Published December 9, 2013

A role for IL-25 and IL-33–driven type-2 innate lymphoid cells in atopic dermatitis

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Type 2 innate lymphoid cells (ILC2s, nuocytes, NHC) require *RORA* and *GATA3* for their development. We show that human ILC2s express skin homing receptors and infiltrate the skin after allergen challenge, where they produce the type 2 cytokines IL-5 and IL-13. Skin-derived ILC2s express the IL-33 receptor ST2, which is up-regulated during activation, and are enriched in lesional skin biopsies from atopic patients. Signaling via IL-33 induces type 2 cytokine and amphiregulin expression, and increases ILC2 migration. Furthermore, we demonstrate that E-cadherin ligation on human ILC2 dramatically inhibits IL-5 and IL-13 production. Interestingly, down-regulation of E-cadherin is characteristic of filaggrin insufficiency, a cardinal feature of atopic dermatitis (AD). ILC2 may contribute to increases in type 2 cytokine production in the absence of the suppressive E-cadherin ligation through this novel mechanism of barrier sensing. Using *Rag1*$^{-/-}$ and RORα-deficient mice, we confirm that ILC2s are present in mouse skin and promote AD-like inflammation. IL-25 and IL-33 are the predominant ILC2-inducing cytokines in this model. The presence of ILC2s in skin, and their production of type 2 cytokines in response to IL-33, identifies a role for ILC2s in the pathogenesis of cutaneous atopic disease.

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Abbreviations used: AD, atopic dermatitis; HDM, house dust mite; ILC, innate lymphoid cell.

Atopic dermatitis (AD) is a common pruritic inflammatory skin disease that is associated with barrier dysfunction and Th2 cell adaptive immune responses to common environmental allergens. It is a disease with complex genetic and environmental susceptibility factors. Although it is likely that many genetic loci are involved, the association of filaggrin-null mutations with AD has provided a major step forward in our understanding of disease pathogenesis (Palmer et al., 2006). Filaggrin is expressed in keratinocytes and is thought to have a role in skin barrier function, cutaneous pH, and hydration (Presland et al., 2001; Sandilands et al., 2009). However, little is known as to how an inherited epidermal abnormality leads to a compromised skin barrier, skin inflammation, and related atopic disorders, although high levels of IL-13 and IL-4 are known to be expressed in lesions of AD (Leung et al., 2004; Kim et al., 2013).

Although Th2 cells have been characterized as producers of the cardinal cytokines IL-4, IL-5, and IL-13 in AD (Leung et al., 2004), the recent discovery of innate lymphoid cells (ILCs) raises the question of their potential involvement as innate sources of type 2 cytokines in

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The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 13 2939-2950
www.jem.org/cgi/doi/10.1084/jem.20130351

Supplemental Material can be found at:
http://jem.rupress.org/content/suppl/2013/12/04/jem.20130351.DC1.html

The Journal of Experimental Medicine

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this disease. Several recent studies have identified a family of CD45-expressing hematopoietic effector ILCs that link the innate and adaptive arms of the immune system (Mjösberg et al., 2011; Spits and Cupedo, 2012; Walker et al., 2013). Such ILCs are found in the blood, spleen, intestine, liver, lung, FALCs (fat-associated lymphoid clusters), and LNs of mice (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010; Mjösberg et al., 2011). An ILC subset that produces type 2 cytokines (IL-5, IL-9, and IL-13), and which is independent of RORγt, has been designated as the type 2 ILC or ILC2 (Spits et al., 2013; Walker et al., 2013). ILC2s are negative for lineage markers of T and B cells, but in mice they express c-Kit (CD117), ST2, CD90, and the hematopoietic and lymphoid markers CD45 and IL-7Rα (CD127). Consistent with their expression of IL-17RB (IL-17BR and IL-25R) and ST2 (IL-33R) receptors, these cells respond to IL-25 and IL-33 by producing type 2 cytokines, and in mice ILC2s have been shown to induce goblet cell hyperplasia and eosinophilia, and contribute to protection against helminth infections (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Spits and Cupedo, 2012).

In mice, lung-resident ILC2s have also been demonstrated to contribute to airway hyper-reactivity, induced by viral or allergen challenge (Moro et al., 2010; Mjösberg et al., 2011; Monticelli et al., 2011; Barlow et al., 2012; Klein Wolterink et al., 2012). However, ILC2s also serve to restore epithelial integrity and lung function after infection with the H1N1 influenza virus, predominantly by producing amphiregulin, a regulator of wound healing (Monticelli et al., 2011). The human counterparts of ILC2 were recently reported in human lung parenchyma and bronchoalveolar lavage fluid, and defined as lineage-negative cells that express IL-7Rα and the ST2 subunit of the IL-33 receptor (Monticelli et al., 2011). More comprehensively, Spits et al. (2013) reported CD45^{hi}, CD127^{+}, and CD117^{+} cells in peripheral blood, fetal gut, and the inflamed nasal polyps of patients with rhinosinusitis. The cells also expressed CRTH2 and CD161 and, in response to epithelial cytokines, produced large amounts of IL-13 and IL-5, but not IL-17A or IL-22 (Mjösberg et al., 2011).

Recently, ILC2-like cells have been reported within mouse and human atopic lesional skin and, at least in mice, recruitment of ILC2-like cells to sites of inflammation was demonstrated to be dependent on TSLP and independent of IL-33 (Kim et al., 2013). However, transgenic mice with IL-33 expressed under the keratin 14 promoter developed a spontaneous AD-like inflammation of the skin which associated with ILC2 infiltration (Imai et al., 2013). A further murine study identified ILC2s in the skin which were present at a 30% frequency of T cells and were IL-7 and IL-2 dependent. Using intravital microscopy, it was shown that these ILC2s interacted with mast cells and produced IL-13 (Roediger et al., 2013). It is therefore unclear to what extent human and murine skin-derived ILC2s are dependent on IL-33. This is an important question for understanding disease and for directing future therapeutic activity. Herein, we define a lineage-negative, IL-7Rα^{+}CRTH2^{+}c-kit^{+}ICOS^{+}CD161^{+}CD25^{+}CCR4^{+}CCR10^{+}NKp46^{-}CD56^{-}, RORα^{+}GATA3^{+} ILC2 in human skin that is elevated in biopsies from AD patients and shows elevated ST2, IL-17BR, TSLPR, and KLRG1 expression. These cells respond potently to IL-33 by releasing type 2 cytokines and amphiregulin. Furthermore, human ILC2 can be inhibited by their ligation of E-cadherin, suggesting a novel mechanism for barrier sensing. Investigation of ILC2s in a BALB/c mouse model of dermatitis demonstrated their

