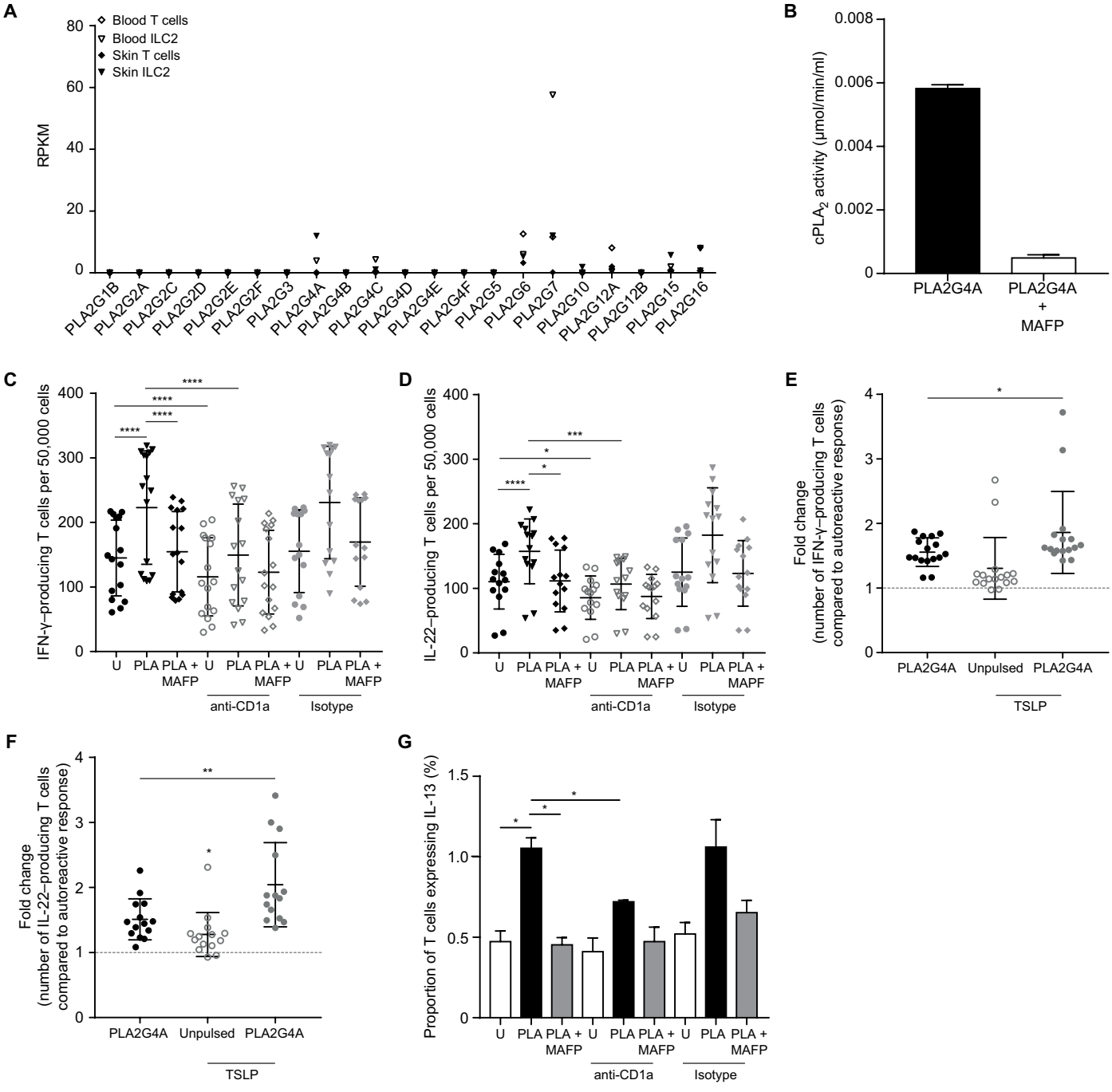

Fig. 5. ILC2 express PLA2G4A, which generates CD1a ligands. (**A**) PLA_2 gene expression analysis of skin- and blood-derived ILC2 and T cells determined by RNA sequencing and measured in RPKM. (**B**) $cPLA_2$ activity of recombinant PLA2G4A irreversibly inhibited by 1 μM MAFP measured using free thiol detection. (**C** and **D**) Autologous ILC2 and T cells were isolated from donor PBMCs by FACS and CD3 MACS bead separation, respectively. Before coculture with autologous T cells, ILC2 were either unpulsed (U) or pulsed with PLA2G4A (1 μg/ml) or PLA2G4A (1 μg/ml) inhibited with 1 μM MAFP. IFN-γ (C) and IL-22 (D) production was detected by ELISpot in the presence or absence of anti-CD1a blocking antibody (10 μg/ml) or isotype control (10 μg/ml) (n = 8 donors; one-way ANOVA; data represent at least six independent experiments). (**E** and **F**) ILC2 were cultured in FCS and stimulated in the presence or absence of TSLP for 72 hours before pulsing with PLA2G4A. Effect of the presence or absence of previous stimulation of ILC2 with TSLP upon the number of IFN-γ–producing (P = 0.0226; E) or IL-22–producing (P = 0.0029; F) T cells induced by coculture. Fold change was calculated relative to the unpulsed autoreactive response in the absence of TSLP (represented by dashed line). Statistics calculated