

Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells

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Type 2 immunity is critical for defense against cutaneous infections but also underlies the development of allergic skin diseases. We report the identification in normal mouse dermis of an abundant, phenotypically unique group 2 innate lymphoid cell (ILC2) subset that depended on interleukin 7 (IL-7) and constitutively produced IL-13. Intravital multiphoton microscopy showed that dermal ILC2 cells specifically interacted with mast cells, whose function was suppressed by IL-13. Treatment of mice deficient in recombination-activating gene 1 (*Rag1*^{-/-}) with IL-2 resulted in the population expansion of activated, IL-5-producing dermal ILC2 cells, which led to spontaneous dermatitis characterized by eosinophil infiltrates and activated mast cells. Our data show that ILC2 cells have both pro- and anti-inflammatory properties and identify a previously unknown interactive pathway between two innate populations of cells of the immune system linked to type 2 immunity and allergic diseases.

The skin is the outermost barrier between the environment and internal organs that contains cells of the immune system deployed as sentinels that serve as a first line of defense against microbial attacks. The epidermis of mice has two relatively uniform populations of such cells, Langerhans cells and dendritic epidermal T cells (DETCs), whereas the dermis contains a large variety of leukocytes, including dermal dendritic cells (DDCs), macrophages, mast cells and $\alpha\beta$ and $\gamma\delta$ T cells. It is believed that most of the productive immune responses to invading microbes are initiated in the dermis, although the precise lineage relationships of dermal leukocyte subsets and their exact functions during homeostasis and disease are yet to be defined.

Type 2 cytokine-dependent responses are essential for protection against helminth infections but also underlie the development of inflammatory allergic diseases, such as atopic dermatitis¹. Although overproduction of epidermis-associated innate cytokines, such as thymic stromal lymphopoietin (TSLP)², has been associated with the development of cutaneous allergic sequelae, the cellular events in the skin that could be responsible for biasing subsequent immune responses to a T helper type 2 (T_H2; atopic) phenotype have not been identified. In particular, there is no information about whether an innate type 2 sensor is present in the dermis.

A class of innate lymphoid cells (ILCs), the group 2 ILCs (ILC2 cells)³, has been identified in fat-associated lymphoid tissue and mucosa-associated tissues (the respiratory and intestinal tracts)

on the basis of their responsiveness to interleukin 25 (IL-25) and the production of type 2 cytokines, the foremost being IL-13 (refs. 4–8). Cells that belong to the ILC2 family include natural helper cells⁷, nuocytes⁸ and innate helper 2 cells⁹. ILC2 cells are defined as lineage-negative (Lin⁻) CD45⁺CD90⁺CD127(IL-7R α)⁺ICOS⁺ with variable expression of the stem-cell-factor receptor CD117 (c-Kit), the lineage marker Sca-1 and the IL-33 receptor ST2. Nuocytes have been shown to differentiate from common lymphocyte progenitors and to depend on the transcription factor ROR α ¹⁰. In adult mice, ILC2 cells are a rare population that expands in response to IL-25 or IL-33 or during helminth infection⁸ and represent a chief source of IL-13, a pleiotropic cytokine that mediates a variety of effects, including suppression of cytokine production by monocytes¹¹, mucus production by goblet cells and the recruitment of eosinophils by epithelial cells^{12–14}. ILC2 cells can also produce IL-5, which has been linked to eosinophil recruitment, as well as IL-9 (ref. 15), which is prominent during helminth infections^{16,17} and has been linked to atopic disease¹⁸. ILC2 cells have been shown to be involved in immunity to helminths as well as in allergic conditions such as asthma and chronic sinusitis^{4,5,7,8} but are also probably important for maintaining tissue homeostasis⁶. Despite such advances, very little is understood about the activity of ILC2 cells *in situ*, and it remains unclear what other cell types ILC2 cells interact with *in vivo*.

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Here we describe the phenotype, developmental requirements and *in situ* activity of a unique population of CD103⁺ ILC2 cells in the skin, which we have called 'dermal ILC2 cells' (dILC2 cells). These cells depended on IL-7 for their survival and produced IL-13 in steady-state conditions. The dILC2 cells continuously screened the dermis, where they 'preferentially' interacted with skin-resident mast cells. We further found that dILC2 cells responded to systemic treatment with complexes of IL-2 and antibody to IL-2 (anti-IL-2) by proliferating and producing IL-5, which in turn promoted eosinophil influx and cutaneous inflammation. Thus, dILC2 cells emerge as distinct dermal residents with the potential to initiate type 2 immune responses as well as to exert regulatory function on other dermal populations of immune-system cells.

RESULTS

Identification of skin-resident CD103⁺ ILC2 cells

We sought to determine whether mouse skin might contain ILC2 cells defined, at least in part, by the absence of lineage markers and expression of the cell-surface protein CD90 (Thy-1) and the inducible costimulator ICOS⁸. We used CD2 expression to exclude natural killer (NK) cells and NKT cells (Supplementary Fig. 1) and identified in the skin of wild-type mice a population of CD45⁺CD11b[−]CD90^{hi}CD3[−]CD2[−] ILCs (Fig. 1a) that localized mainly to the dermis at approximately one third the abundance of T cells (Fig. 1b). These cells expressed ICOS (Fig. 1c), consistent with an ILC2 phenotype.

The same staining strategy also identified an equivalent population in the mesentery (Fig. 1c) that most probably corresponded to the natural helper cells described before⁷. However, unlike the mucosal populations, skin ILC2 cells uniquely expressed CD103 (Fig. 1d), an integrin ($\alpha_E\beta_7$) expressed by some skin-resident leukocytes, particularly T cells¹⁹. Further phenotypic analysis of this population showed a lack of key markers of T cells and NK cells, together with expression of markers associated with ILC2 cells, notably the high-affinity IL-2 receptor CD25, Sca-1 and ST2 (Supplementary Fig. 2). In contrast to the ILC2 populations described in other tissues, skin ILC2 cells did not have detectable expression of CD117 (c-Kit), but they did express the IL-25 receptor IL-17BR (Supplementary Fig. 2 and data not shown). We therefore called these 'dermal ILC2 cells' (dILC2 cells). These CD45⁺CD3[−]CD2[−]CD90^{hi} cells were detectable in other tissues, including blood and skin-draining lymph nodes (Fig. 1e and data not shown), but their abundance in the total leukocyte pool was very low for these tissues, particularly relative to their abundance in the dermis, where dILC2 cells constituted 5–10% of all CD45⁺ cells isolated (Fig. 1f). We concluded that the dermis contains an abundant, phenotypically distinct population of ILC2 cells.

Developmental requirements for dILC2 cells *in vivo*

We next determined the developmental requirements of dILC2 cells *in vivo*. As many skin cells, including Langerhans cells, DETCs and dermal

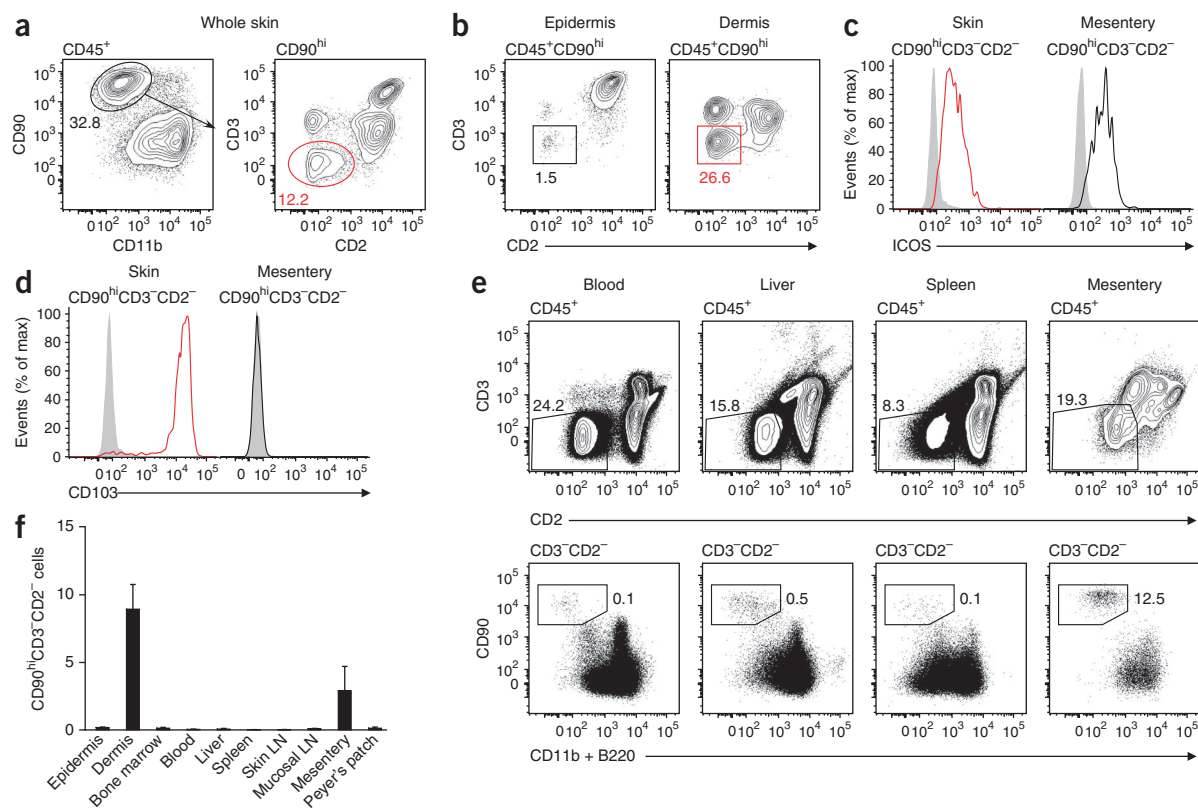


Figure 1 Identification and phenotype of dermal ILC2 cells. (a) Flow cytometry of ILC2 cells from the skin of wild-type mice. Numbers adjacent to outlined areas indicate percent CD90^{hi}CD11b^{lo} cells among CD45⁺ cells (left) or CD3[−]CD2[−] cells among those CD90^{hi}CD11b^{lo}CD45⁺ cells (right). (b) Flow cytometry of CD45⁺CD90^{hi} ILC2 cells from the epidermis (left) and dermis (right) of wild-type mice ($n = 3$). Numbers adjacent to outlined areas indicate percent CD3[−]CD2[−] cells. (c, d) Expression of ICOS (c) and CD103 (d) by CD90^{hi}CD3[−]CD2[−] ILC2 cells from the skin (left; red lines) and mesentery (right; black lines) of wild-type mice ($n = 4$), assessed by flow cytometry. Gray shaded curves (d), fluorescence-minus-one control. (e) Flow cytometry of ILCs in the blood, liver, spleen and mesentery of wild-type mice ($n = 3$). Numbers adjacent to outlined areas indicate percent CD3[−]CD2[−] cells among CD45⁺ cells (top row) or CD90^{hi}CD11b^{lo}B220[−] cells among CD45⁺CD3[−]CD2[−] cells (bottom row). (f) Abundance of ILC in various organs (horizontal axis) of wild-type mice ($n = 3$), relative to total isolated leukocytes. LN, lymph node. Data are representative of >20 (a), three (b) or two (c, d) independent experiments or are from one of (e) or are pooled from (f) two independent experiments (mean and s.d. in f).