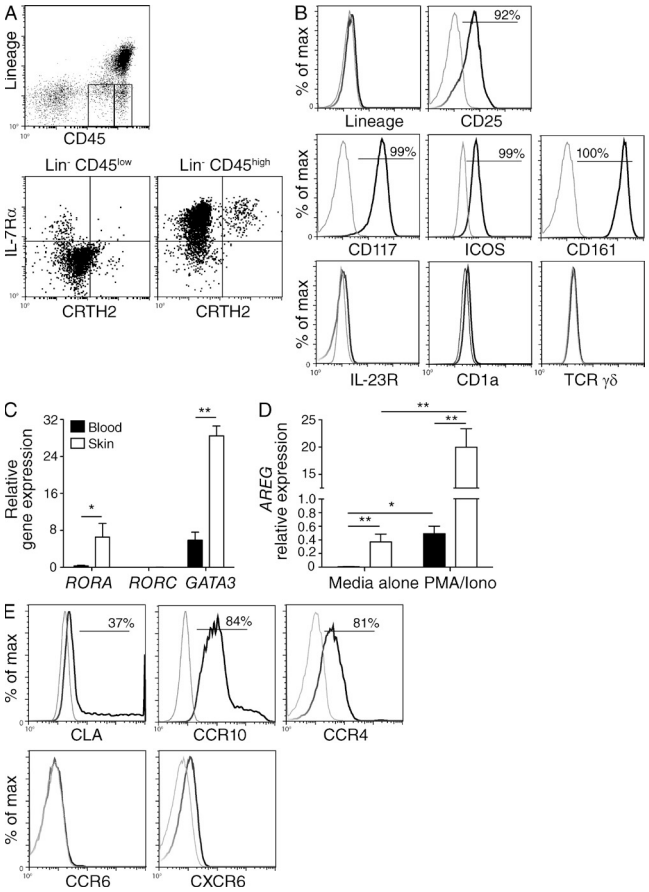

Figure 1. ILC2s are resident in human skin. (A) Freshly isolated leukocytes from the human skin were stained with the combination of ILC surface markers, including lineage markers (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FcεRI, TCR-γδ, TCR-αβ, and CD123), CD45, and IL-7Rα. Flow cytometric analysis of IL-7Rα and CRTH2 expression on lineage-negative CD45^{hi} and lineage-negative CD45^{low} cells (n = 15) is shown. (B) Expression of the indicated cell surface markers on Lin^{-}CD45^{hi}IL-7Rα^{+}CRTH2^{+} cultured ILC2 from the skin (n = 3). Black plots show the relevant marker; grey show the isotype control. Bars show the percentage of cells expressing the relevant marker. (C and D) CD45^{hi}IL-7Rα^{+} CRTH2^{+} cells were sorted and cultured from blood and skin, and *RORA* (n = 8, where n is number of donors), *RORC* (n = 5), *GATA3* (n = 3; C), and *AREG* (n = 3; D) gene expression relative to *GAPDH* was measured by RT-PCR. (E) Lin^{-}CD45^{hi}IL-7Rα^{+}CRTH2^{+} ILC2s were isolated from human skin and expression of the indicated homing receptors was analyzed by flow cytometry. Black plots show the relevant marker; grey show the isotype control. Bars show the percentage of cells expressing the relevant marker. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's t test (mean and SEM in C and D).

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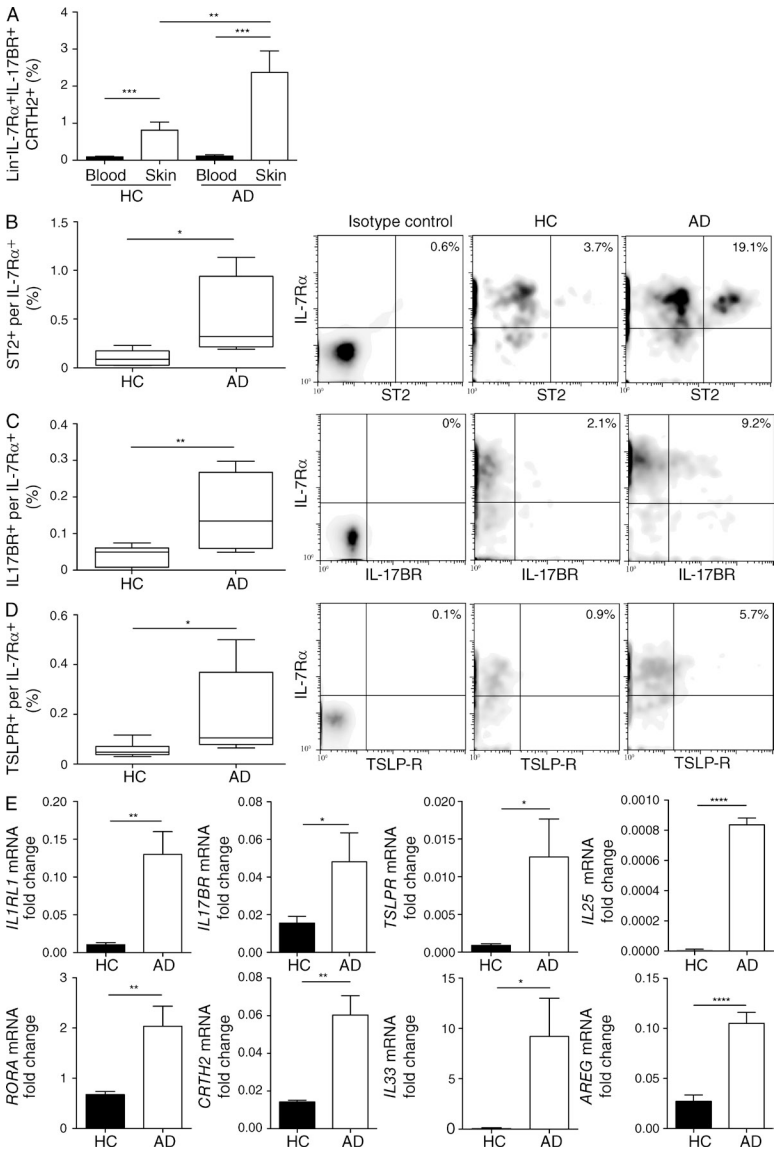

Figure 2. ILC2s are present at higher frequency and with a modified phenotype in the skin of AD patients. (A) Frequency of ILC2 (as a percentage of IL-7Rα$^+$ cells) in the blood and skin of healthy controls (HCs; $n = 15$) or in lesional biopsies of the acutely affected skin of patients with AD ($n = 8$) was measured by flow cytometry. (B–D) ILC2s (Lin$^-$CD45^{high}IL-7Rα$^+$) were isolated from the skin of patients with AD and healthy controls, and expression of ST2 (B), IL-17BR (C), and TSLPR (D) was analyzed by flow cytometry. Representative flow plots are shown on the right. Numbers indicate percentages of cells within the top right quadrant of gated populations. B: patients and controls ($n = 7$); C: patients ($n = 5$), controls ($n = 8$); D: patients ($n = 6$), controls ($n = 7$). (E) Acute lesional skin biopsies were taken from patients with AD ($n = 8$) and healthy controls ($n = 6$), and expression of *IL1RL1* (IL-33R), *IL-33*, *IL17BR*, *IL-25*, *CRTH2*, *TSLPR*, *AREG*, and *RORA* was measured by RT-PCR relative to *GAPDH*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's *t* test (mean and SEM in A and E; min and max in B–D).

contribution to skin inflammation, and further identified primary roles for IL-25 and IL-33 in their regulation, with a lesser role for TSLP in this model. Thus, the ILC2-initiating cytokines IL-25, IL-33, and TSLP all play a role in the regulation of skin inflammation and represent potential therapeutic pathways in AD.

RESULTS

An ILC2 population resides in human skin

To investigate the presence of ILCs in human skin, we isolated lymphoid cells from skin biopsies of healthy adult donors ($n = 15$). After excluding cells that expressed common lineage markers (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FcεRI, TCR-γδ, TCR-αβ, and CD123), we observed that many CD45$^+$ cells (52.78 ± 33.26%) express the lymphoid marker IL-7Rα, and therefore are likely to belong to the ILC family (Fig. 1 A and Fig. S1 A). To specifically identify the ILC2 subpopulation, we stained ILC for the human ILC2 marker CRTH2. Almost all CRTH2-expressing cells exhibited high levels of surface CD45, which is similar to the population of ILC2 reported in fetal and adult human intestines (Mjösberg et al., 2011; Fig. 1 A). Skin-resident ILC2 expressed high levels of c-kit, ICOS, CD161, and CD25, whereas they were negative for IL-23R, CD1a, and γδ (Fig. 1 B). We also analyzed the expression of killer cell lectin-like receptors (KLRs) and killer cell immunoglobulin-like receptors (KIRs) (Averdam et al., 2009). ILC2 did not express any of the KIR receptor family members KIR2DL1/S1, KIR3DL1/S1, and KIR2DL2/S2/L3/S4, the KLR family receptors, NKG2A and NKG2C, or the NK-associated surface markers NKp46 and CD56 (unpublished data).

ILC2 differentiation and expansion is largely dependent on the transcription factors RORα (Wong et al., 2012) and GATA3 (Hoyler et al., 2012). Using quantitative PCR, we analyzed the transcripts of these genes in purified ILC2 isolated from human skin. Skin-resident ILC2 expressed low levels of

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the *RORC* transcripts, while expressing relatively high amounts of *RORA* and *GATA3* transcripts. Interestingly, when compared with similarly purified ILC2 from the blood, *RORA* and *GATA3* expression in skin-resident ILC2 were significantly higher (P = 0.04 and 0.009, respectively; Fig. 1 C).