between the baseline response or as indicated in the figure (n = 8 donors; t test; data represent at least six 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 anti-CD1a blocking antibody (10 μg/ml) or isotype control (10 μg/ml) upon coculture with ILC2 pulsed or unpulsed with PLA2G4A (n = 3 donors; one-way ANOVA; three 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 + SD).

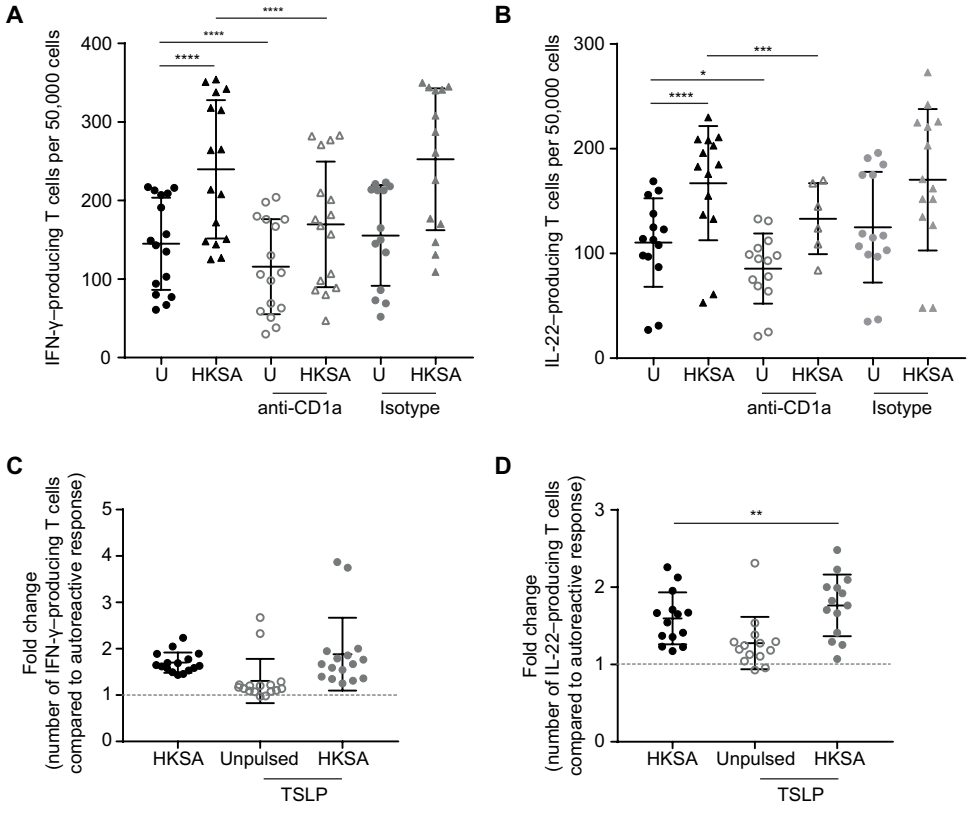

Fig. 6. ILC2 present bacterial lipid ligands derived from *S. aureus*. (**A** and **B**) Autologous ILC2 and T cells were isolated from donor PBMCs by flow cytometric sorting and CD3 MACS bead separation, respectively. Before coculture with autologous T cells, ILC2 were pulsed with heat-killed *S. aureus*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

PLA_2 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 that *S. aureus* induced PLA2G4A and showed that this could be mediated by TLR2 or TLR4 stimulation. The PLA2G4A induction associated with an 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 $cPLA_2$ activity and TLR2 and TLR4 signaling demonstrated that PLA_2 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, although only a minor PLA_2-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; we show that ILC2 derived from atopic dermatitis patients displayed a greater capacity to express PLA2G4A.