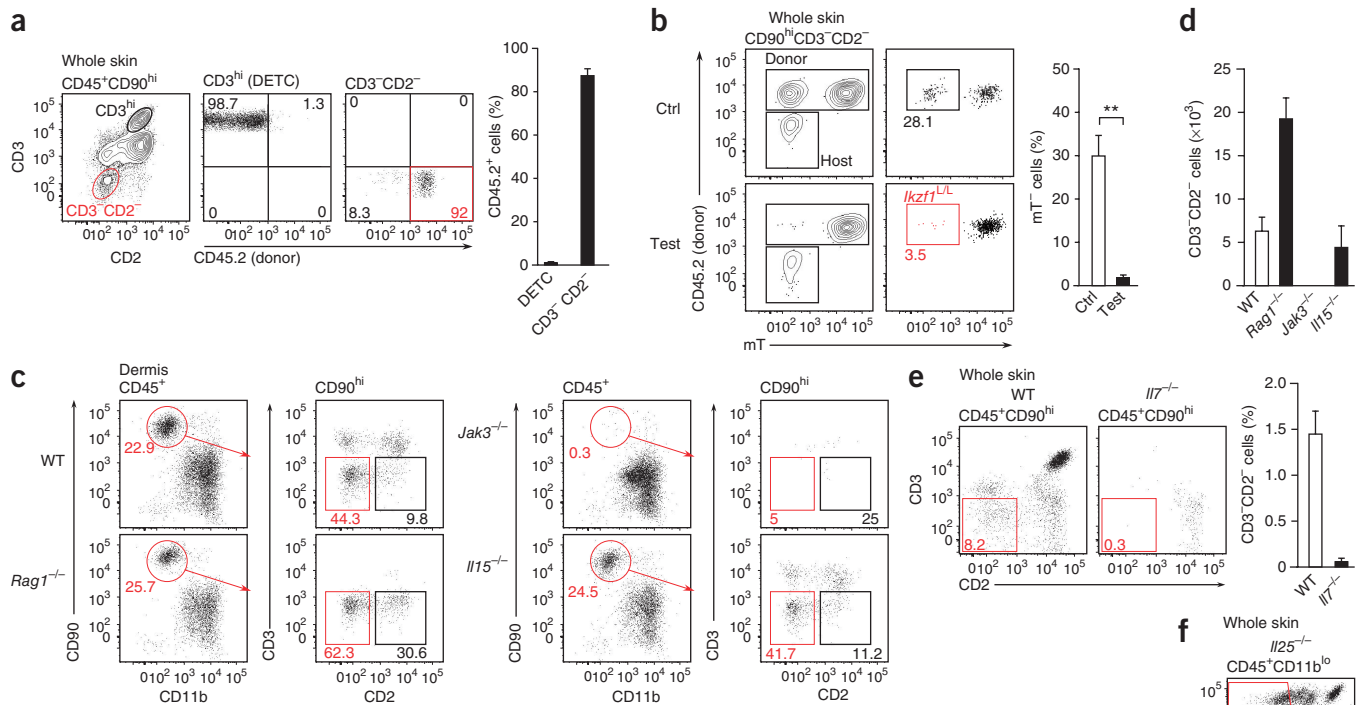
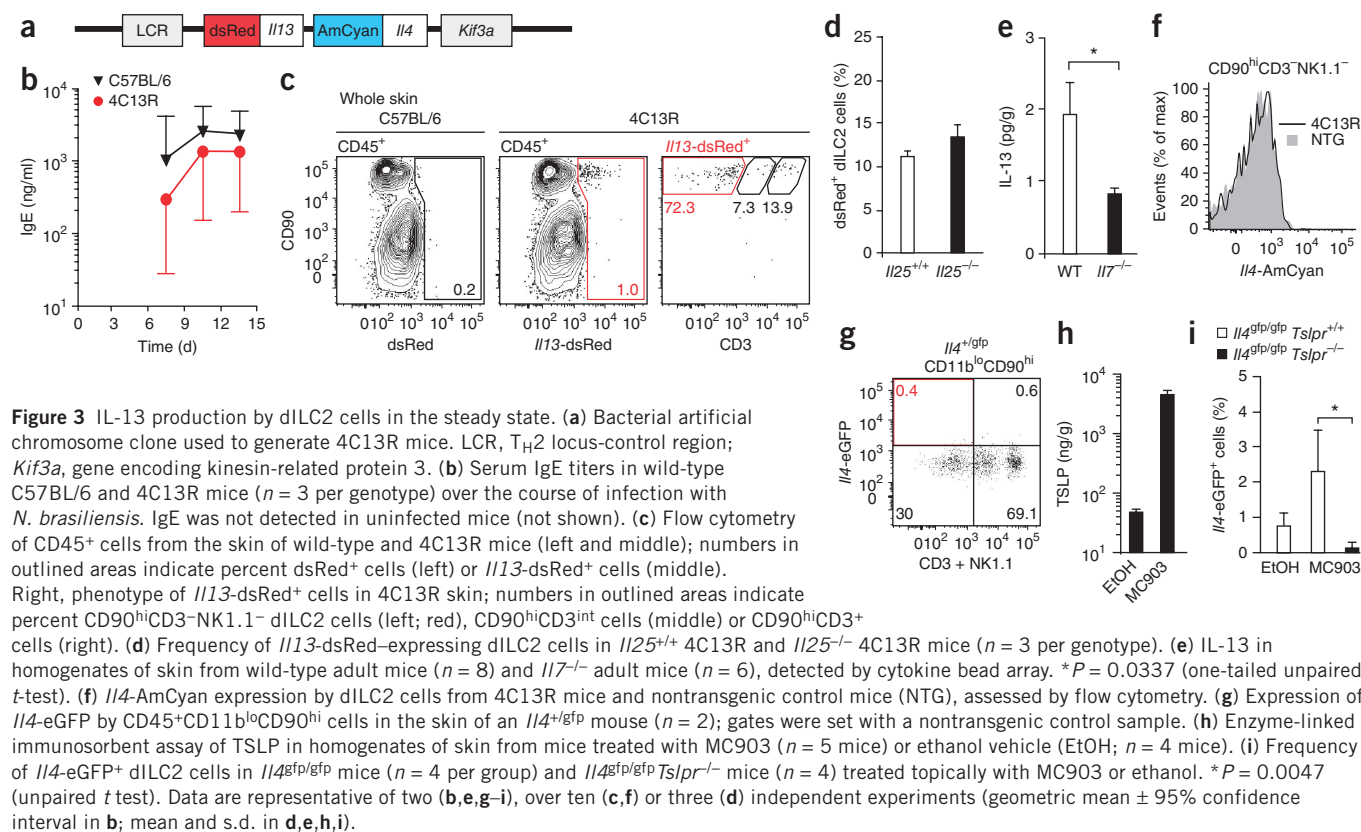


Figure 2 Development and homeostatic requirements of dILC2 cells. **(a)** Flow cytometry of skin cells from CD45.1⁺ mice ($n = 7$) 8 weeks after irradiation and transfer of bone marrow from CD45.2⁺ mice (left); numbers in quadrants indicate percent cells in each throughout. Right, frequency of DETCs and dILC2 cells of donor origin. **(b)** Flow cytometry (left) of cells from wild-type (CD45.1⁺) hosts ($n = 5$) 8 weeks after transfer of a 50:50 mixture of wild-type mT/mG (mT⁺) plus wild-type (CD45.2⁺) bone marrow ($n = 3$ chimeras; control (Ctrl)) or wild-type mT/mG (mT⁺) plus *Ikzf1*^{L/L} (CD45.2⁺) bone marrow ($n = 2$ chimeras; Test). Right, contribution of CD45.2⁺ donor cells to dILC2 cells, assessed as the frequency of mT⁻ cells. **(c)** Flow cytometry of cells from the dermis of wild-type, *Rag1*^{-/-}, *Jak3*^{-/-} and *Il15*^{-/-} mice ($n = 3$ per genotype). Numbers adjacent to outlined circles indicate percent CD90^{hi}CD11b^{lo} cells among CD45⁺ cells; numbers below outlined squares indicate percent CD3⁻CD2⁻ (dILC2) cells (red) or CD3⁻CD2⁺ cells (black). **(d)** Absolute number of dILC2 cells in the skin of wild-type, *Rag1*^{-/-}, *Jak3*^{-/-} and *Il15*^{-/-} mice ($n = 3$ per genotype; two ears each). **(e)** Flow cytometry of CD45⁺CD90^{hi} cells from the skin of wild-type and *Il17*^{-/-} mice ($n = 3$ per genotype; left); numbers in red outlined areas indicate percent CD3⁻CD2⁻ (dILC2) cells. Right, frequency of CD3⁻CD2⁻ (dILC2) cells. **(f)** Flow cytometry of CD45⁺CD11b^{lo} cells from the skin of an *Il25*^{-/-} mouse ($n = 4$ mice). Number in red outlined area indicates percent CD90^{hi}CD3⁻NK1.1⁻ dILC2 cells. Data are pooled from **(a)** or are representative of **(b–f)** two independent experiments (mean and s.d. in **a,b,d,e**).

$\gamma\delta$ T cells, are largely or partially radioresistant¹⁹, we first addressed the radiosensitivity of dILC2 cells. We sublethally irradiated CD45.1⁺ recipient mice and reconstituted them with CD45.2⁺ bone marrow. At 8 weeks after irradiation, dILC2 cells were predominantly of donor origin, in contrast to radioresistant CD3^{hi} DETCs (Fig. 2a). As nuocytes have been shown to be derived from common lymphoid progenitors¹⁰, we determined whether dILC2 cells were dependent on the transcription factor Ikaros, which is essential for the generation of common lymphoid progenitors and subsequent development of 'lymphoid' cells²⁰. We made use of *Ikzf1*^{L/L} mice, in which the β -galactosidase reporter is inserted into the locus encoding Ikaros (*Ikzf1*)²¹. *Ikzf1*^{L/L} mice are not completely deficient in Ikaros but are nevertheless unable to achieve wild-type Ikaros activity. To address the reliance of dILC2 cells on Ikaros, we sublethally irradiated wild-type (CD45.1⁺) mice and reconstituted them with a 50:50 mixture of mT/mG bone marrow (which in these experiments had ubiquitous expression of membrane-targeted red fluorescent protein (RFP) Tomato (mT)²² and wild-type bone marrow ('control chimeras') or a 50:50 mixture of mT/mG bone marrow and *Ikzf1*^{L/L} bone marrow ('test chimeras') and examined the chimeric recipients 6–8 weeks later. Test chimeras had a significantly lower frequency of donor-derived (CD45.2⁺) mT⁻ dILC2 cells than did control chimeras (Fig. 2b), which indicated that dILC2 cells were dependent on Ikaros for their development, consistent with a lymphoid origin for these cells.

To further explore the developmental and homeostatic requirements of dILC2 cells, we evaluated these cells in various genetically deficient mice. We found dILC2 cells in mice deficient in recombination-activating gene 1 (*Rag1*^{-/-}), mice of the nonobese diabetic, severe combined immunodeficiency strain and thymus-deficient *Foxn1*^{nu/nu} (nude) mice (Fig. 2c, Supplementary Fig. 3 and data not shown), which confirmed that they were not a T cell population and did not require the thymus for their development. We found no dILC2 cells in mice deficient in the kinase Jak3 (*Jak3*^{-/-})²³ (Fig. 2c,d), which indicated a requirement for signaling through the common γ -chain for their development or maintenance. Although dILC2 cell numbers were unaffected in mice deficient in IL-15 or IL-2 (Fig. 2c,d and data not shown), they were absent from IL-7-deficient skin (Fig. 2e). Collectively these data indicated that dILC2 cells shared the same developmental requirements that nuocytes¹⁰ and natural helper cells⁷ have. Of note, *Rag1*^{-/-} mice and other T cell-deficient mice had more dILC2 cells than did wild-type mice (Fig. 2d and Supplementary Fig. 3), possibly due to the greater availability of IL-7. Given the apparent propensity of nuocytes to undergo population expansion in response to IL-25 (ref. 8), we also determined whether dILC2 cells were present in *Il25*^{-/-} mice. Lack of IL-25 had no effect on dILC2 cell numbers (Fig. 2f), which indicated that IL-25 alone was dispensable for the homeostasis of dILC2 cells in the steady state.



Production of IL-13 by dILC2 cells

Studies of reporter mice and intracellular cytokine staining have reported that nuocytes and other innate helper cells produce IL-13 (refs. 7–9) and small amounts of IL-4 (ref. 24). To determine whether dILC2 cells might similarly produce IL-13 and IL-4, we used the 4C13R dual reporter mouse, which has transgenic expression of the cyan fluorescent protein AmCyan under the control of *Il4* regulatory elements and the red fluorescent protein dsRed under the control of *Il13* regulatory elements (Fig. 3a). In 4C13R mice, cellular expression of *Il4* and *Il13* is reported without an effect on the endogenous production of IL-4 and IL-13. We found that 4C13R mice were healthy and viable and had a robust immunoglobulin E (IgE) response to infection with *Nippostrongylus brasiliensis* (Fig. 3b), and AmCyan and dsRed fluorescence was readily detectable in 4C13R T cells cultured under T_H2 -inducing conditions (data not shown).

When we examined the skin of 4C13R mice, we found that dsRed-expressing cells were exclusively CD45⁺CD90^{hi} cells that were mostly CD3⁻NK1.1⁻ dILC2 cells and some epidermal CD3^{hi} DETCs; the latter had lower expression of I13-dsRed than did dILC2 cells (Fig. 3c and data not shown). I13-dsRed expression by dILC2 cells was unaffected in I125^{-/-} mice (Fig. 3d). Furthermore, we consistently detected IL-13 protein in all wild-type skin samples but found significantly less IL-13 protein in I17^{-/-} mice (Fig. 3e), which indicated that dILC2 cells produced IL-13 in the steady state.