Activation of murine ILC2 by epithelial cytokines can induce type 2 cytokine production and initiate allergic responses (Barlow et al., 2012; Klein Wolterink et al., 2012) while they can concurrently induce amphiregulin, a wound-healing regulator which aids tissue repair (Monticelli et al., 2011). Therefore, we compared the transcript levels of the amphiregulin gene (*AREG*) in skin-resident ILC2s and those purified from the blood. Interestingly, the expression of *AREG* was also significantly higher in the skin-resident ILC2 (P value = 0.02) and was further up-regulated in response to stimulation (Fig. 1 D). These data on differential expression of *AREG* in skin and blood ILC2 infer that the activation state and/or function of ILC2 is distinct at the different anatomical sites.

It has been proposed that ILC2s derive from common lymphoid progenitors in the bone marrow and mature ILC2s circulate in the blood and home to the tissues (Mjösberg et al., 2011; Wong et al., 2012). As the skin is a major site of antigen encounter and long-term immune surveillance, recruited leukocytes in the skin are imprinted with tissue-specific homing receptors, such as cutaneous lymphocyte antigen (CLA) and chemokine receptors including CCR4, CCR6, and CCR10, which provide effective and quick trafficking of memory cells to sites of inflammation (McCully et al., 2012). We found that a significant proportion of ILC2s derived from healthy donors express the skin-homing receptors CLA, CCR10, and CCR4 (Fig. 1 E). Collectively, these data indicate that skin-infiltrating/-resident ILC2 may have a phenotype that is distinct from circulating ILC2.

It is noteworthy that compared with the paucity of circulating ILC2s in the blood, ILC2s are highly enriched in naive human skin tissue (0–0.18% vs. 0.04–2.94% of lymphoid cells in blood and skin, respectively; Fig. 2 A). To examine whether ILC2 cells might contribute to the pathogenesis of AD, we examined peripheral blood and acute lesional skin of adults with AD for the presence of ILC2. There were significantly (P < 0.005) more ILC2s detected in lesional skin biopsies from atopic patients relative to healthy individuals (Fig. 2 A). In contrast, there was a similar frequency of circulating ILC2 in the peripheral blood of both groups (Fig. 2 A). Disruption of epithelial barrier integrity in the lung and skin induces the production of IL-25, IL-33, and TSLP (Islam and Luster, 2012). Consistent with their activation in response to IL-25, IL-33, and TSLP, human atopic skin-derived ILC2s express IL-17RB (IL-25R), ST2 (IL-33R), and TSLP receptors (Fig. 2, B–D). Notably, all three receptors were further up-regulated in freshly isolated ILC2 from the skin of patients with AD as compared with healthy controls (Fig. 2, B–D). Gene expression analysis on skin samples isolated from either noninvolved skin or AD-involved skin clearly demonstrated the up-regulation of genes encoding the IL-17RB (IL-25R), ST2 (IL-33R), and TSLP receptors in the AD skin samples, and this was

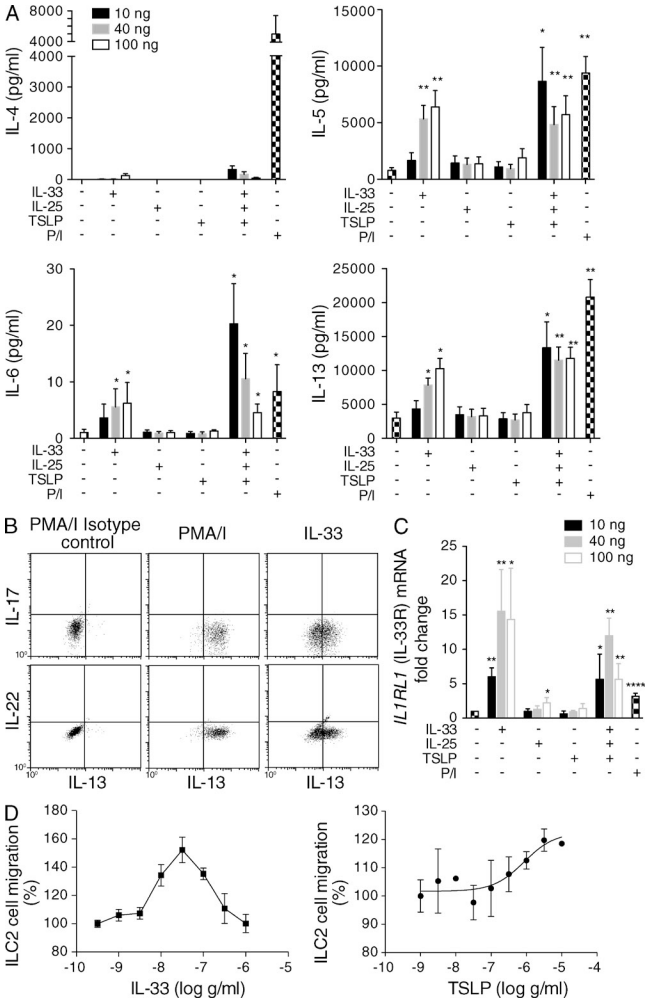

Figure 3. Human skin ILC2s stimulated with IL-33 produce high levels of type-2 cytokines. (A) Cultured skin ILC2s were stimulated with PMA/ionomycin or IL-25, IL-33, and TSLP alone or in the indicated combinations and concentrations. Supernatant was collected after 24 h. Levels of IL-4, IL-5, IL-6, and IL-13 were measured by multiplex cytokine analysis. Statistical comparisons are compared with the negative (no cytokine) control. (B) Skin-derived ILC2s were stimulated with PMA/ionomycin or IL-33, and levels of IL-13, IL-17A, and IL-22 expression were measured using intracellular cytokine staining. Data are representative of four independent experiments on cultured cells. (C) ILC2s were stimulated for 16 h with IL-25, IL-33, or TSLP in isolation or combination at the indicated combinations. Expression of *IL1RL1* (IL-33R) was measured by RT-PCR. Data are representative of three independent experiments. Statistical comparisons are compared with the negative (no cytokine) control. (D) Chemotaxis of skin-derived ILC2s toward indicated concentrations of IL-33 and TSLP, relative to baseline, was measured after 1 h using transmigration across a 5 µM pore membrane. Data are representative of three independent experiments (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001, unpaired Student's t test (mean and SEM in A, C, and D).

accompanied with elevated expression of *CRTH2*, *RORA*, and *AREG* mRNA (Fig. 2 E). Analysis for cytokine gene expression also detected increased levels of *IL33* and *IL25* in the AD samples (Fig. 2 E). Thus, skin ILC2s were more prevalent in the lesional skin of AD patients and showed a coincident

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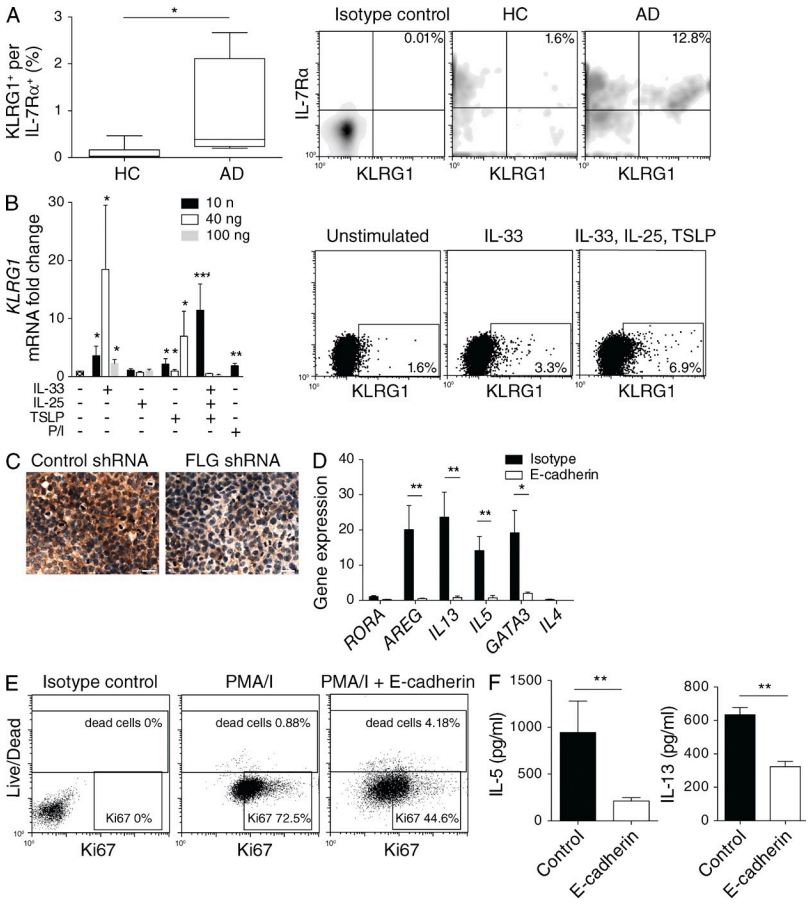