Although 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 to 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, patient numbers become limiting. The scarcity of ILC2 in the skin and suction blisters leads to modeling 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 that 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 patients. 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 crosstalk 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.

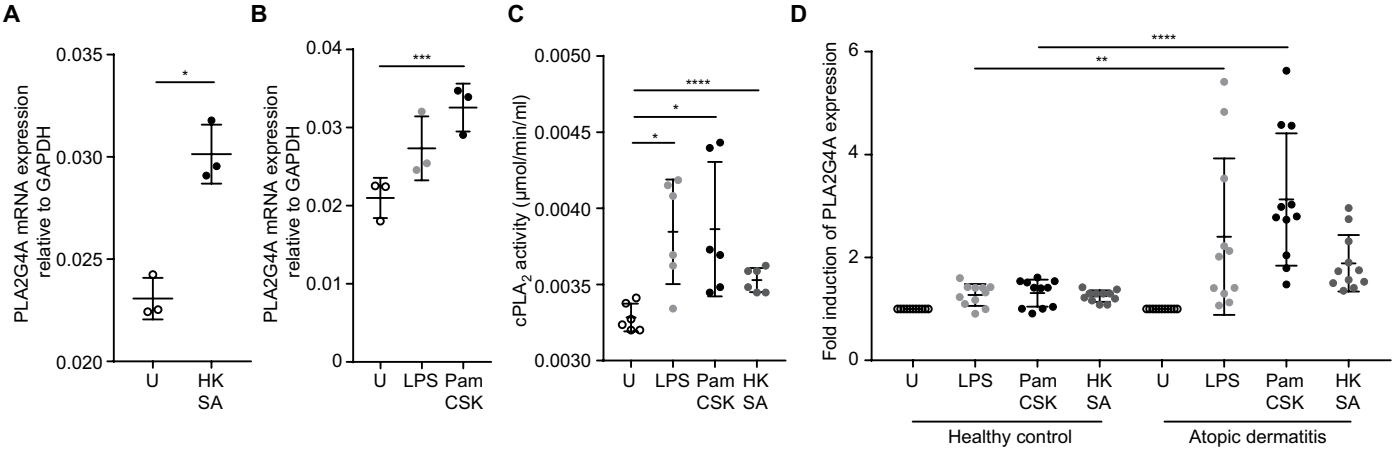

Fig. 7. Bacterial components can stimulate ILC2 to produce cPLA$_2$. (**A**) Real-time PCR analysis of PLA2G4A gene expression by ILC2 after stimulation with heat-killed *S. aureus* preparation ($n = 3$; $P = 0.0258$, *t* test; data representative of three independent experiments). (**B**) Real-time PCR analysis of PLA2G4A gene expression by ILC2 after stimulation with TLR2 (PamCSK; 10 μg/ml) and TLR4 (LPS; 1 μg/ml) ligands ($n = 3$; *t* test; data representative of three independent experiments). (**C**) cPLA$_2$ activity was measured in the supernatant of ILC2 stimulated with TLR2 and TLR4 ligands or heat-killed *S. aureus* (10^8 cells/ml) ($n = 6$; *t* test; data representative of three independent experiments). (**D**) Real-time PCR analysis of PLA2G4A gene expression by ILC2 from healthy controls or atopic dermatitis patients after stimulation with TLR2 (PamCSK; 10 μg/ml) and TLR4 (LPS; 1 μg/ml) ligands or heat-killed *S. aureus* (10^8 cells/ml) ($n = 5$ donors; one-way ANOVA; data representative of four independent experiments). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ (mean ± SD).