In contrast to their expression of I13-dsRed, dILC2 cells from 4C13R mice did not express AmCyan (Fig. 3f), which indicated that these cells did not produce IL-4 in the naive state. We found that dILC2 cells from mice with sequence encoding enhanced green fluorescent protein (eGFP) knocked in to one *Il4* allele (I14^{+/gfp})²⁵ to report *Il4* expression were similarly eGFP⁺ (Fig. 3g). As it has been demonstrated that human ILC2 cells are able to produce IL-4 in response to TSLP *in vitro*²⁶, we

assessed whether dILC2 cells might similarly respond to TSLP *in vivo*. We examined eGFP expression by dILC2 cells from I14^{gfp/gfp} mice after topical application of the vitamin D analog MC903, which has been shown to induce TSLP expression by skin keratinocytes²⁷. We found more production of TSLP in MC903-treated ears than in vehicle-treated control ears (Fig. 3h). After exposure to MC903, a small but reproducible population of dILC2 cells from TSLP receptor-sufficient mice expressed I4-eGFP, but dILC2 cells from TSLP receptor-deficient mice did not (Fig. 3i). IL-4 protein was not detectable in MC903-treated skin (data not shown), whereas IL-13 production by dILC2 cells was greater in MC903-treated skin than in vehicle-treated control skin, although this did not reach statistical significance (Supplementary Fig. 4). We concluded that dILC2 cells produced IL-13 but not IL-4 in the naive state but generated IL-4 under defined inflammatory conditions.

Visualization of dILC2 cells *in vivo*

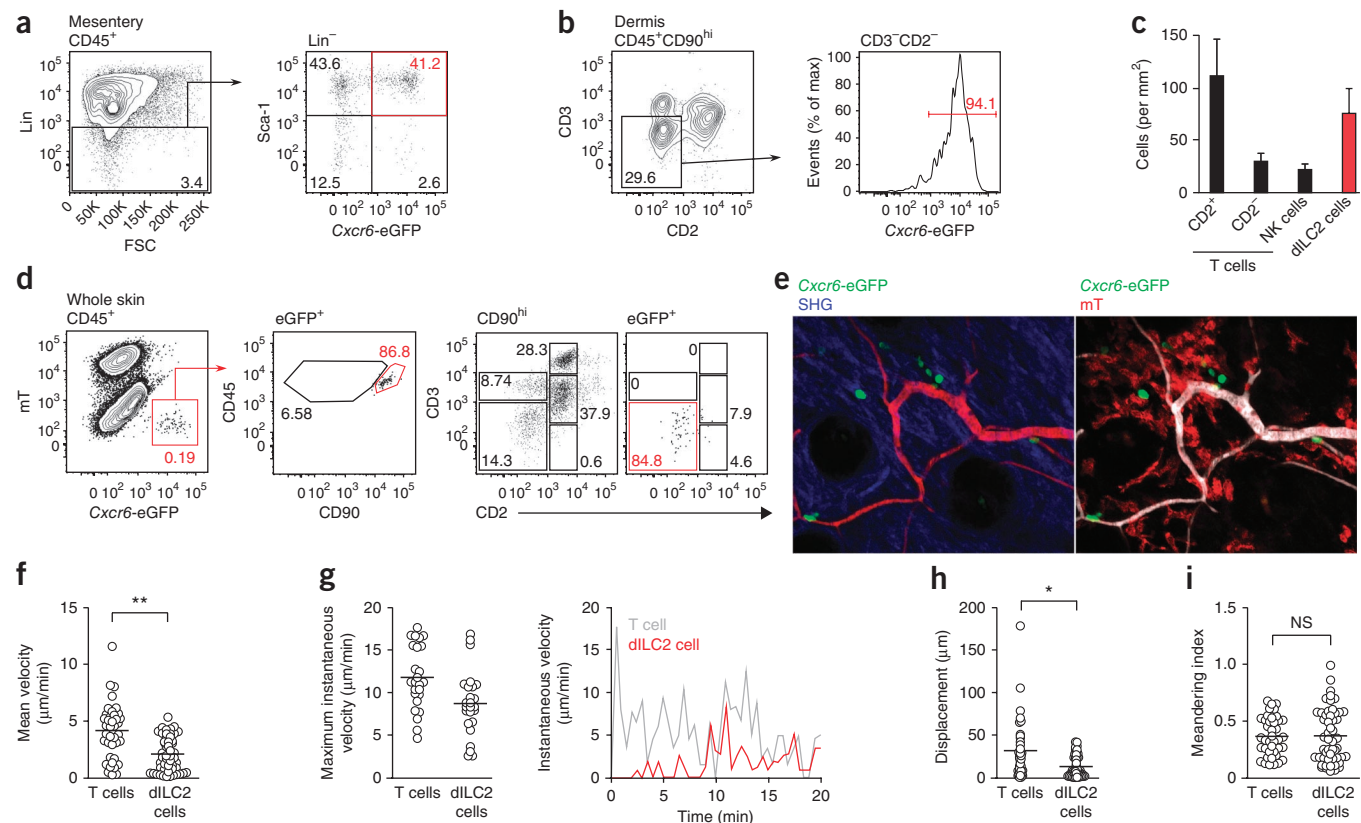
To visualize dILC2 cells *in situ*, we made use of mice with sequence encoding eGFP knocked in to one *Cxcr6* ('Bonzo') allele (which encodes the chemokine receptor CXCR6; *Cxcr6*^{+/gfp} mice)²⁸. In these mice, various lymphoid populations express eGFP, including some T cell subsets and ROR γ ⁺ ILCs²⁹. Flow cytometry of Lin⁻Sca-1⁺ natural helper cells⁷ in the mesentery of *Cxcr6*^{+/gfp} mice demonstrated bright fluorescence in approximately 50% of these cells (Fig. 4a). In the skin, almost all dILC2 cells expressed eGFP (Fig. 4b). The ease with which eGFP⁺ cells can be imaged in the skin of *Cxcr6*^{+/gfp} mice¹⁹ enabled us to determine that density of dILC2 cells in *Cxcr6*^{+/gfp} mouse skin was 50–100 cells per mm² (Fig. 4c).

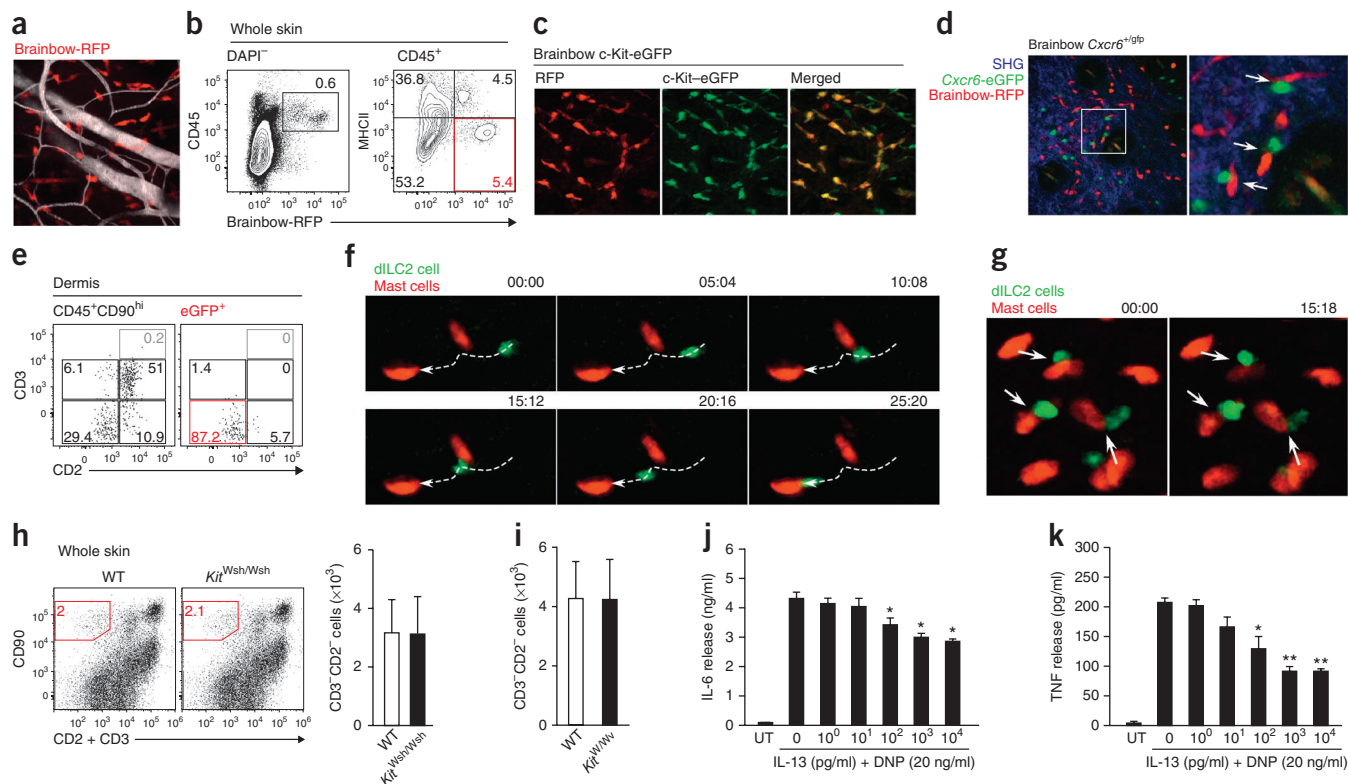
To visualize dILC2 cells exclusively, we exploited their radio-sensitivity and generated mixed-bone marrow chimeras by injecting *Rag1*^{-/-}*Cxcr6*^{+/gfp} bone marrow plus *Rag1*^{+/+} GFP-mT⁺ mT/mG bone marrow into irradiated wild-type recipient mice. We also generated

control chimeras by injecting irradiated recipients with a mixture of a small number of *Cxcr6*^{+/gfp} bone marrow cells with a large number of nonfluorescent *Rag1*^{-/-} bone marrow cells, so that eGFP expression was restricted mainly to donor-derived T cells (data not shown). Flow cytometry confirmed that eGFP expression in the skin of recipient mice was confined to CD90^{hi}CD3⁻CD2⁻ dILC2 cells, with only a minor contribution from CD3⁻CD2⁺ NK cells (Fig. 4d). We found that eGFP⁺ dILC2 cells were readily detectable by multiphoton microscopy (Fig. 4e), by which they showed a distinct migratory phenotype (Supplementary Video 1). In contrast to the movement of cutaneous T cells, the average speed of dILC2 cells rarely exceeded 5 $\mu\text{m}/\text{min}$ and was characterized by brief migratory periods followed by extended pauses (Fig. 4f and Supplementary Video 2). Thus, whereas the instantaneous speed of dILC2 cells was able to exceed 10 $\mu\text{m}/\text{min}$ (Fig. 4g), their average speed was similar to that of migratory DDCs³⁰. The total displacement of dILC2 cells within a 15-minute time frame was also attenuated relative to

that of the faster T cells (Fig. 4h), whereas their mean meandering indices were almost identical (Fig. 4i). These data demonstrated that despite their lower migratory speed, dILC2 cells nevertheless patrolled their local environment, consistent with a potential immunosurveillance function.

The presence of donor-derived mT-expressing cells in the mixed chimeras described above enabled us to address whether dILC2 cells interacted with radiosensitive leukocytes in the skin, particularly dermal macrophages and DCs (Fig. 4e). Despite the high prevalence of mT⁺ mT/mG bone marrow-derived antigen-presenting cells (APCs) in the ears of mixed chimeras, the interactions of dILC2 cells with those cells were infrequent and transient (Supplementary Videos 3 and 4). Similarly, in triple chimeras generated by a combination of the mixed chimeras described above with the inclusion of bone marrow from CD11c-eYFP mice (which express enhanced yellow fluorescent protein (eYFP) from the gene encoding the common DC marker CD11c) for the visualization of DDCs, we did not observe





sustained interactions of dILC2 cells with eYFP⁺ DDCs or mT⁺ APCs (Supplementary Video 5 and data not shown).

Although we did not observe many interactions of dILC2 cells with APCs, the frequent stationary status of dILC2 cells nevertheless suggested that these cells did form stable contacts with another, presumably radioresistant cell population. Although that population could have included nonhematopoietic cells such as endothelial cells and fibroblasts, we were particularly interested in determining whether dILC2 cells were interacting with mast cells, given their role as an effector population in type 2 immune responses³¹ and their resistance to irradiation (data not shown). To explore that possibility, we exploited the observation that Brainbow mice (with transgenic expression of a set of fluorescent proteins)³² can be used as mast-cell reporter mice. We found not only expression of RFP (mT)

in central and peripheral neurons in Brainbow mice but also RFP expression by some skin leukocytes (Fig. 5a), including a population of RFP^{dim}MHCII^{hi} migratory DDCs and a nonmigratory population of predominantly perivascular RFP^{hi}MHCII^{lo} cells (Fig. 5b and Supplementary Video 6). Phenotypic analysis by flow cytometry demonstrated that these RFP^{hi} cells were CD11b^{lo}FcR1⁺c-Kit⁺ mast cells (data not shown), a result confirmed by the coexpression of RFP and eGFP in the progeny of Brainbow mice crossed with c-Kit-eGFP mast-cell reporter mice³³ (Fig. 5c).