Figure 4. E-cadherin can regulate type 2 cytokine production by human skin ILC2. (A) ILC2s were isolated from the skin of patients with AD ($n = 4$) and healthy controls ($n = 8$), and expression of KLRG1 was analyzed by flow cytometry. Representative flow plots are shown on the right. Numbers indicate percentages of cells within the top right quadrant of gated populations. (B) ILC2s were stimulated with IL-33, IL-25, and TSLP, and levels of KLRG1 gene and protein expression were measured by RT-PCR and flow cytometry. Statistical comparisons are compared with the negative (no cytokine) control. Numbers on the flow cytometry plots indicate the proportion of gated cells that express KLRG1 ($n = 4$). (C) E-cadherin expression by keratinocytes after control (left) or filaggrin (right) shRNA knockdown (bars, 50 µm; $n = 3$). (D) Expression of *RORA*, *AREG*, *IL-13*, *IL-5*, *GATA3*, and *IL*-4 mRNA relative to *GAPDH* by ILC2 activated using PMA/I before and after culture with rhE-cadherin (recombinant human E-cadherin) for 24 h ($n = 7$), as measured by PCR. (E) Ki67 and live/dead staining of ILC2 incubated with PMA/ionomycin in the presence or absence of E-cadherin. Data are representative of three independent experiments. Numbers indicate percentages of cells within the indicated gates. (F) IL-5 and IL-13 concentration in supernatant from ILC2 activated with IL-25 and IL-33 and incubated in the presence or absence of rhE-cadherin ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's *t* test (min and max in A; mean and SEM in B, D, and F).

up-regulation of the receptors for the key ILC2-inducing cytokines IL-25, IL-33, and TSLP in an environment in which *IL25* and *IL33* gene expression was also elevated.

IL-33 is a potent stimulus for skin-derived ILC2

We next assessed the responses of ILC2, isolated from healthy skin, to treatment with the cytokines IL-25, IL-33, and TSLP because a defining feature of ILC2s is their ability to produce IL-13 and IL-5, but not IL-17A/IL-22, in response to stimulation with IL-25 and IL-33. Using multiplex analysis, we compared the cytokine production after stimulation with PMA/ionomycin or cytokine combinations at different concentrations (Fig. 3). IL-33 stimulation elicited significant levels of IL-13, IL-6, and IL-5 production (Fig. 3 A) but induced little IL-4 (Fig. 3 A). No IL-17A or IL-22 expression was detected (unpublished data). TSLP and IL-25 did not induce significant IL-4, IL-13, IL-6, or IL-5 production when present in isolation but interestingly were able to enhance cytokine production when present in combination. Increased type 2 cytokine gene expression indicated that this was not simply due to the release of preformed cytokines (unpublished data). We also evaluated the cytokine production of IL-25– and IL-33–activated cells using intracellular cytokine staining. Although skin-derived ILC2s produced high levels of IL-13 after stimulation with PMA/ionomycin or IL-33, they did not express detectable IL-17A and IL-22 (Fig. 3 B). IL-25 failed to induce IL-13 in these assays (unpublished data). The responsiveness to IL-33 also coincided with an increase of IL-33 receptor expression (Fig. 3 C).

Using trans-migration assays, we also determined that IL-33, in contrast to IL-25, elicited significant migration of skin-derived in vitro–cultured ILC2 (Fig. 3 D and not depicted). Although migration toward TSLP was also observed, this occurred only at high concentrations (Fig. 3 D). Thus, IL-33 is a potent stimulus for the activation of human skin-derived ILC2, inducing the up-regulation of its own receptor, enhancing the expression of type-2 cytokine expression, and increasing the migratory capacity of these cells.

E-cadherin ligation can inhibit ILC2 cytokine secretion

We noted that KLRG1 (killer cell lectin-like receptor G1) was also more highly expressed by ILC2s that were freshly isolated from AD samples, as compared with healthy controls (Fig. 4 A), and that IL-33 and TSLP both up-regulated KLRG1 gene and protein expression on cultured ILC2s (Fig. 4 B). Although KLRG1 has been studied recently as a marker of ILC2, in the context of mouse ILC2 development within the bone marrow and the periphery (Hoyler et al., 2012), the functional role of KLRG1 on ILC2 has not been investigated. Interestingly, KLRG1 has been reported to act as an inhibitory receptor on a proportion of NK and effector $CD8^+$ T cells (Hofmann et al., 2012) and KLRG-1 binds to

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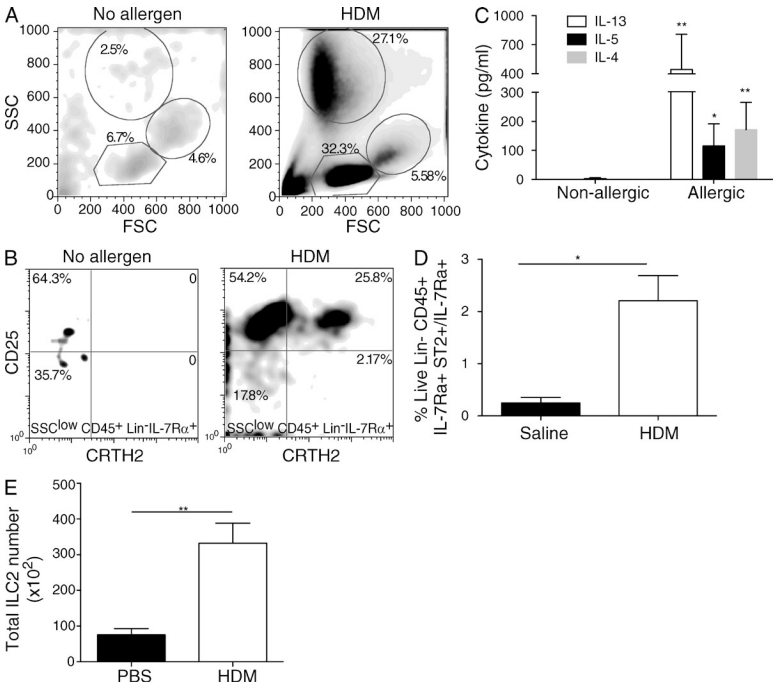

Figure 5. ILC2s localize to the skin during an allergic response in vivo. An intraepidermal injection of HDM was given to an HDM-allergic individual, and 2 h later suction blisters were formed over the site of injection or on an uninjected area of skin. Fluid was extracted 26 h later, and flow cytometry was performed to analyze total cell (A) and ILC2 (B) accumulation expressed as percentage of cells within the plots (shown as numbers within each gated region). (C) Levels of IL-13, IL-5, and IL-4 within the blister fluid isolated from allergic ($n = 5$) and nonallergic donors ($n = 3$) were quantified 26 h after HDM administration into the skin. Statistical comparisons are made between cytokine levels from allergic and nonallergic donors. (D) Infiltration of ST2-expressing ILC2 into human skin 26 h after HDM or saline challenge ($n = 4$). (E) ILC2 (defined as $Lin^-CD45^+IL\text{-}7R\alpha^+ICOS^+c\text{-}Kit^+$ cells) infiltration into the skin of C57BL/6 strain mice after subcutaneous administration of HDM allergen or PBS was measured using flow cytometry ($n = 3$–4). Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$, unpaired Student's *t* test (mean and SEM in C–E).

the cell adhesion molecule E-cadherin, which is widely expressed on keratinocytes and Langerhans cells (Gründemann et al., 2006), thereby inhibiting cellular proliferation (Rosshart et al., 2008). Notably, E-cadherin expression is down-regulated from the surface of keratinocytes in the lesional skin of patients with AD (Trautmann et al., 2001). Moreover, we have determined that E-cadherin is down-regulated from human keratinocytes after shRNA knockdown of the AD-associated filaggrin gene (Fig. 4 C). We hypothesized that null mutations in the filaggrin gene, which are found in 20–40% of patients with severe AD, may contribute to the down-regulation of E-cadherin, leading to a failure to turn off ILC2 proliferation and cytokine expression.