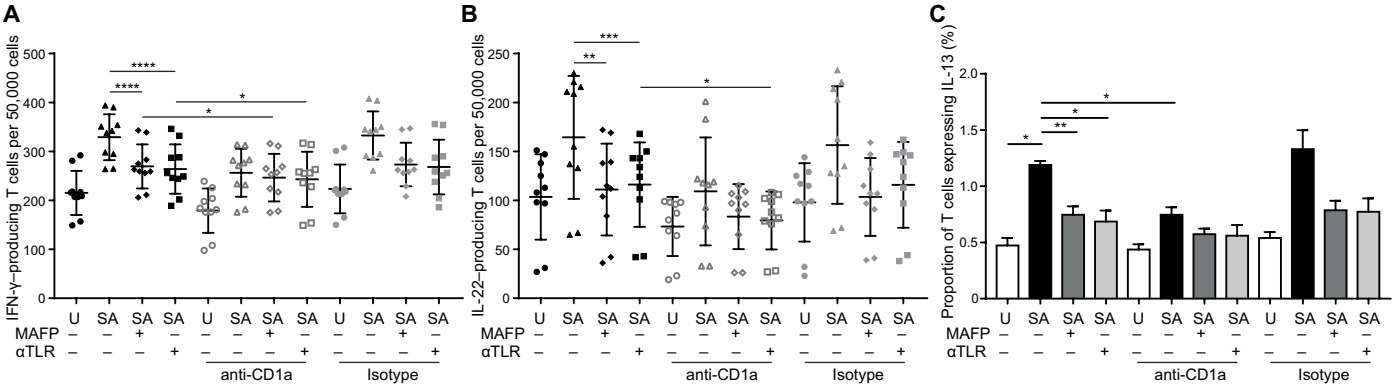

Fig. 8. TLR stimulation of ILC2 by *S. aureus* induces PLA2G4A and generation of lipid ligands, which can be presented to T cells by CD1a. Autologous ILC2 and T cells were isolated from donor PBMCs by flow cytometric sorting and CD3 MACS microbead separation, respectively. Before coculture with autologous T cells, ILC2 were pulsed with heat-killed *S. aureus* (SA; 10^8 cells/ml) with or without inhibition of cPLA$_2$ (1 μM MAFP) or TLR2 and TLR4 signaling [anti-TLR2 (10 μg/ml) and anti-TLR4 (10 μg/ml)]. (**A** to **C**) IFN-γ (A) and IL-22 (B) production was detected by ELISpot, and IL-13 was detected by flow cytometry (C) in the presence or absence of anti-CD1a blocking antibody (10 μg/ml) or isotype control (10 μg/ml). ELISpot data represent at least six independent experiments and $n = 8$ donors. IL-13 FACS data represent $n = 3$ donors and three 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 + SD).

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 patients were only excluded if they were on systemic immunosuppression or topical calcineurin inhibitors. Clinic patients and controls were recruited sequentially; blinding and randomization were not required because there was no intervention. Thus, variation between the functional responses of different donors is expected because cells were isolated from individuals of different age, gender, ethnicity, and medical history, although broadly defined as healthy controls. Sample size was determined on the basis of previous studies of CD1a-reactive T cell response frequencies in humans (*62*). 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 was constructed as previously described (PMID 25502201). Sf9 cells (1.0 liter) were infected with the *PLA2G4A* baculovirus and harvested after 72 hours. Recombinant His-tagged PLA2G4A was purified from the cell lysate

by a combination of immobilized metal affinity chromatography and gel filtration.

Antibodies and flow cytometry

For fluorescence-activated cell sorting (FACS) surface staining, the cells were labeled with the following anti-human antibodies purchased from BioLegend, unless stated otherwise: CD3 (OKT3), 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), and 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 and healthy individuals at a vacuum pressure of 250 mmHg. Blisters were generated over the site of an HDM intradermal injection or over unchallenged skin. Blisters were formed within 30 to 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, 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 used for RNA sequencing analysis.

RNA sequencing

Suction blister fluid– and blood-derived peripheral blood mononuclear cells (PBMCs) were centrifuged at 1500 rpm for 5 min at 4°C to pellet the blister-infiltrating cells, which were resuspended in phosphate-buffered saline (PBS). Blister and blood cell populations were isolated by flow cytometry and collected directly into TRIzol LS gating on subsets of T cells ($CD3^+CRTH2^+$) and ILC2 ($CD45^+$/ $Lineage^-/CRTH2^+/IL7Ra^+$). 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 RNeasy 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. After quality control analysis with the fastQC package (www.bioinformatics.babraham.ac.uk/projects/fastqc), reads were aligned using STAR (*63*) against the human genome assembly [NCBI (National Center for Biotechnology Information) build37 (hg19) UCSC (University of California, Santa Cruz) transcripts]. Nonuniquely 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 (reads per kilobase of transcript per million mapped reads) values were generated using the edgeR package (*67*).