To determine whether dILC2 cells interacted with mast cells *in vivo*, we generated Brainbow *Cxcr6*^{+/GFP} mice and assessed their skin by multiphoton microscopy. We observed eGFP⁺ cells in close proximity to RFP^{hi} mast cells in these mice (Fig. 5d). Those apparent interactions often lasted the entire recording session of 10–30 min

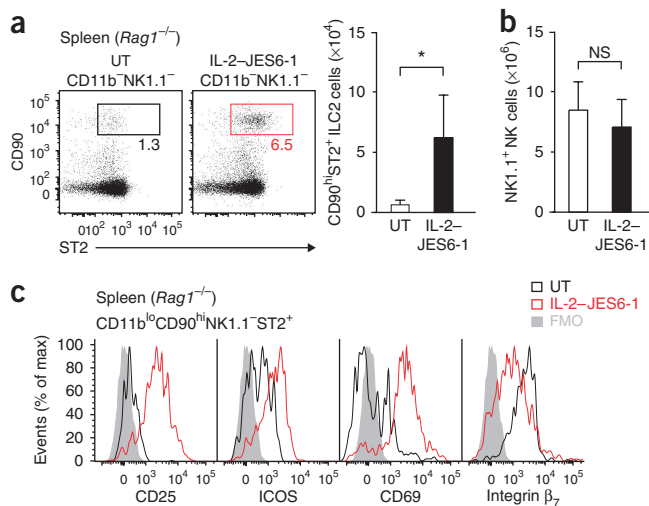


Figure 6 IL-2-induced activation and proliferation of ILC2 cells *in vivo*. (a) Flow cytometry (left) of CD11b-NK1.1⁻ splenocytes from *Rag1*^{-/-} mice left untreated (*n* = 4 mice) or treated with IL-2-JES6-1 (*n* = 5 mice); numbers below outlined areas indicate percent CD90^{hi}ST2⁺ ILC2 cells. Right, absolute number of splenic ILC2 cells from mice at left. **P* = 0.0180 (unpaired *t*-test). (b) Absolute number of splenic NK1.1⁺ NK cells in mice as in (a). (c) Surface marker expression by splenic ILC2 cells from mice as in (a) (*n* = 2 mice). FMO, 'fluorescence-minus-one' control. Data are representative of three independent experiments (a,b; mean and s.d.) or two independent experiments (c).

(Supplementary Video 7). To visualize dILC2 cells exclusively, without the presence of eGFP⁺ T cells, we generated mixed-bone marrow chimeras by injecting *Rag1*^{-/-}*Cxcr6*^{+/gfp} bone marrow together with *Rag1*^{+/+} GFP⁻ bone marrow into irradiated Brainbow mice. As before, eGFP expression was restricted mainly to dILC2 cells (Fig. 5e), whereas RFP expression was restricted to radioresistant mast cells. Intravital multiphoton imaging of these mice demonstrated not only that dILC2 cells migrated in close proximity to skin-resident mast cells (Fig. 5f and Supplementary Videos 8 and 9) but also that dILC2 cells formed stable interactions with mast cells that lasted over 20–30 min (Fig. 5g and Supplementary Video 10). Despite those sustained interactions, mast cells did not seem to be important for the occupation by and/or survival of dILC2 cells in the skin, as both the frequency and absolute number of dILC2 cells in the skin of mast cell-deficient *B6-Kit*^{W-sh/W-sh} and *WBB6F1-Kit*^{W/W-v} mice³⁴ did not differ from that of their respective control counterparts (Fig. 5h,i).

To determine how interactions between dILC2 cells might affect mast-cell biology, in particular the role of ILC2 cell-produced cytokines such as IL-13 and IL-9, we used a well-established *in vitro* model in which we measured IgE-dependent release of IL-6 and tumor-necrosis factor (TNF) by presensitized bone marrow-derived mast cells in the presence or absence of recombinant IL-13 or IL-9 (ref. 35). Coincubation with IL-13 had a dose-dependent suppressive effect on the IgE-dependent release of cytokines by mast cells (Fig. 5j,k). This suppression was specific, as coincubation with IL-9 conversely enhanced the production of IL-6 and TNF achieved by the same protocol (Supplementary Fig. 5). These data suggested that dILC2 cells had the potential to modulate mast-cell function through the production of IL-13.

IL-2-induced activation and proliferation of ILC2 cells *in vivo*

We sought to address the function of dILC2 cells *in vivo*. Although antibody-depletion approaches have been used successfully to evaluate

the role of lung ILC2 cells *in vivo*^{4,36}, dILC2 cells seemed resistant to such strategies, as treatment with anti-CD90.2 or anti-CD25 did not result in the depletion of dILC2 cells in naive *Rag1*^{-/-} mice, even when splenic ILC2 cells were effectively cleared (Supplementary Fig. 6). We therefore sought to activate dILC2 cells directly *in vivo* to stimulate cytokine production. In contrast to NK cells and NKp46⁺ ILCs, ILC2 cells were unique in their expression of CD25 (Supplementary Fig. 7) and were the main CD25-expressing hematopoietic population in *Rag1*^{-/-} mice. As ILC2 cells have been shown to respond to IL-2 *in vitro* by producing IL-5, IL-9 and IL-13 (refs. 4,15,36), we assessed whether IL-2 might also activate these cells *in vivo* by injecting *Rag1*^{-/-} mice with complexes of IL-2 and JES6-1 (a monoclonal antibody specific for IL-2) every second day for 1–3 weeks. Coadministration of IL-2 plus JES6-1 has been shown to selectively expand populations of CD25-expressing regulatory T cells in wild-type mice without activating cells such as NK cells that express the low-affinity IL-2 receptor³⁷. After 2 weeks of such treatment, splenic ILC2 populations were expanded approximately fivefold in *Rag1*^{-/-} mice (Fig. 6a), which indicated that IL-2 stimulated the proliferation of these cells. As expected, NK cells were unaffected (Fig. 6b and data not shown). IL-2-expanded ILC2 populations had an activated phenotype, characterized by higher surface expression of CD25, ICOS, the activation marker CD69 and ST2, whereas the expression of some adhesion molecules (integrin β_7 and CD49d) was downregulated (Fig. 6c and Table 1).

In the skin, the population expansion of dILC2 cells in response to IL-2-JES6-1 was much greater, with approximately tenfold greater absolute numbers after 2 weeks of treatment (Fig. 7a). To visualize these cells, we treated *Rag1*^{-/-}*Cxcr6*^{+/gfp} mice with IL-2-JES6-1 for 2 weeks and then assessed the skin by multiphoton microscopy. We found many more *Cxcr6*-eGFP⁺ dILC2 cells that were located mainly in close proximity to blood vessels (Fig. 7b). Intravital imaging of these mice showed that activated dILC2 cells had the same heterogeneous migratory phenotype observed in the steady state, such that some dILC2 cells migrated but a large proportion remained stationary throughout the imaging period (Supplementary Video 11).

Of note, we routinely observed vascular leakage of Evans blue when imaging IL-2-treated mice, indicative of inflammation (Supplementary Fig. 8a). We therefore assessed the cytokine profile of skin from IL-2-treated mice and found more production of IL-5 and IL-13 protein, consistent with the population expansion and activation of dILC2 cells (Fig. 7c), but also more inflammatory cytokines such as IL-6, TNF and CXCL1 (Fig. 7d). We did not detect

Table 1 Surface profile of ILC2 cells

	UT	IL-2-JES6-1
CD25	+	hi
ICOS	+	hi
ST2	lo	hi
CD69	lo	hi
Integrin β_7	hi	lo
CD49d	lo	–
CD49e	lo	lo
KLRG1	hi	hi
NKG2D	–	–
NKp46	–	–
CD84	+	+
CD103	–	–
IL-13R α 1	+	+
Fc ϵ R1	–	–

Surface expression of various proteins (left column) on ILC2 cells obtained from untreated and IL-2-JES6-1 treated mice (*n* = 4 per group) and activated *in vivo*, assessed by flow cytometry. –, not detected; +, positive; hi, high expression; lo, low expression. Data are from two independent experiments.

IL-3, IL-4, IL-9, IL-17 or interferon- γ in either IL-2-treated mice or untreated mice, whereas the concentrations of TSLP and IL-25 were similar in the two groups (data not shown). Consistent with its higher

concentrations of IL-5 and CXCL1, IL-2-treated skin contained dermal leukocyte infiltrates consisting of eosinophils and neutrophils (Supplementary Fig. 8b and data not shown). To determine whether

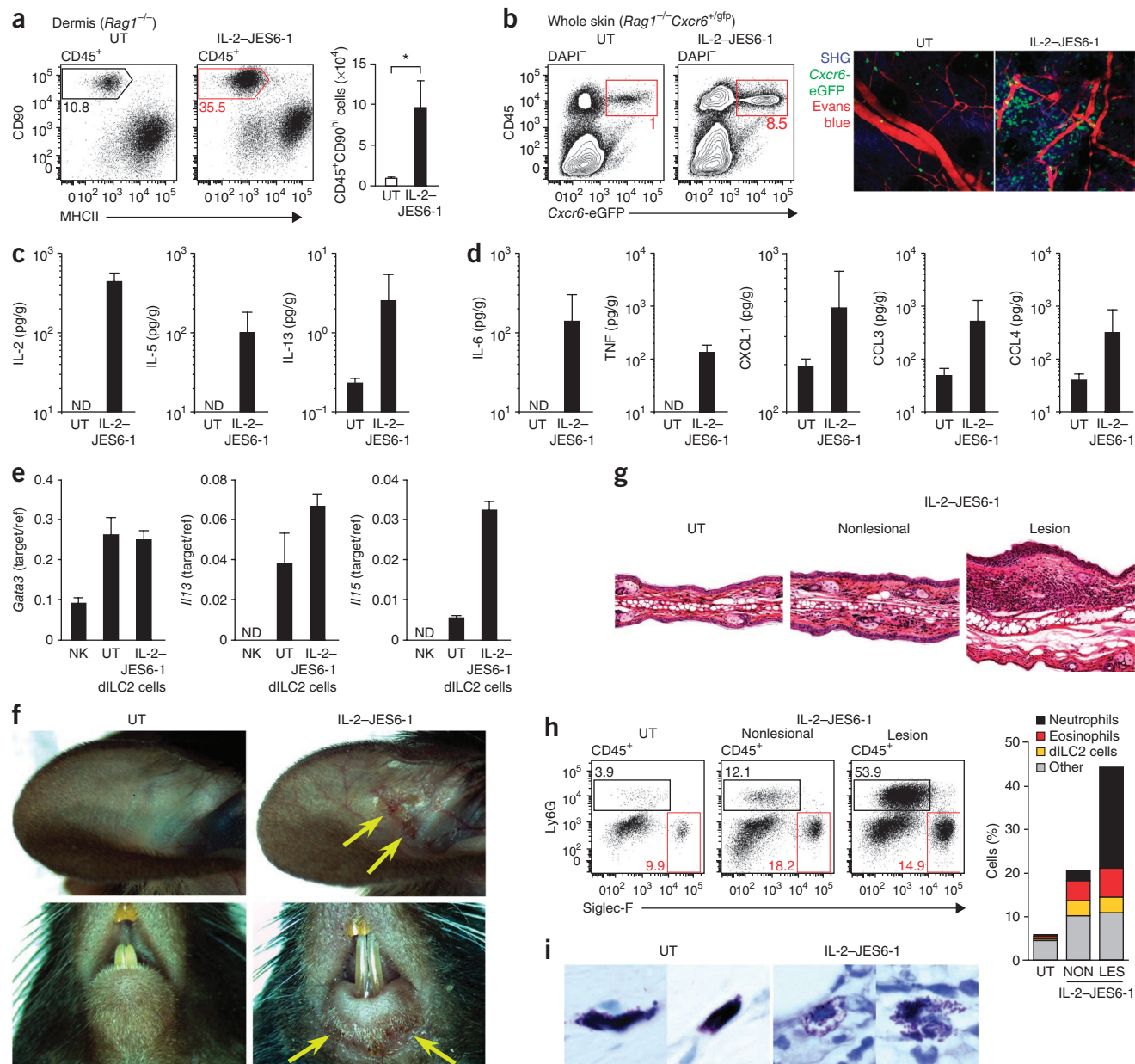


Figure 7 IL-2 stimulation of dILC2 cells *in vivo*. **(a)** Flow cytometry (left) of dermal leukocytes isolated from ears of *Rag1*^{-/-} mice left untreated ($n = 4$ mice) or treated with IL-2-JES6-1 ($n = 5$ mice); numbers below outlined areas indicate percent CD90^{hi} dILC2 cells. Right, absolute number of CD45⁺CD90^{hi} dILC2 cells from mice at left. * $P = 0.0015$ (unpaired t -test). **(b)** Flow cytometry (left) of skin cells isolated from *Rag1*^{-/-}*Cxcr6*^{+/gfp} mice ($n = 4$) left untreated or treated with IL-2-JES6-1; numbers below outlined areas indicate percent eGFP⁺ dILC2 cells. Right, intravital multiphoton microscopy of dILC2 cells (green) in skin from mice as at left; each image is a z-projection through a volume of 88 μ m in the dermis (extracellular matrix and blood vessels detected as in Fig. 4e). **(c,d)** Concentration of cytokines (c) or cytokines and chemokines (d) in homogenates of tail skin from adult *Rag1*^{-/-} mice left untreated ($n = 9$ mice) or treated with IL-2-JES6-1 ($n = 12$ mice), detected by cytokine bead array. **(e)** Real-time PCR analysis of *Gata3*, *Il13* and *Il15* mRNA in splenic NK cells (NK) and in dILC2 cells sorted from *Rag1*^{-/-} mice left untreated or treated with IL-2-JES6-1; results (for target genes) are presented relative to those of three reference genes (ref; *Gapdh*, *Actb* and *Hprt*). ND, not detected. **(f)** Presence (yellow arrows) or absence of spontaneous skin lesions on the ears (top row) and mouth area (bottom row) of *Rag1*^{-/-} mice left untreated or treated with IL-2-JES6-1. **(g)** Ear sections from mice as in a, stained with hematoxylin and eosin. **(h)** Flow cytometry (left) of leukocytes isolated from an untreated *Rag1*^{-/-} mouse and from lesional and nonlesional skin of IL-2-treated *Rag1*^{-/-} mice; numbers adjacent to outlined areas indicate percent Ly6G⁺ neutrophils (black) and Siglec-F⁺ eosinophils (red). Right, proportion of neutrophils, eosinophils and dILC2 cells in skin as at left. **(i)** Microscopy of tail sections from untreated and IL-2-treated *Rag1*^{-/-} mice, stained with toluidine blue. Original magnification, $\times 150$ (b) or $\times 100$ (g) or $\times 1,000$ (i). Data are representative of two (a,b,e,h,i) or four (f,g) independent experiments (mean and s.d. in a,e) or are pooled from three independent experiments (c,d; geometric mean and 95% confidence interval).