We therefore investigated whether a KLRG1–E-cadherin interaction may alter ILC2 function and act as a suppressive mechanism for dampening the ILC2 response, PMA/ionomycin-activated human ILC2s were then cultured with recombinant plate-bound E-cadherin for 24 h. Supporting the inhibitory role of E-cadherin binding, we detected down-regulation of the expression of *GATA3*, as well as transcripts for the ILC2 signature cytokines *IL13* and *IL5*, and *AREG* (Fig. 4 D), and reduced ILC2 proliferation (Fig. 4 E). Furthermore, in the presence of plate-bound E-cadherin, the production of IL-13 and IL-5 protein was reduced in IL-25– and IL-33–activated ILC2 (Fig. 4 F). Thus, in tissues expressing E-cadherin, the up-regulation of KLRG1 on ILC2 may represent an important mechanism for ameliorating the ILC2-mediated type 2 cytokine-driven inflammatory response, but when E-cadherin is down-regulated, for example in AD, this signal is absent or reduced, resulting in unhindered ILC2 cytokine production. This would represent a novel mechanism of skin barrier sensing.

Allergen challenge can induce ILC2 infiltration into human and mouse skin

House dust mite (HDM) extract is one of the most common aeroallergens that associate with exacerbation of AD symptoms (Crack et al., 2012). To examine ILC2 recruitment to allergen-provoked skin, we used suction blisters to sample skin cells before and after HDM allergen delivery in the epidermis of humans. We extracted the cells infiltrating the blister 26 h after intraepidermal administration of HDM extract to the skin. After HDM administration, there was a significant infiltration into blisters from allergic individuals compared with nonallergic individuals for granulocytes (39.9 vs. 4.53, $P < 0.01$), monocytes (8.84 vs. 1.86, $P < 0.01$), and lymphoid cells (13.57 vs. 2.55, $P < 0.01$). Infiltrating lymphoid cells included CD3, CD56, and ILC2 populations (Fig. S1 B). ILC2s ($Lin^-CD45^+CD127^+CD25^+CRTH2^+ST2^+$) were clearly observed after allergen challenge (Fig. 5 [A and B] shows a representative experiment of cell accumulation in blisters). Higher concentrations of IL-13, IL-5, and IL-4 were detected in the blister fluid of allergic donors 26 h after HDM allergen challenge, whereas in nonallergic individuals there were no detectable type 2 cytokines (Fig. 5 C). Furthermore, we observed that ST2-expressing ILC2 infiltrated the skin 26 h after HDM challenge of humans (Fig. 5 D).

To support our human studies, we also investigated the impact of HDM extract treatment on the presence of ILC2 in mouse skin. Subcutaneous administration of HDM resulted in a significant ($P < 0.05$) increase in skin-associated ILC2 (defined as $Lin^-CD45^+IL\text{-}7R\alpha^+ICOS^+c\text{-}Kit^+$ cells; Neill et al., 2007, 2010) in both C57BL/6 (Fig. 5 E) and BALB/c (not depicted) mice. Thus, in both human and mouse skin, ILC2 numbers increase in response to allergens such as HDM extract.

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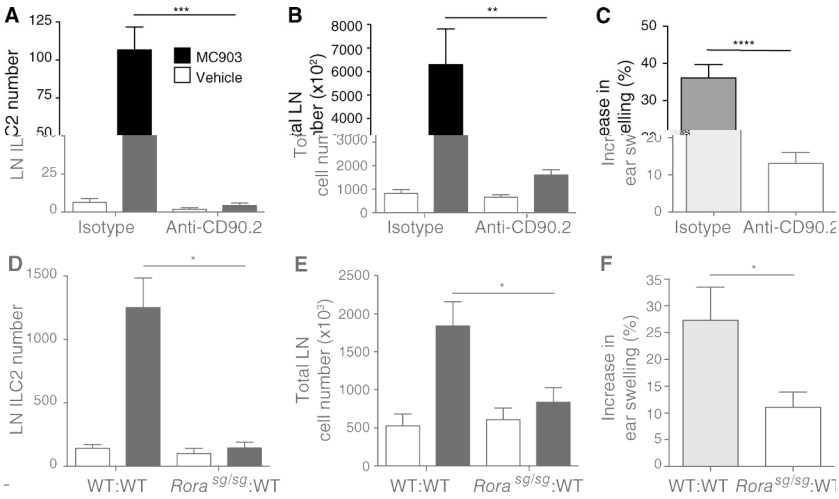

Figure 6. Depletion of ILC2 significantly reduces ear swelling and inflammation in mice. MC903 or vehicle was applied topically for 4 d to the ear of *Rag1*$^{-/-}$ mice with or without prior treatment of anti-CD90.2 antibody or isotype control. In A, B, D, and E, vehicle is shown as open bars and MC903 is shown as filled bars. Accumulation of LN ILC2 (A); total LN cell number (B); and increase (percent) in ear thickness after MC903 application (C). After 4 d of topical application of MC903 or vehicle to the ear of wild-type or *Rora*$^{sg/sg}$ bone marrow chimera mice, the accumulation of LN ILC2 (D), total LN cell number (E), and increase (percent) in ear thickness (F) were documented. n = 5–6 mice per group. Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's t test with Welch's correction (mean and SEM).

ILC2s accumulate in calcipotriol-challenged mouse skin and are critical for inflammation

To provide in vivo evidence for the functional importance of ILC2 in skin inflammation, we used a mouse model of calcipotriol-induced skin inflammation. Calcipotriol (MC903) is a synthetic form of active vitamin D_3 that induces AD-like lesions in mice (Li et al., 2006), which associates with TSLP induction in the skin. After four daily topical applications of MC903 to the ear pinna of BALB/c mice, ear thickness was found to increase compared with the control vehicle-treated ear. Coincident with this thickening, cell infiltration in the draining LN also increased significantly and included an enlarged population of Lin$^-$CD45$^+$IL-7Rα^+ICOS$^+$c-Kit$^+$ cells consistent with the surface phenotype of previously described ILC2 in mice (unpublished data; Neill et al., 2010).

To determine the potential contribution of ILC2 to the initiation of inflammation, we assessed whether MC903 continued to induce inflammation in *Rag1*$^{-/-}$ mice lacking B and T cells when treated with anti-CD90.2 to deplete ILC2 (Monticelli et al., 2011). *Rag1*$^{-/-}$ mice were treated with MC903 in the presence of either an anti-CD90.2 antibody or an isotype control antibody. MC903-induced inflammation was still detected in isotype control-treated *Rag1*$^{-/-}$ mice (Fig. 6, A–C), but administration of the anti-CD90.2 antibody ablated ILC2 (Fig. 6 A) and correlated with significantly reduced cellular infiltration and ear swelling (Fig. 6, B and C). These data demonstrate a role for ILC2 in dermatitis in mice, and that recruitment of ILC2 to inflamed skin is independent of T and B lymphocytes but dependent on CD90.2$^+$ cells.

RORα is required for the induction of MC903-dependent skin inflammation in mice

Because CD90.2 is expressed on several innate cells, we used an additional in vivo strategy to investigate skin inflammation using mice lacking the transcription factor RORα, which we have demonstrated to have a profound deficit in ILC2 development but to have intact CD4$^+$ T cells (Wong et al., 2012). Initially, we observed that RORα-deficient mice developed significantly lower MC903-induced ear inflammation and cell infiltration compared with wild-type littermate controls (unpublished data). However, because RORα-deficient (Staggerer) mice also have defects in neuronal development (Wong et al., 2012) we wished to exclude the possibility that such immune-independent factors might be affecting skin inflammation. To achieve this, we generated bone marrow chimaeras in which irradiated wild-type C57BL/6 recipients were reconstituted with wild-type or RORα-deficient bone marrow. After 6 wk, the mice were treated with MC903 and inflammation assessed. Mice receiving wild-type bone marrow had increased numbers of ILC2 in the draining LN of ears administered with MC903 as compared with levels in vehicle-treated ears (Fig. 6 D). In contrast, MC903 failed to induce ILC2 in mice receiving the RORα-deficient bone marrow (Fig. 6 D). In the absence of ILC2 the total cellular infiltrate was ameliorated (Fig. 6 E), and this corresponded with significantly reduced ear swelling in comparison to the wild-type group (Fig. 6 F). These findings strongly suggest that ILC2s can infiltrate murine skin and that they are critical for the development of AD-like cutaneous inflammation.