Cell sorting and culture

PBMCs were isolated from healthy adult donors under local ethics approval [Oxford C, 09/H0606/71, National Research Ethics Service (NRES) Committee South Central]. ILC2 were isolated and cultured as previously described (*7*). Briefly, the lineage (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, CD123, and $FcεRI)^-$, $CD45^+$, $IL7Rα^+$, and $CRTH2^+$ ILC2 population was sorted into 96-well plates at 100 cells per well and resuspended in mixed leukocyte reaction of γ-irradiated PBMCs from three healthy volunteers (2×10^6 cells/ml) coupled with IL-2 (100 IU/ml) and phytohemagglutinin. After 4 to 6 weeks, the growing cells were tested by flow cytometry staining to ensure a pure population of $lineage^-CRTH2^+IL\text{-}7Rα^+$ ILC2 was obtained (fig. S1, E and F). Autologous T cells were isolated from donor PBMCs before isolation of ILC2 using magnetic-activated cell sorting (CD3 MicroBeads, Miltenyi Biotec).

Cytokines and TLR agonists

For stimulation studies, ILC2 were incubated in culture with TSLP (50 ng/ml; PeproTech), PamCSK (10 μg/ml; Invivogen), LPS (1 μg/ml; Invivogen), or heat-killed *S. aureus* (10^8 cells/ml; Invivogen) for 6 to 72 hours at 37°C, as noted in the figure legends. Cells were then centrifuged at 1500 rpm for 5 min at 4°C, the supernatant was saved for enzyme activity, and cells were used for rtPCR analysis.

Quantitative rtPCR

mRNA extraction was performed using a TurboCapture 96 mRNA kit (72251, Qiagen) following the manufacturer's instructions. Complementary DNA was prepared from the mRNA using M-MLV reverse transcriptase (Invitrogen). TaqMan probes for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Hs02786624_g1), PLA2G4A (Hs00996912_m1), and PLA2G4C (Hs01003754_m1) were used to analyze the 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 good clinical practice 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 into <0.5-mm pieces using a scalpel and scissors in petri dishes and incubated in collagenase P (1 mg/ml; Roche)–containing medium overnight at 37°C in the petri dish. After overnight digestion, the remaining tissue was homogenized with a Pasteur pipette, and endonuclease deoxyribonuclease I (200 Kunitz unit/ml; 10104159001, Roche) was added for 15 min 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 the skin were placed epidermis down in a petri dish containing dispase (5 U/ml) at 4°C overnight. The epidermal layer was then peeled from the dermis with forceps, chopped up, and placed in an Eppendorf tube with 0.5% trypsin and 0.02% EDTA at 37°C for 15 min. The samples were homogenized with a Pasteur pipette, strained through a 40-μm filter, and diluted and washed in FCS-containing medium. 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.) 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), or heat-killed *S. aureus* (10^8 cells/ml) overnight and were

then washed and resuspended in FCS ILC2 medium (RPMI supplemented with 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml), sodium pyruvate, nonessential amino acids, β-mercaptoethanol, Hepes, plus 10% FCS). The plates were washed six times with RPMI-Hepes and blocked for 1 hour with FCS ILC2 medium. A total of 50,000 T cells were plated per well to which 50,000 ILC2 were added for coculture with anti–human leukocyte antigen (HLA)–A,B,C (10 μg/ml; W6/32, eBioscience) and HLA-DR (L243, eBioscience) antibodies to inhibit ILC2-MHC-TCR interaction.

In some wells, anti-CD1a blocking antibody (OKT-6; 10 μg/ml), immunoglobulin G1 (IgG1) isotype control (10 μg/ml), $cPLA_2$ 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 the addition of T cells. Wells were set up in duplicate or triplicate. PMA (10 ng/ml) and ionomycin (500 ng/ml) stimulation was included as positive controls for T cells and ILC2, and T cells alone in the absence of ILC2 and ILC2 alone were included as negative controls. After overnight incubation at 37°C and 5% CO_2, culture supernatants were recovered, and plates were washed six times in 0.05% PBS-Tween-20 and incubated with biotin-linked anti–IFN-γ or anti–IL-22 monoclonal antibody (1 μg/ml; Mabtech AB) for 2 hours. After washing six times in 0.05% PBS-Tween-20, the plates were incubated for 1 hour with streptavidin-alkaline phosphatase (Mabtech AB). Spots were visualized using an alkaline phosphatase conjugate substrate kit (Bio-Rad) and enumerated using an automated ELISpot reader (Autimmun Diagnostika gmbh ELISpot Reader Classic). The number of IFN-γ– 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 IFN-γ or IL-22 spots in the T cell alone as 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 calculated the CD1a-dependent response by analyzing the differential signal of unblocked versus anti–CD1a-mAb–blocked conditions. The variability of human data and of the efficacy and completeness of neutralizing antibody experiments was accounted for by careful replication and analysis of a number of donors with experiments completed on different occasions. The concentration of OKT6 used for blocking has been previously optimized (*51*). Previous incubation of ILC2 with anti-CD1a preceding antigen pulsing was undertaken to aid CD1a blocking.