the higher concentrations of IL-5 and IL-13 were due to more production of these cytokines by dILC2 cells, we sorted CD45⁺CD90^{hi}CD2⁻ dILC2 cells from untreated and IL-2-treated *Rag1*^{-/-} mice and examined the abundance of *Il5* and *Il13* mRNA by quantitative RT-PCR. The expression of both *Il13* and *Il5* by dILC2 cells was much higher after treatment with IL-2-JES6-1, which indicated that IL-2-expanded dILC2 populations were functionally distinct from steady-state dILC2 cells, whereas the expression of *Gata3* (which encodes the transcription factor GATA-3)^{26,38} remained unchanged (Fig. 7e).

We observed that over 50% of *Rag1*^{-/-} mice spontaneously developed clinically overt inflammatory skin lesions within 2–3 weeks of treatment with IL-2-JES6-1, particularly on or around the ears, eyes, mouth and tail (Fig. 7f and data not shown). Histologically, these lesions showed epidermal hyperplasia, dermal thickening and extensive influx of eosinophils and neutrophils throughout the dermis and epidermis (Fig. 7g,h and Supplementary Fig. 8c). Additionally, skin mast cells had a degranulated morphology in IL-2-treated skin (Fig. 7i), particularly in regions with leukocyte infiltration (Supplementary Fig. 8d). We concluded that IL-2-activated dILC2 cells promoted cutaneous inflammation, mediated at least in part by enhanced production of IL-5 and recruitment of eosinophils.

DISCUSSION

The skin is important for the early recognition of infection or environmentally derived antigens and for the appropriate bias for development into the T_H1, T_H2 or T_H17 subset of helper T cells; these processes are critical for resolution of the challenge. Furthermore, given the link of type 2 immunity to many important allergic and parasitic diseases, better understanding of the regulatory cues involved in its generation is imperative. In contrast to the well-established type 1-initiating cells in the dermis, such as IL-12-producing migratory DDCs and IL-17 producing $\gamma\delta$ T cells¹⁹, the existence of an equivalent dermal leukocyte subset that drives downstream T_H2 immunity has remained speculative. Type 2 cytokine-producing ILC2 cells in the lung and gut have emerged as early responders after systemic parasitic infection and have also been linked to the pathogenesis of allergic diseases such as asthma^{4,24}. Here we have identified a previously unknown population of skin-resident CD103⁺ dermal ILC2 cells. In contrast to the previously described ILC2 cells in mucosa-associated tissues, we have demonstrated that the dermal ILC2 were abundant in the naive state and constitutively produced IL-13, but after activation they were also able to undergo population expansion and switch to a proinflammatory phenotype characterized by enhanced production of IL-5. The dILC2 cells were responsive to TSLP and were able to express *Il4*, which further emphasizes their likely importance as a type 2 sensor in the skin.

The nearly exclusive production of IL-13 in the skin by dILC2 cells may have relevance for atopic skin conditions, given the evidence that IL-13 has a major role in allergic inflammation, including atopic dermatitis³⁹. Although the steady-state production of IL-13 by dILC2 cells would indicate a homeostatic function for this cytokine, it is nevertheless conceivable that dILC2 cell-derived IL-13 also has a role in atopic disease under more pathological conditions, particularly when the cytokine has better access to other cell populations such as endothelial cells and keratinocytes⁴⁰.

It remains unclear whether dILC2 cells are a subset of circulating ILC2 cells⁹ or whether they are a permanent skin-resident population. In the former case, ILC2 cells would seem to be highly skin trophic, given their greater abundance in the dermis than in blood or other organs. Indeed, we estimate that there are more dILC2 cells in two mouse ears than can be found in the entire spleen. Thus, ILC2 cells are not as rare as believed but do have a greater propensity for

nonlymphoid tissues, particularly the skin. The greater prevalence of dILC2 cells in *Rag1*^{-/-} mice and much lower abundance in *Il7*^{-/-} mice suggests that ILC2 cells compete with T cells for IL-7, analogous to the greater prevalence of NK cells in *Rag1*^{-/-} mice due to improved IL-15 bioavailability⁴¹.

An important aspect of the function of leukocytes is their migratory activity and interactions in intact tissues. Our *in vivo* imaging experiments have shown that despite their developmental and phenotypic similarities, dILC2 cells differed considerably from T cells in their activity in the skin. The dILC2 cells characteristically migrated in bursts, followed by extensive pauses. These periods of migratory arrest seemed to result from encounters with skin-resident mast cells. The physical proximity of these two cell types supports the idea that ILC2 cells can directly influence mast-cell function and vice versa and that such influence need not be restricted to secreted cytokines but could result from receptor-ligand interactions. Thus, intravital imaging has identified an unexpected partnership between two type 2 cells of the immune system *in vivo* that is probably important in both steady-state and inflammatory conditions.

Finally, we have demonstrated that ILC2 cells underwent direct activation and population expansion after treatment *in vivo* with IL-2-anti-IL-2 complexes, which enabled us to directly compare the phenotype of steady-state ILC2 cells with that of activated ILC2 cells *in vivo* and to assess the effect of the stimulation of dILC2 cells on cutaneous inflammation. We found that *in vivo*-stimulated ILC2 cells had a distinct phenotypic signature characterized by a greater abundance of CD25, ICOS and ST2 at the surface. In the skin, activated dILC2 cells produced much more IL-5, which resulted in eosinophil recruitment and predisposed the mice to spontaneous dermatitis. The occurrence of spontaneous lesions in sites routinely exposed to body fluids (tears and/or saliva) or minor trauma (ears and tail) is consistent with the existence of an external trigger, which presumably initiates a cascade of immunological events after keratinocyte activation.

The relevance of the responsiveness of ILC2 cells to IL-2 in immunocompetent mice requires additional investigation. It is likely that ILC2 cell function *in vivo* is regulated at least in part by T cells, as the main source of IL-2 in wild-type mice is activated T cells and the bioavailability of IL-2 is in turn influenced by the number of regulatory T cells⁴². By extension, ILC2 cells could represent an important downstream effector mechanism of the adaptive immune response. Indeed, evidence indicating that the population expansion of ILC2 cells during helminth infection depends on T cells has already been reported⁸, although IL-2 was not suggested as the underlying mechanism. In any case, our data have demonstrated that activation of dILC2 cells by IL-2 alone was sufficient to promote cutaneous inflammation and that these cells represented a potent effector population under the appropriate conditions.

The distinct phenotypic and functional differences between untreated and IL-2-treated dILC2 cells demonstrated that these cells had at least two functionally distinct states *in vivo*. In the steady state, dILC2 cells probably took on an immunoregulatory role in which dILC2 cell-produced IL-13 served to moderate mast cell activity. In contrast, once stimulated, dILC2 cells became proinflammatory and were able to promote eosinophil influx and mast-cell activation. Together, these data demonstrate that dILC2 cells represent an abundant, phenotypically unique population of group 2 innate lymphoid cells in the skin with an important role in the regulation of cutaneous inflammation. These findings may be of relevance to various inflammatory skin conditions in which eosinophil recruitment is a defining feature, including atopic skin disease and parasitic infection.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary information is available in the [online version of the paper](#).

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AUTHOR CONTRIBUTIONS

B.R., W.W. and G.L.G. conceived of the idea for this project and wrote the paper; B.R., R.K. and N.S. did immunology and flow cytometry experiments; M.A.G. provided expertise in mast-cell biology and, together with K.H.Y., did mast cell-stimulation experiments; B.R., R.J. and P.L.T. designed and did imaging experiments; E.F.-B., A.J.M., S.S.T., T.V.G., H.A.B., B.S.K., D.A. and B.F.d.S.G. contributed to experimental design and did experiments; X.C. and W.E.P. generated the 4C13R mice; and all authors discussed the results and commented on the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Mice with transgenic expression of AmCyan under *Il4* regulatory elements and destabilized dsRed (dsRed-DR) under *Il13* regulatory elements (4C13R mice) were prepared by bacterial artificial chromosome (BAC)–recombinant engineering technology with selection by *galk* (*Escherichia coli* gene encoding galactokinase). A BAC (RP23.97H11) containing the T_H2 locus-control region and genes or regions encoding IL-13, CNS-1, IL-4, VA and kinesin-II subunit (*Kif3a*) was obtained from the Children's Hospital of Oakland Research Institute. The color plasmids p-AmCyan1-N1 and p-dsRed-Express-DR were from Clontech. The starting ATG codon of *Il4* in BAC RP23.97H11 was targeted with a *galk* cassette containing 5' and 3' homology arms from the *Il4* gene. In the resultant '*Il4 galk*' BAC, *galk* was then targeted with an AmCyan construct, which replaced *galk* and thus placed the AmCyan into the correct site in *Il4* in the BAC. By the same procedure, the *galk* cassette was targeted with the starting ATG codon of *Il13* of '*Il4-AmCyan*' BAC, which contains 5' and 3' homology arms of *Il13*. In the resultant '*Il13 galk-Il4-AmCyan*' BAC, *galk* was targeted with a dsRed-DR construct, which replaced *galk*. This placement was verified by full sequencing of the region between 5' and 3' homology regions on the *Il13*-dsRed-DR-*Il4-AmCyan* BAC. The BAC was made linear by digestion with *AscI*, and the size of the fragment containing T_H2 *Lcr*, *Il13*-dsRed-DR, CNS-1, *Il4*-AmCyan, VA and *Kif3a* was verified by PCR. The fragment was then microinjected into C57BL/6 oocytes. Those oocytes were subsequently transferred into pseudopregnant foster mothers on the C57BL/6 background. Southern blot analysis was used to identify which offspring expressed the construct. The genomic DNA of potentially transgenic pups was isolated. DNA was digested with *Bam*HI, separated by electrophoresis through a 0.8% agarose gel, transferred to nylon membrane and probed with a 916–base pair PCR fragment spanning the 5' and 3' homology arms of the *Il13*-dsRed-DR construct. As a positive control, the purified *Bam*HI-digested *Il13*-dsRed-Express-DR–BAC RP23.97H11 construct that was used for initial microinjections was analyzed in parallel with the genomic DNA samples.