A predominant role for IL-25 and IL-33, but not TSLP, in regulating mouse skin ILC2 in BALB/c mice

To address the relative importance of the ILC2-inducing cytokines IL-33, IL-25, and TSLP in regulating ILC2 numbers and pathology in response to skin inflammation, BALB/c strain mice deficient in these cytokine pathways were challenged with MC903. Strikingly, the greatest reduction in skin inflammation was observed in those mice lacking IL-25 (Fig. 7, A and B). This correlated with a marked decrease in the numbers of ILC2 in the ear tissue (Fig. 7 C) and also the skin-draining LN (Fig. 7 D). The absence of IL-33 signaling also resulted in a significant decrease in ear swelling (Fig. 7 A) that also correlated with a significant reduction in ILC2 numbers in the ear tissue (Fig. 7 C) and draining LN (Fig. 7 D). The absence of TSLP signaling resulted in a modest reduction in ILC2 numbers in inflamed ear tissue (Fig. 7 C), total infiltrating cell numbers (Fig. 7 B), and ear swelling (Fig. 7 A). We have also analyzed the relative role of these cytokines in

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MC903-induced skin inflammation in mice on a C57BL/6 strain background. Similar to BALB/c mice (Fig. 7 A), the degree of skin inflammation was ameliorated in the absence of signaling by each cytokine, with skin inflammation in C57BL/6 strain background being markedly dependent on TSLP (not depicted), as recently reported (Kim et al., 2013). These data suggest a hierarchical role for these three initiating cytokines in the regulation and recruitment of mouse ILC2 in the genesis of skin inflammation. The differences also highlight the importance of concurrent human data in the context of clinical disease.

DISCUSSION

Here, we have shown that type 2 ILCs represent a resident population of ILCs in the skin of both humans and mice. Similar to their counterparts in other tissues, skin ILC2s are characterized by a lack of lineage markers but expression of IL-7Rα, CRTH2, and CD45 (Mjösberg et al., 2011) and the ILC2-specific transcription factors RORα and GATA3 (Halim et al., 2012; Hoyler et al., 2012; Spits and Cupedo, 2012; Wong et al., 2012). Interestingly CRTH2 was expressed on a greater proportion of ILC2s taken from the fetal gut (Mjösberg et al., 2011) as compared with the ILC2s here taken from the skin, which could represent differential organ-specific CRTH2 expression. Furthermore, the lack of RORγt and NKp46 further confirms that they are distinct from other members of the ILC family: ILC3, LTi, or conventional NK cell populations (Spits and Cupedo, 2012; Walker et al., 2013). Although it remains possible that ILC2s can express the latter receptors under specific conditions, their expression levels are low or absent on skin-derived ILC2s. Human skin ILC2s have also recently been described by Kim et al. (2013); however, our data presented here go on to show that human skin-derived ILC2s express the cytokine receptors ST2 (IL-33R), TSLPR, and IL-17BR (IL-25R). Furthermore, ILC2s were also positive for skin-homing markers, which would support the possibility that in addition to skin residence, these cells can also home to human skin in inflammatory settings. We observed that ILC2s were enriched within AD acute lesional skin and such cells expressed significantly greater levels of ST2, IL17BR, TSLPR, and KLRG1 as compared with ILC2 present in the skin of healthy controls. Furthermore, IL-33, but not IL-25, induced significant migration and production of type 2 cytokines and amphiregulin by skin-derived ILC2. Although TSLP could also induce cytokine production and migration, the levels were significantly lower than those observed with IL-33.

It is notable that amphiregulin has previously been implicated in barrier repair mechanisms in dermatitis (Iordanov et al., 2005) and evidence supports the role of innate immune responses in the pathogenesis of AD (McGirt and Beck, 2006; De Benedetto et al., 2009; Shiohara et al., 2011). AD lesions in humans are associated with down-regulation of E-cadherin expression. We investigated ILC2 activity in the presence and absence of E-cadherin, an adhesion protein pivotal for maintaining the integrity of epithelia. Activated skin-resident ILC2s express high levels of the inhibitory KLRG1 receptor that, upon interaction with E-cadherin, down-regulates expression of transcription factors and production of IL-13, IL-5, and amphiregulin. In the inflamed skin lesions of patients with AD, cleavage of E-cadherin (Trautmann et al., 2001) and production of epithelial cytokines may result in discontinuation of the inhibitory signal and potentially allow engagement of activating receptors. This may lead to the unrestricted release of type 2 cytokines and to the overproduction of wound healing regulators by ILC2. Thus, this KLRG1–E-cadherin model of ILC2 regulation may represent a novel mechanism for barrier sensing in the skin. It is of interest that E-cadherin promotes DC maturation and E-cadherin–stimulated DCs can induce T regulatory cells in mice (Jiang et al., 2007), whereas E-cadherin–expressing DCs promote Th17 responses and disease in a colitis model

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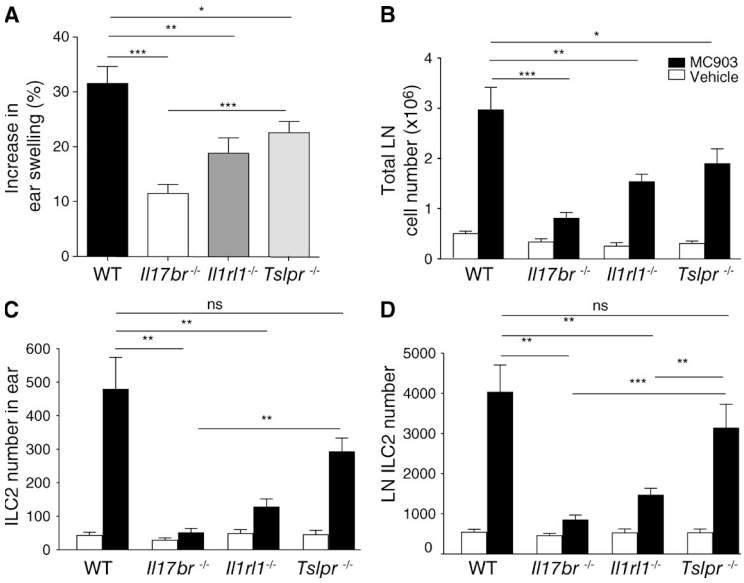

Figure 7. Roles of IL-25, IL-33, and TSLP in skin swelling and inflammation and ILC2 infiltration in BALB/c mice strains. MC903 or vehicle was applied topically for 4 d to the ear of wild-type or knockout mice as indicated. Increase (percent) in ear thickness (A), total LN cell number (LN; B), number of ILC2 in the ear sample (C), and number of ILC2 in the LN (D) were documented. $n = 9–10$ per group. Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, unpaired Student's *t* test with Welch's correction (mean and SEM).

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(Siddiqui et al., 2010). These findings are consistent with E-cadherin having broader relevance beyond a purely physical function, but having a critical role in signaling epithelial events to resident and recruited immune cells.

Although these data would support a role for ILC2 in the inflammation associated with AD, it is possible that they represent a late event in established disease that does not contribute to the primary inflammation. In addition, some of our experiments required the in vitro culture of ILC2s to obtain enough numbers for investigation. Although this technique is very useful, and has been successfully used in the mouse system to first discover and then investigate ILC2s (Neill et al., 2010), it may mean that some aspects of ILC2 biology are not truly reflected in ex vivo systems. Therefore, to investigate ILC2s in the human in vivo and to look at the timing of their entry into allergic sites, we characterized the associations of ILC2 infiltration after allergen challenge in the skin of allergic individuals. The ST2-expressing ILC2 population was enriched in the skin after intraepidermal delivery of HDM allergen. Allergic donors had immediate local urticarial responses with marked type 2 cytokine production within the skin blister in response to the intraepidermal administration of antigen. Indeed, products of mast cell degranulation and epithelial cytokines, such as IL-33, may contribute directly to ILC2 infiltration. It is noteworthy that in a recent study on murine skin, ILC2s were found to associate with mast cells (Roediger et al., 2013). The increase in ILC2 number over 26 h is unlikely to be explained purely by local proliferation of ILC2 and suggests that ILC2 can be recruited from peripheral blood in response to allergen challenge. Indeed we also demonstrated that in mice, intradermal administration of HDM also induces infiltration of ILC2 into the skin.