PLA_2 biochemical activity assays

$cPLA_2$ and $sPLA_2$ activity of recombinant PLA2G4A, heat-killed *S. aureus* preparation, and ILC2 culture supernatant was measured using either a $cPLA_2$ kit (Cayman Chemical) or a $sPLA_2$ kit (Cayman Chemical) 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 PLA_2, cleavage of the substrate (diheptanoyl thio-PC) at the sn-2 position results in the release of the thiol group. In both assays, absorbance was measured with a spectrophotometer (wavelength, 414 nm; CLARIOstar) to give a measure of PLA_2 activity.

Statistical analysis

One-way ANOVA tests with Tukey's multiple comparison tests ($\alpha = 0.05$) and paired and unpaired two-tailed *t* tests were performed using GraphPad Prism version 6.00 (GraphPad Software), as indicated in the figure legends. Error bars represent SD of the mean. The number of biological replicates and the number of times the experiments were independently performed are recorded in the figure legends. Exact *P* values are given in table S1.

SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/2/18/eaan5918/DC1

Fig. S1. Gating strategy for analysis of ILC2.

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

Table S1. Source data.

Table S2. RPKM expression values for RNA sequencing data.

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Acknowledgments: We thank the staff of the Weatherall Institute of Molecular Medicine FACS facility, all research patients and controls, and nurses, M. Westmoreland, and T. Mackenzie. **Funding:** This study was supported by the National Institute for Health Research (NIHR) Clinical Research Network, British Association of Dermatologists, British Skin Foundation, Misses Barrie Charitable Trust, Medical Research Council (CF7720, U105178805, and MR/K018779/1), Wellcome Trust (090532/Z/09/Z), and NIHR Oxford and Leicester Biomedical Research Centres. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health. **Author contributions:** C.S.H., M.S., V.J.J., Y.-L.C., J.L.B., D.J.C., and G.O. performed the experiments. E.R. carried out the bioinformatics analysis of RNA sequencing data. D.J. provided samples. A.N.J.M., D.J.C., R.J.O., R.J., and J.L.B. provided feedback and supervised the aspects of the study. G.O. conceived the study and wrote the manuscript with C.S.H. C.S.H. completed the statistical analysis of the data. **Competing interests:** G.O. has served on advisory boards or holds consultancies or equity with Eli Lilly, Novartis, Janssen, Orbit Discovery, and UCB Pharma and has undertaken clinical trials for Atopix, Regeneron/Sanofi, Roche, and AnaptysBio. A.N.J.M. has received grant support from MedImmune/AstraZeneca and GSK. All other authors declare that they have no competing interests.

Submitted 5 May 2017
Accepted 7 November 2017
Published 22 December 2017
10.1126/sciimmunol.aan5918

Citation: C. S. Hardman, Y.-L. Chen, M. Salimi, R. Jarrett, D. Johnson, V. J. Järvinen, R. J. Owens, E. Repapi, D. J. Cousins, J. L. Barlow, A. N. J. McKenzie, G. Ogg, CD1a presentation of endogenous antigens by group 2 innate lymphoid cells. *Sci. Immunol.* **2**, eaan5918 (2017).

Science Immunology

CD1a presentation of endogenous antigens by group 2 innate lymphoid cells

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Sci. Immunol. **2**, eaan5918.
DOI: 10.1126/sciimmunol.aan5918

A new look at lipid surveillance

Human group 2 innate lymphoid cells (ILC2) play roles in maintaining homeostasis and defending against pathogens, but dysregulated ILC2 responses have been linked to asthma and allergic responses. Hardman *et al*. now use an in vivo human skin challenge model to show that ILC2 express CD1a, which is regulated by TSLP, and that CD1a$^+$ILC2 can present endogenous lipid antigens to CD1a-reactive T cells and induce inflammatory responses. CD1a$^+$ILC2 expressed the phospholipase PLA2G4A, which contributed to CD1a-mediated T cell activation, and this pathway was involved in sensing *Staphylococcus aureus*–associated skin inflammation. These results provide insight into lipid sensing by skin-resident ILC2 and how this pathway may contribute to atopic skin inflammation and pathogen surveillance.