All mice used were on the C57BL/6 background, unless specified otherwise. BALB/c mice, C57BL/6 mice, B6.SJL/Ptprc^a (CD45.1) mice, *Rag1*^{−/−} mice, NOD-SCID (NOD.CB17-Prkdc^{scid}/ARC) mice and *Foxn1*^{nu/nu} (nude) mice were from the Animal Research Centre of Perth, Australia. *Tcrb*^{−/−} mice, *Tcrd*^{−/−} mice, mT/mG mice²², *Cxcr6*^{+/gfp} mice²⁸, *Il2*^{−/−} mice and Brainbow (B6;CBA-Tg(Thy1-Brainbow1.0)Llch/J) mice³² were from The Jackson Laboratory. *Rag2*^{−/−} mice were from Taconic. The c-Kit-eGFP mice³³ were provided by P. Besmer. *Irf1*^{L/L} mice have been described⁴³. *Il7*^{−/−} mice⁴⁴, *Il15*^{−/−} mice⁴⁵ and *Jak3*^{−/−} mice²³ were provided by J.H. Cho and J. Sprent. *Il25*^{−/−} mice⁴⁶ were provided by M. Kleinschek. *Il3*^{−/−} mice (BALB/c) have been described⁴⁷. *Il4*^{gfp/gfp} mice, *Il4*^{+/gfp} mice (BALB/c) and *Tslpr*^{−/−} mice⁴⁸ were from the US National Institutes of Health. B6-Kit^{W-sh/W-sh} and WBB6F₁-Kit^{W/W-v} mice³⁴ were bred and maintained at the Institute of Veterinary and Medical Science animal facility, Adelaide^{34,35}. CD11c-eYFP mice have been described³⁰. C57BL/6-C^{2J} (albino B6) mice have been described⁴⁹. *Il25*^{−/−} 4C13R mice and *Il4*^{gfp/gfp}*Tslpr*^{−/−} mice⁴⁸ were bred and maintained by the Malaghan Institute of Medical Research animal facility in Wellington, New Zealand. C57BL/6-C^{2J} Brainbow (albino Brainbow) mice, Brainbow c-Kit-eGFP mice, Brainbow *Cxcr6*^{+/gfp} mice and *Rag1*^{−/−}*Cxcr6*^{+/gfp} mice were generated at the Centenary Institute animal facility. All animal experiments were done with the approval of the Animal Ethics Committee at the University of Sydney (Sydney, Australia), the Victoria University Animal Ethics Committee (Wellington, New Zealand) or the Institute of Medical and Veterinary Science Animal Ethics Committee (Adelaide, Australia).

Tissue processing and flow cytometry. Spleens, Peyer's patches and skin-draining (inguinal, axillary and brachial) and mucosal (mesenteric) lymph nodes were digested for 30 min at 20–22 °C with collagenase IV (0.5 mg/ml; Sigma) before filtration through an 80-μm stainless-steel mesh to obtain single-cell suspensions. Spleen cells were washed in running buffer (5% FCS and 5 mM EDTA in PBS), followed by lysis of red blood cells before cells were counted and stained with antibodies. Spleen and lymph node samples were counted with a Sysmex XS-1000i Automated Hematology Analyzer (Toa Medical Electronics). Mesentery, as defined before⁷, was carefully dissected and digested for 30 min at 20–22 °C with collagenase IV (0.5 mg/ml; Sigma)

before filtration through an 80-μm stainless-steel mesh to obtain single-cell suspensions. Isolation of skin cells involved one of two processes. For whole-skin preparation, ears were split into dorsal and ventral halves with forceps and were digested for 60 min at 37 °C with 2–5 mg/ml collagenase type IV in PBS (Sigma-Aldrich) to release cells. For some whole-skin preparations, release of leukocytes was achieved by incubation of skin for 60 min at 37 °C in 0.5 mg/ml Liberase TL ResearchGrade (Roche) in PBS instead of collagenase IV, followed by processing as described above. Alternatively, for dermal preparations, ears were split into dorsal and ventral halves with forceps and first digested for 90 min at 37 °C with 5 U/ml dispase I in PBS (BD Biosciences) to allow separation of the epidermis and dermis. After epidermal separation, dermal halves were then digested for 45 min at 37 °C in 2–5 mg/ml collagenase type IV in PBS. Tissues were filtered through an 80-μm stainless-steel mesh to obtain single-cell suspensions. Cells were washed in running buffer (5% FCS and 20 mM EDTA in PBS) and counted volumetrically with AccuCount beads (Spherotech). For isolation of lamina propria cells, small intestines were excised from mice after ventricle perfusion with Hank's balanced-salt solution (HBSS; Life Technologies). The mesentery and Peyer's patches were removed, and intestinal contents flushed thoroughly with 25 ml HBSS containing 5% FCS. Segments 2 cm in length were sliced longitudinally and rinsed in serum-free HBSS. For removal of epithelial sheets, flushed intestinal sections were agitated by hand for 1 min in HBSS containing 1 mM EDTA, followed by rocking for 15 min at 20–22 °C (100 r.p.m. (approximately 1.12g); Ratek Orbital Shaker Model OM11). The removal of epithelial sheets was repeated before the tissue was diced and digested for 30 min at 37 °C with collagenase (1 mg/ml Collagenase IV (Sigma) in HBSS containing 5%FCS) (150 rpm, approx. 1.68g Ratek Orbital Shaker). Digested contents were triturated and filtered through 40-μm cell strainers. Cells were washed twice and pelleted at 500g before being stained. Single-cell suspensions were incubated with anti-CD16/32 (2.4G2; BD Biosciences) for blockade of nonspecific binding and were stained with fluorochrome-conjugated antibodies prepared in flow cytometry buffer (PBS containing 5% FCS, 2 mM EDTA and 0.02% sodium azide). Fluorochrome-conjugated antibodies to the following cell-surface molecules were used: CD2 (RM2-5), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CD25 (PC61), CD38 (90/CD38), CD43 (S7), CD44 (IM7), CD45 (30-F11), CD45R (RA3-6B2), CD45.2 (104), CD49b (DX5), CD49d (9C10 (MFR4.B)), CD69 (H1.2F3), CD117 (2B8), CD132 (TUGm2), integrin β₇ (M293), MHC class II (2GB), NK1.1 (PK136) and ST2 (DJ8; all from BD Biosciences); CD5 (53-7.3), CD27 (LG.7F9), CD39 (24DMS1), CD90.2 (53-2.1), CD103 (2E7), CD122 (TM-b1), CD244.2 (eBio244F4), ICOS (7E.17G9), IL-13Ra1 (13MOKA), IL-15Ra (DNT15Ra), KLRG1 (2F1), NKG2D (CX5), NKp46 (29A7.4), FcR1 (MAR-1) and Sca-1 (D7; all from eBioscience); and CD84 (MCD84.7), IL-13Ra1 (5H10-27(MFR5); all from BioLegend). Rabbit polyclonal anti-IL-17RB (06-1071) was from Millipore. IgE-specific antibodies (6HD5 and cR1E4) were purified in-house from hybridoma cell lines at the Malaghan Institute. DNP-specific antibodies (SPE-7) were purified in-house from the hybridoma cell line at Centre for Cancer Biology. For flow cytometry, cell suspensions were resuspended in running buffer containing 0.5 μg/ml DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Molecular Probes, Invitrogen) for exclusion of dead cells. For analysis of phenotype, the presence of other leukocytes bearing the marker of interest was a requisite internal positive control before dILC2 cells were considered to lack expression, to exclude possible misinterpretation due to enzyme cleavage artifacts. Expression was compared with that of fluorescence-minus-one controls. Cells were analyzed on a FACSCanto (BD), an Accuri C6 (BD) or a four-laser, five-laser or custom ten-laser LSR II (BD). Flow cytometry data were analyzed with FlowJo software (Tree Star).

Generation of bone marrow chimeras. For the creation of bone marrow chimeras, B6.SJL/Ptprc^a (CD45.1) hosts were irradiated with 750 cGy (Gammacell 40 Exactor dual-source ¹³⁷Cs; Nordion International). Then, 24 h later, the irradiated hosts received 8 × 10⁶ to 10 × 10⁶ bone marrow cells from C57BL/6 (CD45.2) donor mice. For competitive repopulation experiments, B6.SJL/Ptprc^a (CD45.1) hosts were sublethally irradiated with 750 cGy and, 24 h later, received a bone marrow graft (8 × 10⁶ to 10 × 10⁶ cells) consisting of mT/mG bone marrow mixed at a ratio of 1:1 with either wild-type C57BL/6 (CD45.2) bone marrow (control chimeras) or *Irf1*^{L/L} bone marrow (for mixed (test

chimeras). C57BL/6-C^{2J} (albino B6) mice or C57BL/6-C^{2J} Brainbow (albino Brainbow) mice were irradiated with 750 cGy and received a bone marrow graft (8×10^6 to 10×10^6 cells) from the appropriate donor mice. All chimeric mice were allowed to reconstitute for at least 6 weeks before use in experiments.

Measurement of cytokines in tissues. Cytokines of tissue homogenate were measured by Cytometric Bead Array (CBA) assay (BD) or enzyme-linked immunosorbent assay (TSLP, IL-25 and IL-33 DuoSet; R&D Systems), with minor modifications of the manufacturer's protocol. Tissues were mechanically homogenized with a rotor stator homogenizer (Polytron) in PBS containing protease inhibitors (Halt™ protease inhibitor; Thermo Scientific) and 5 mM EDTA. The homogenate was then centrifuged at 15,000g for 10 min at 4 °C. The resulting supernatant was then used for cytokine quantification, with all sample and reaction volumes 'scaled down' to either 10% (CBA) or 25% (enhanced-sensitivity CBA) of the recommended volume.

Cytokine concentrations were interpolated from standard curves generating with recombinant proteins (BD or R&D Systems). The amount of cytokine per gram of tissue was calculated as follows: [(volume of homogenate (ml) + tissue volume (ml)) × cytokine concentration (pg/ml)] / (tissue weight (g)), with the assumption of a tissue density of 1 mg/ml and 100% efficient release of cytokines into the homogenate.

Analysis of IgE after infection with *N. brasiliensis*. Wild-type and 4C13R mice were infected by subcutaneous injection of 600 live *N. brasiliensis* third-stage larvae. Blood was collected from the lateral vein on days 6, 9, 12 and 15 after infection and was spun at 1,800g for 4 min, then serum was collected and stored at -20 °C. Serum IgE was measured by enzyme-linked immunosorbent assay. Plates were coated overnight at 4 °C with 100 µl anti-IgE (6HD5; Malaghan Institute) at a concentration of 5 µg/ml. Nonspecific binding of samples was blocked by preincubation of plates for 2 h with 10% FCS in PBS. Diluted samples and an IgE standard (12-3; prepared in-house) were added to coated plates, followed by incubation for 2 h at 20–22 °C, and then extensive washing and incubation for 2 h at 20–22 °C in 100 µl biotinylated anti-IgE (3 µg/ml; R1E4; Malaghan Institute). Samples were then incubated for 1 h at 20–22 °C in 100 µl HRP Streptavidin (1:1000 dilution; GE Healthcare Life Sciences), and plates were developed with 100 µl BD OptEIA TMB Substrate (BD). Development was stopped by the addition of 0.25 M H₂SO₄.

Multiphoton intravital imaging of skin and image analysis. Preparation of animals and imaging were done as described^{19,30}. Mice were anesthetized by intraperitoneal injection of ketamine-xylazine (80–10 mg per kg body weight), with repeated half-dose injections as required. The ears were treated with Nair depilatory cream (Church and Dwight) for removal of hair. Mice were then placed onto a custom-built stage for positioning of the ear on a small metal platform for multiphoton imaging. The ear was immersed in a solution of PBS and glycerol (70/30, vol:vol) and was covered with a coverslip. The body temperature was maintained at 37 °C with a heating pad placed underneath the mouse, and the temperature of the platform was maintained independently at 35 °C. In some experiments, mice were injected with Evans blue for the detection of blood vessels. Multiphoton intravital imaging was done with a LaVision BioTec TriMScope attached to an Olympus BX-51 fixed stage microscope equipped with a diode-pumped, wideband mode-locked Ti:Sapphire femtosecond laser (MaiTai, SpectraPhysics) and an APE Optical Parametric Oscillator (OPO). Samples were imaged through a 20× or 16× water-immersion objective (actual magnification, ×220 or ×150, respectively). For detection of fluorophores, samples were simultaneously exposed to polarized laser light at a wavelength of 920 nm for the excitation of GFP, YFP and Evans blue and second-harmonic generation and 1,080-nm light from the OPO for excitation of mT or phycoerythrin. Three-dimensional (x, y, z) images of the ear skin were acquired (spacing of 2–4 µm in z-axis over the appropriate depth) every 30–60 s for a period of 0.5–2 h. Chromatic aberration between Ti:Sapphire- and OPO-excited fluorophores was determined before imaging experiments with autofluorescent beads and was adjusted for post-acquisition in ImageJ software. In experiments involving CD11c-eYFP, the GFP and YFP channels were spectrally unmixed post-acquisition with ImageJ. Migration parameters were determined with Volocity (PerkinElmer) as described^{19,30}. Image stacks were transformed into movies with ImageJ.