To address the role of ILC2 in skin inflammation experimentally, we used MC903-induced dermatitis as a model of AD-like inflammation. We show that MC903-induced dermatitis is associated with infiltration of ILC2 into inflamed skin of mice. Furthermore, using two different mouse models of ILC2 deficiency, anti-CD90.2 mAb treatment of *Rag1*$^{-/-}$ mice (Monticelli et al., 2011) and RORα-deficient chimera mice (Wong et al., 2012), we observed significantly reduced skin inflammation. Although each model has limitations, collectively they indicate a role for ILC2 in skin inflammation. To examine the relative contribution of IL-25, IL-33, and TSLP to skin inflammation, we performed parallel experiments using gene knockout mice deficient in the respective cytokine receptors on the BALB/c background. We demonstrate that IL-25 and IL-33 are the predominant ILC2-inducing cytokines in response to skin challenge, with TSLP having a less marked role in comparison. This would be consistent with the fact that an increase in IL-33 mRNA is reported in the lesional skin biopsies of patients with AD compared with healthy individuals (Miller, 2011), and IL-25 is also found in higher concentrations in these patients (Wang et al., 2007; Hvid et al., 2011). In the current study, we also show that *IL33* and *IL25* are overexpressed in acute lesional AD skin. It is relevant that a recent report has proposed that TSLP is the primary cytokine inducing ILC2 in the skin and that the ILC2 response was independent of IL-33 (Kim et al., 2013). One difference between Kim et al. (2013) and this study was their use of the C57BL/6 strain mouse. Indeed, our additional analysis of ST2-deficient, IL-17BR–deficient, and TSLP-deficient mice that had been backcrossed to the C57BL/6 strain did suggest a greater role for the TSLP pathway on the C57BL/6 background as compared with the BALB/c background, but we continued to find significant roles for both IL-25 and IL-33 (unpublished data). Thus, using mice on the C57BL/6, we also observed that TSLP played a more influential role in skin responses to MC903, but that IL-25 and IL-33 were equally influential. Certainly, transgenic mice expressing IL-33 under the keratin 14 promoter also developed an antigen-independent dermatitis which was associated with ILC2 infiltration (Imai et al., 2013). These differences confirm the importance of relating such findings to human ILC2 and clinical disease. The human data within the current study strongly suggest that skin-derived ILC2s are dependent on IL-33 and, therefore, upstream or downstream components of this pathway may present opportunities for therapeutic intervention.

Collectively, these data show that ILC2s are resident in human skin but the frequencies of ILC2s are elevated in the setting of AD and expanded after allergen challenge. Skin ILC2 can be isolated and expanded in vitro for further functional assays. Ex vivo skin ILC2s retain responsiveness to IL-33, with IL-33 promoting ILC2 migration and type 2 cytokine production. KLRG1–E-cadherin interactions exert an inhibitory effect on ILC2 activation, suggesting that settings associated with low E-cadherin expression may promote ILC2 activation. Barrier dysfunction is a key early event in the pathogenesis of AD which is further compounded by type 2 cytokine-mediated inflammation. Therefore, the current findings implicate a novel mechanism of barrier sensing. Although a population of ILC2-like cells has recently been reported in human and mouse skin, the role of IL-33 has been questioned. We now demonstrate that human skin-derived ILC2s express ST2 (IL-33R) and respond to IL-33 by producing type 2 cytokines. Furthermore, our experiments in mice indicate that the initiating cytokines IL-25 and IL-33 are both important for the development of allergic skin inflammation through their regulation of ILC2. It is likely that IL-25, IL-33, and TSLP are all involved in the regulation of ILC2 and allergic skin inflammation and such redundancy may have important therapeutic implications (Wahl et al., 1987; Rand et al., 1991; Cherry et al., 2008; Mun et al., 2010; Kim et al., 2013).

MATERIALS AND METHODS

Isolation of immune cells from the skin of human and mice. Human skin samples were obtained from healthy donors undergoing plastic surgery or biopsies from lesional skin of atopic patients. Samples were taken under GCP guidance with ethical approval of the NRES Committee South Central. After removal of subcutaneous fat, the samples were dissected and incubated in collagenase P (Roche). After overnight digestion at 37°C, the remaining tissue was passed through a 70-µm strainer (VWR) and washed with cold 10 mM EDTA solution. After spinning, the pellet was resuspended

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in cold RPMI and passed through a 40-μm strainer. The samples were further purified using Ficoll density gradient purification. Leukocytes were isolated from mice after overnight incubation at 4°C in dispase solution (STEMCELL Technologies), followed by collagenase digestion at 37°C for 2 h with vigorous shaking. The subsequent steps were similar to the isolation of cells from human skin tissue.

FACS analysis and cell sorting. For FACS surface staining, the cells were labeled by the following anti–human antibodies (purchased from BioLegend unless stated otherwise): CD3 (SK7; BD), CD19 (SJ25C1; BD), CD123 (FAB301C; R&D Systems), CD11b (DCIS1/18), CD11c (BU15; Abcam), CD8 (RPA-T8), FcεRI (AER-37 ([CRA-1]), CD14 (MφP9; BD), CD4 (MEM-241), CD45 (H130), ICOS (C398.4A), CD56 (B159), CRTH2 (BM16; Miltenyi Biotec), IL-7Rα (A019D5), CD25 (BC96; eBioscience), and live/dead violet (Invitrogen). The following anti–mouse antibodies were used for FACS analysis: CD3 (145-2C11), B220 (RA3-6B2), CD19 (1D3), CD4 (GK1.5), CD8a (53–6.7), CD11b (M1/70), Gr-1 (RB6-8C5), CD11c (N418), Sca-1 (D7), TER119 (TER-119), c-Kit (2B8), NKp46 (29A1.4), IL-7Ra (A7R34), CD45.1 (A20), CD45.2 (104), TCR-γδ (GL3), ICOS (C398.4A), and IL17BR (D9.2). The samples were acquired using FACSDiva or Summit software on LSRFortessa or CyAn flow cytometers, respectively. FlowJo and Summit software were used for further data analysis. ILC2s were purity-sorted after depletion of T cells using CD3 micro beads (Miltenyi Biotec) and staining with the above antibodies on a MoFlo XDP cell sorter.

Cell stimulation, intracellular cytokine staining, and multiplex bead array. Cells were stimulated with one or a combination of the following cytokines: 10, 40, or 100 ng/ml of recombinant human IL-25 (PeproTech), 10, 40, or 100 ng/ml of IL-33 (PeproTech), 10, 40, or 100 ng/ml of IL-7 (PeproTech), 10 ng/ml PMA (Sigma-Aldrich), and 2 μmol/liter ionomycin (Sigma-Aldrich) for specified time periods. For intracellular cytokine staining (ICS), 3 μmol/liter monensin was added for the last 6 h of the culture to the medium. After cell surface staining, the cells were permeabilized using Cytofix/Cytoperm kit (BD) and stained for production of cytokines using IL-13 (BD), IL-22 (BD), and IL-17A (eBioscience). Multiplex bead arrays were performed according to manufacturer's instructions (Millipore). In brief, 25 μl of culture supernatant was incubated for 1 h with anti-cytokine antibody-coupled beads. After three washes, samples were incubated with biotinylated detection antibody for 1 h before Streptavidin-phycoerythrin incubation for a further 30 min. Fluorochrome-conjugated beads were enumerated using a MAGPIX multiplexing instrument and MILLIPLEX analysis 5.1 software (Millipore).

ILC2 cell culture. Purified ILC2s were sorted into 96-well plates at the density of 100 cells/well and resuspended in mixed lymphocyte reaction (MLR) of gamma-irradiated peripheral blood mononuclear cells (PBMCs) from three healthy volunteers (2×10^6 cells/ml) coupled with 100 IU/ml IL-2 in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat inactivated human serum and 2 mM L-glutamine and 10 ml/liter penicillin-streptomycin (Sigma-Aldrich; R10H). Every 2–3 d thereafter, half of the medium was replaced with equal volumes of R10H and 100 IU IL-2. Every 4 wk, the cultures were fed with MLR mixed with IL-2.

Quantitative RT-PCR. RNA extraction, reverse transcription, preamplification, and qPCR were performed using Ambion Single Cell-to-CT kit (Life Technologies) according to manufacturer's instructions. The following gene expression assays were purchased from Applied Biosystems: *GATA3* (Hs00231122_m1), *IL5* (Hs01548712_g1), *TSLPR* (Hs00845692_m1), *KLRG1* (Hs00929964_m1), *CRTH2* (Hs00173717_m1), *RORC* (Hs01076122_m1), *IL13* (Hs00174379_m1), *GAPDH* (Hs99999905_m1), *IL1RL1* (HS00545033-m1), *IL4* (Hs00174122_m1), and *RORA* (HS00536545_m1). Reactions were performed in a 7500 Fast Thermal Cycler (Applied Biosystems).

Chemotaxis assay. Transmigration assays were performed in a ChemoTx microplate (NeuroProbe 106–5; 5 μm pore size). 4×10^4 purity-sorted ILC2s were placed in the upper chamber and cytokines were added to the lower chambers. After a 1-h incubation at 37°C with 5% CO_2, the membranes containing top chamber liquid and cells were carefully removed. The cells in the lower chamber were transferred into a white microplate (Perkin-Elmer) and then were quantified by reaction with a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega), followed by a measurement using a FLUOstar OPTIMA luminescence plate reader (BMG LabTech).

Suction blister technique. HDM extract was delivered by intraepidermal skin prick test to the upper arm of the volunteers. Allergic reactivity was defined on the basis of an immediate urticarial response. After ~2 h, suction blister cups were applied to the site of injection with vacuum pressure of 250–450 mmHg. Blisters were formed within 30–90 min. At defined time points, fluid was aspirated using a 30-gauge needle. Fluids were then centrifuged at 1,500 rpm for 5 min at 4°C, and cell pellets were resuspended in R10H and counted using 0.4% trypan blue.

E-cadherin plate-bound experiments. Streptavidin-coated microwell plates (Millipore) were first coated with biotinylated Fc receptor (eBioscience) and then used to immobilize recombinant human E-Cadherin Fc Chimera (R&D Systems) or protein control. Each step was performed at the concentration of 1 μg/ml overnight at 4°C. 5×10^4 PMA/ionomycin-activated ILC2s were cultured on E-cadherin– or protein control–coated plates. After 24 h, cells were washed and RNA was extracted.

Statistical analyses. Student's *t* tests were performed using Prism (version 6.00; GraphPad Software).

Mice. BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. Recombination-activating gene 1–deficient (*Rag1*$^{-/-}$) mice and *Rora*$^{sg/sg}$ mice (Staggerer) were backcrossed to the BALB/c and C57BL/6 background, respectively. *Il17br*$^{-/-}$, *Il1rl1*$^{-/-}$ (Neill et al., 2010), and *Tslpr*$^{-/-}$ mice were also used, backcrossed to both BALB/c and C57 BL/6 backgrounds as indicated. Animals were kept and bred in specific pathogen-free conditions. All animal experiments were performed according to the UK Home Office or Irish Department of Health and Children regulations and approved by the Trinity College Dublin's BioResources ethical review board.

Bone marrow transplantation. Bone marrow transplantation was performed as described previously (Wong et al., 2012).

Oxazolone and HDM treatment of mice. To induce inflammatory lesions, 50 μl oxazolone (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich) dissolved in ethanol (4%) was topically applied to shaved mouse flank skin of BALB/c and C57BL/6 strain mice. Simultaneously, 25 μl (50 μg) HDM extract (Greer Laboratories) dissolved in saline, or saline alone, was injected subcutaneously into the same site. 1 wk later, the sites of injection were challenged subcutaneously with 25 μl (50 μg) of HDM extract or saline for 4 consecutive days. On day 10, skin was isolated, weighed, and processed as described above.

MC903 and anti-CD90.2 treatment of mice. To induce AD lesions, 2 nmol MC903 or ethanol (vehicle) was painted on both ears for 4 consecutive days. 24 h after the last challenge, mice were euthanized, and the ear thickness was measured using a thickness gauge micrometer (Mitutoyo). The percent increase in swelling between vehicle- and MC903-treated ears was determined. The leukocytes were then extracted as described earlier. For ablation of the ILC2 population, 200 μg anti-CD90.2 (3H12) antibody was administered intraperitoneally every 2 d starting 2 d before the application of MC903. Skin was processed as described previously (Fallon et al., 2009). Flow cytometry on skin-draining LNs or ear tissue was performed as reported previously (Neill et al., 2010).

Online supplemental material. Fig. S1 shows gating strategies for the identification of human ILC2 in blood and skin using flow

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cytometry. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130351/DC1.

We are grateful to all suction blister, blood, and skin donors. We thank Craig Waugh, Kevin Clark, and Paul Sopp for their help in flow cytometry and cell sorting, and Jennifer Walker for critical reading of the manuscript.

We are grateful for funding from the MRC, Barrie Trust, National Institute of Health Research Biomedical Research Centre Program and Comprehensive Local Research Network. P.G. Fallon is supported by the Wellcome Trust, Science Foundation Ireland, and National Children's Research Centre. J.L. Barlow, S.T. Scanlon, and A.N.J. McKenzie were supported by the MRC and the American Asthma Foundation.

The authors have no conflicting financial interests.

Submitted: 15 February 2013
Accepted: 13 November 2013

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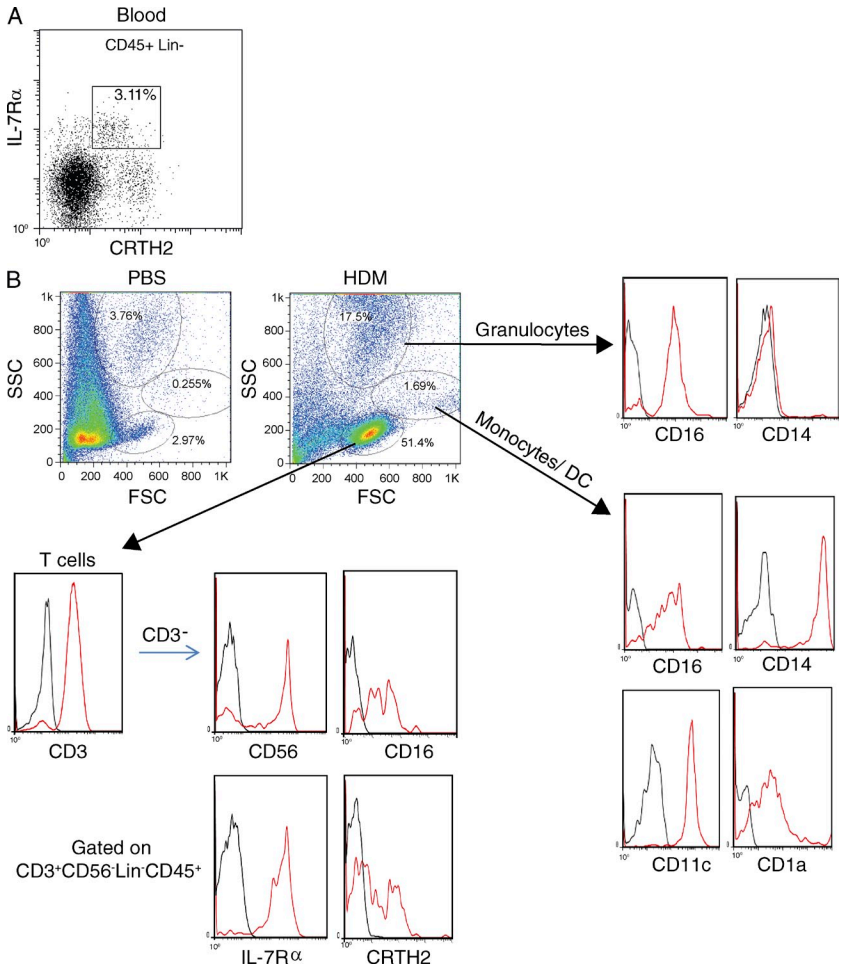

Figure S1. Gating strategies for the identi□cation of human ILC2 in blood and skin using □ow cytometry. (A) Gating strategy for sorting of ILC2 from the blood. Total PBMCs were stained for CD45, lineage markers, IL-7Rα, and CRTH2, and ILC2s were gated as $CD45^{+}Lin^{-}IL\text{-}7R\alpha^{+}CRTH2^{+}$. Data are representative of five independent experiments (n = 18 donors). (B) Cellular infiltrate into the skin after HDM challenge in allergic and nonallergic donors. Shown is a gating strategy to show different populations of cells that infiltrated human skin blisters after HDM allergen or PBS challenge.

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