Counting of dILC2 cells density *in vivo*. Image stacks from *Cxcr6*^{+/GFP} mice were acquired by multiphoton microscopy as described above. Images were collected every 2 µm in the z-axis throughout the entire depth of the ear (approximately 150 µm from the top of the epidermis). Mice were then killed and the ears were digested with dispase as described above. After separation of the epidermis from dermis and digestion with collagenase, dermal samples were stained with anti-CD45 (30-F11), anti-CD3 (145-2C11), anti-CD2 (RM2-5), anti-CD90 (53-2.1) and anti-CD11b (M1/70), and T cells, NK cells and dILC2 cells were then assessed for GFP fluorescence by flow cytometry. Quality of epidermal removal was assessed by the degree of contamination with CD3^{hi} DETCs, and samples were considered sufficiently pure if DETCs constituted less than 1% of total CD45⁺ cells. Multiphoton image stacks were processed with ImageJ. Dermal GFP⁺ cells were identified by presence of the SHG signal³⁰ and were counted manually with the 'point' and 'multi-point' selection tools to determine the density of GFP⁺ cells within the skin. With the data obtained by flow cytometry, the density of individual cell subsets was determined with the following formula: density of cell type Y = [density GFP⁺ cells] × [percentage of GFP⁺ cells of cell type Y] / [percentage of cell-type-Y that are GFP⁺].

Mast-cell stimulation and cytokine measurement. As described³⁵, bone marrow-derived cultured mast cells were obtained by culture of bone marrow progenitor cells from femurs and tibias of female mice for 4–6 weeks in 20% conditioned medium from the WEHI-3 mouse myelomonocytic cell line (which contains mouse IL-3). The purity of mast-cell populations (>95%) was determined by May-Grunwald-Giemsa staining and by flow cytometry (FcεR1⁺c-Kit⁺). Bone marrow-derived cultured mast cells were incubated with IL-13 or IL-9 (0–10,000 pg/ml; Shenandoah Biotechnology) and were sensitized overnight at 37 °C with IgE antibody to dinitrophenyl (generated from mouse SPE-7 hybridoma cells that produce IgE monoclonal antibody; hybridoma cell line provided by Z. Eshar) at 2 µg/ml in WEHI-3 cell-conditioned medium. Bone marrow-derived cultured mast cells were centrifuged for 5 min at 200g and resuspended at a density of 1×10^6 cells per ml in DMEM containing 1% (vol/vol) BSA (Sigma), and then were stimulated for 6 h with 20 ng/ml of antigen specific for 2,4-dinitrophenyl-human serum albumin (Sigma) in the presence of IL-13 or IL-9. IL-6 and TNF in the supernatants were measured by enzyme-linked immunosorbent assay in accordance with the manufacturer's instructions (BD Biosciences).

Depletion of ILC2 cells by monoclonal antibodies *in vivo*. For depletion studies, *Rag1*^{-/-} mice were injected intraperitoneally daily for 2 d with anti-CD25 (PC61) or anti-CD90.2 (30H12) at a dose of 200 µg antibody per day, then were killed and organs were collected. Hybridoma clones PC61 and 30H12 were grown and purified in-house at the Malaghan Institute of Medical Research.

***In vivo* treatment with IL-2 and anti-IL-2.** For expansion of ILC2 populations, *Rag1*^{-/-} mice were treated with IL-2-JES6-1 complexes as described³⁷. Each injection consisted of 1 µg IL-2 (PeproTech) in complex with 5 µg JES6-1 (Walter and Eliza Hall Institute of Medical Research Monoclonal Antibody Facility), which corresponded (approximately) to a molar ratio of 2:1 (cytokine/antibody). IL-2-JES6-1 complexes were prepared by mixture of IL-2 and JES6-1 at the appropriate ratio, followed by incubation for 30 min at 37 °C. Aliquots were then frozen at -70 °C until use. Aliquots were resuspended in PBS immediately before use and were injected intraperitoneally in a volume of 100 µl.

Quantification of gene expression in sorted ILC2 cells. Cells were sorted for gene-expression analysis by quantitative RT-PCR as described⁵⁰, with minor modifications. Ears and spleens from *Rag1*^{-/-} mice were prepared and stained for flow cytometry as described above. The dILC2 cells were identified as DAPI⁺CD45⁺CD11b⁺CD90^{hi}CD2⁻ and splenic NK cells were sorted as DAPI⁺CD11b^{lo}CD90⁺NK1.1⁺. Cells (1×10^3 to 2×10^3 , depending on the experiment) were sorted with a custom ten-laser Influx sorter (BD) into Buffer RLT Plus lysis buffer (Qiagen). RNA was extracted with an RNeasy mini kit (Qiagen) and was reverse-transcribed with Moloney mouse leukemia virus-RT (Ambion) primed with random hexamers (GeneWorks). Quantitative PCR was done with TaqMan Gene Expression Master Mix and the following Taqman assays (Life Technologies assay identifier in parentheses): *Gapdh* (Mm99999915_g1),

Actb (Mm00607939_s1), *Hprt* (Mm00446968_m1), *Il5* (Mm00439646_m1), *Il13* (Mm00434204_m1), *Gata3* (Mm00484683_m1). Target-gene abundance was determined as a ratio of target-gene expression normalized to the geometric mean of three reference genes (*Gapdh*, *Actb* and *Hprt*).

Histological analysis of IL-2-treated skin. Ear pinnae and tail skin of untreated and IL-2-treated mice were fixed in 10% neutral buffered formalin. After fixation in formalin, tissues were embedded in paraffin, and sections 5 μ m in thickness were cut and then stained with hematoxylin and eosin or toluidine blue.

Statistical analysis. An unpaired Student's *t* test (normally distributed) was used for comparison of independent experimental groups unless otherwise specified. Statistics were computed with Prism5 software (GraphPad). A difference with a *P* value of less than 0.05 was considered significant.

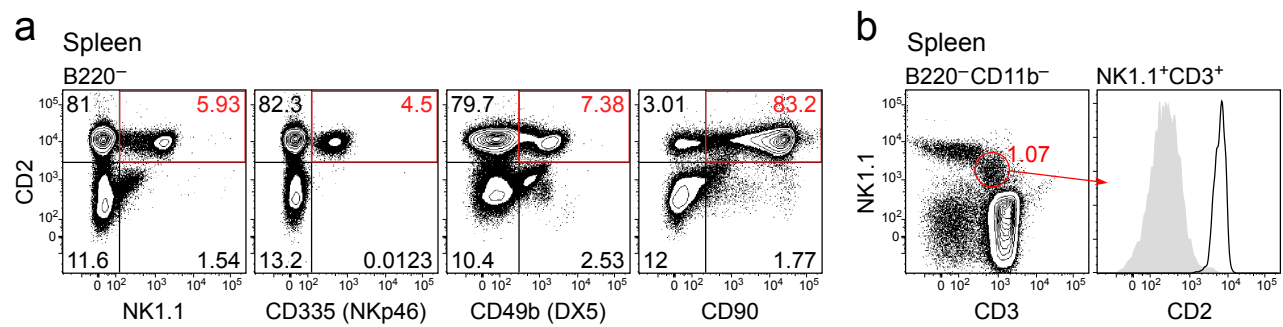
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Supplementary Information

Cutaneous immuno-surveillance and regulation of inflammation by group 2 innate lymphoid cells

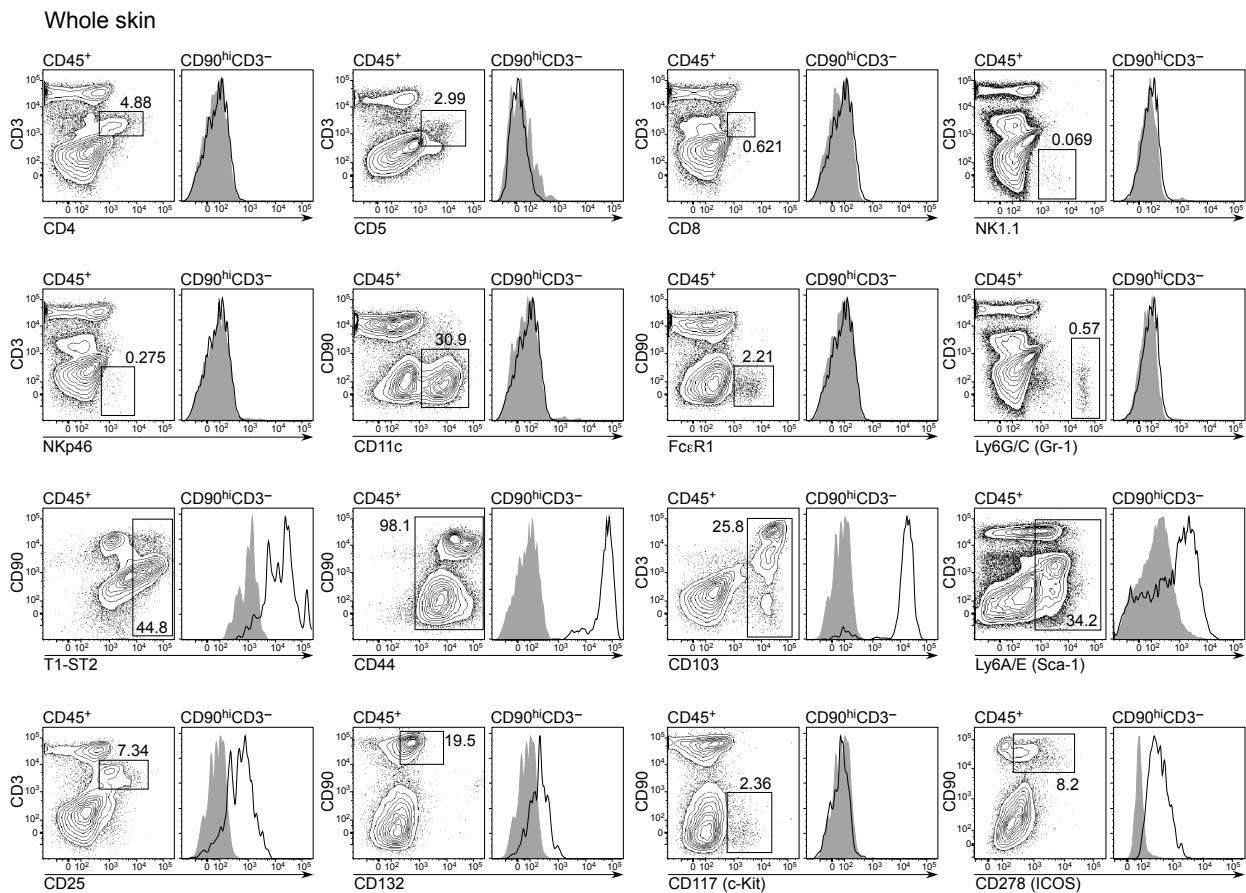
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Supplementary Figure 1



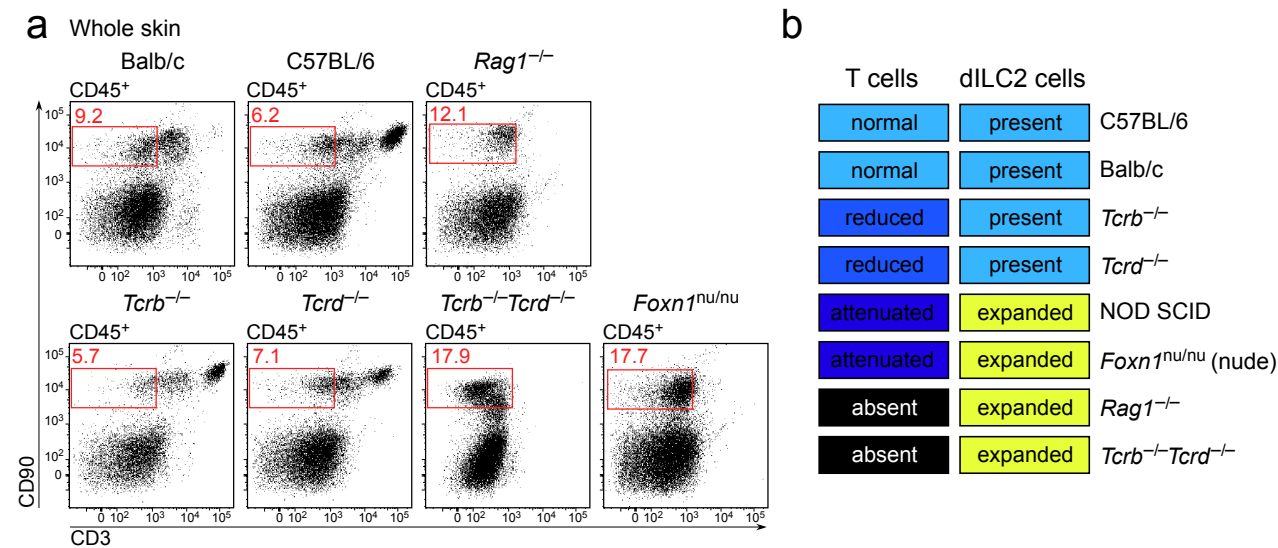
Supplementary Figure 1. CD2 expression by natural killer (NK) and NKT cells. (a) Representative dotplots depicting CD2 expression by NK1.1⁺, Nkp46⁺, DX5⁺ and CD90⁺ NK cells in wild-type C57BL/6 mice. (b) CD2 expression by splenic CD3⁺ NK1.1⁺ NKT cells. Results are representative of 2 independent experiments (*n* = 4).

Supplementary Figure 2



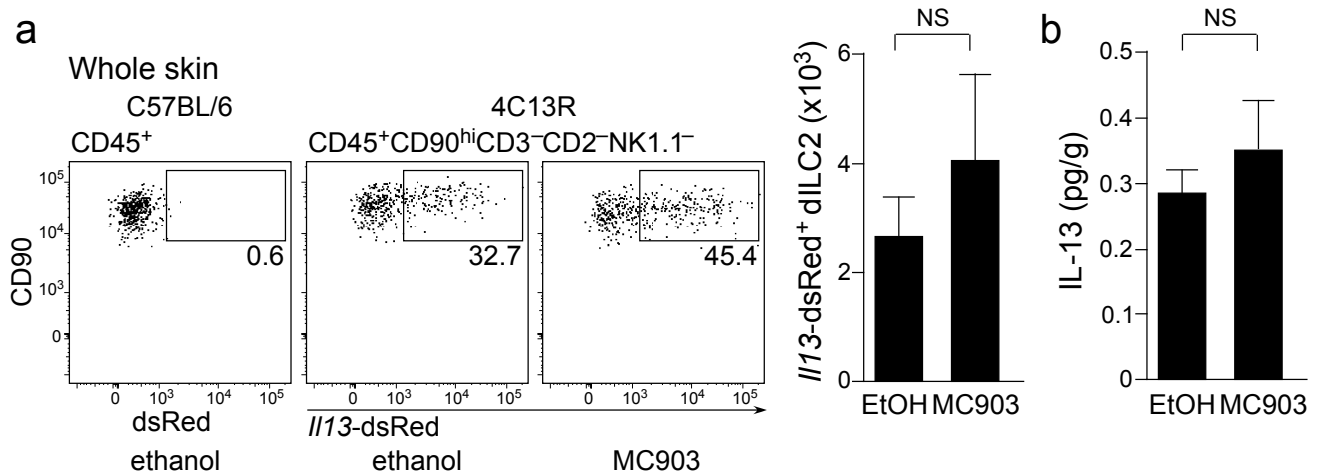
Supplementary Figure 2. Phenotype of skin ILC2. Representative histograms depicting surface expression of indicated markers by CD45⁺CD90^{hi}CD3⁻ dILC2 isolated from the skin of wild-type mice. Contour plots indicate the presence of other leukocytes bearing the marker of interest within the same sample, a requisite internal positive control to exclude possible misinterpretation due to enzyme cleavage artifacts. Results are compiled from 6 independent experiments ($n \geq 2$ per marker).

Supplementary Figure 3



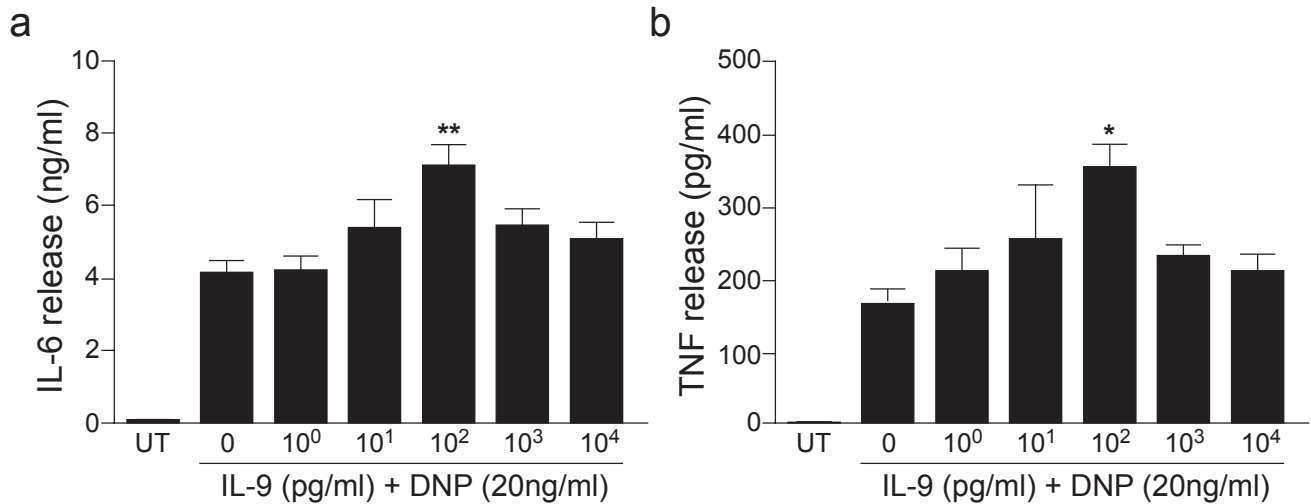
Supplementary Figure 3. Relationship between T cells and ILC in the skin. (a) Representative dotplots of CD45⁺ cells in the skin of indicated mouse strains. Gates indicate CD90^{hi}CD3⁻ ILCs. Numbers indicate percent positive cells within each gate. (b) Summary of T cell and ILC composition in indicated mouse strains. Data are representative of at least two independent experiments for each strain.

Supplementary Figure 4



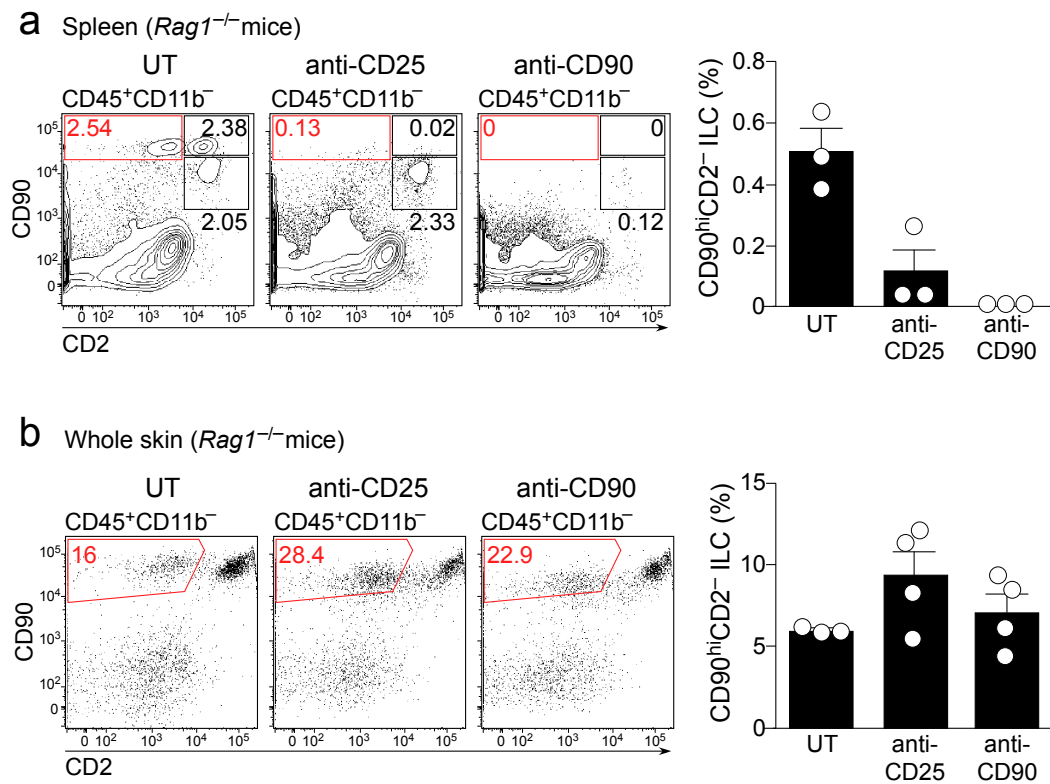
Supplementary Figure 4. IL-13 production by dILC2 in response to MC903 *in vivo*. (a) Left, representative dotplots of dILC2 in 4C13R transgenic mice topically treated with MC903 or vehicle (ethanol, EtOH). Right, absolute number of *IL13*-dsRed⁺ dILC2 in 4C13R mice topically treated with MC903 or EtOH. Data are mean $\pm$ s.d. and were pooled from 2 independent experiments ($n = 6$ for MC903, $n = 4$ for EtOH) (b) IL-13 protein detection within skin homogenates of MC903-treated mice and vehicle (ethanol, EtOH) treated controls, as detected by cytokine bead array. Data are mean $\pm$ s.d. ($n = 5$ for MC903, $n = 4$ for EtOH). NS, not significant.

Supplementary Figure 5



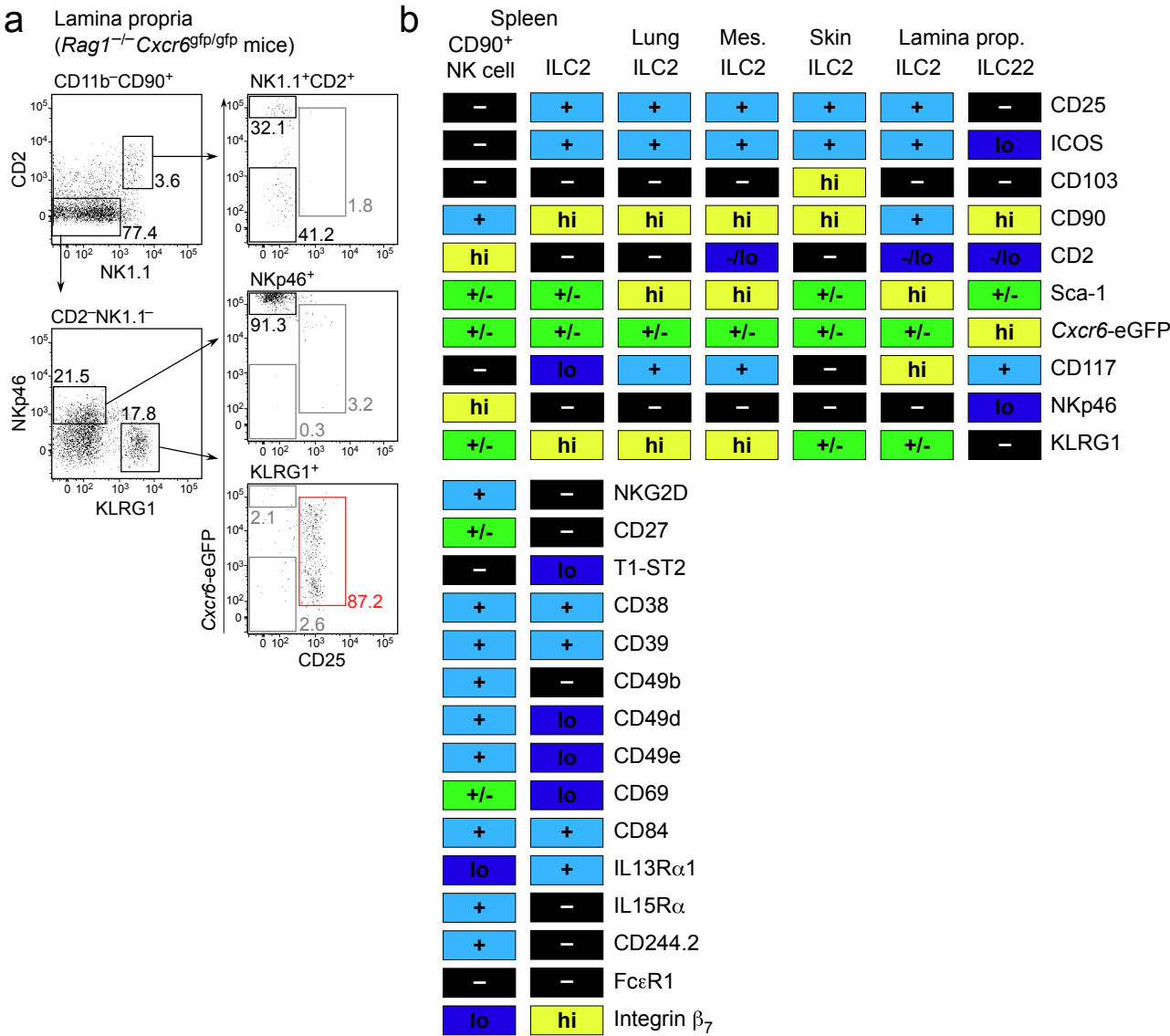
Supplementary Figure 5. Role of ILC2-derived cytokines on mast cell function. (a,b) Effect of exogenous IL-9 on cytokine production by mast cells *in vitro*. Mast cells were pre-incubated with rmIL-9 (0 – 10,000 pg/ml) and sensitized with anti-DNP IgE (2 μ g/ml) for 16 h. Cells were washed and then stimulated with DNP-HSA (20ng/ml) in the presence of rmIL-9 (0 – 10,000 pg/ml) for 6 h. (a) IL-6 and (b) TNF in supernatant were measured by ELISA. Data in (a) and (b) are expressed as mean $\pm$ s.e.m. from 3 independent experiments. Significant differences in cytokine release by DNP alone and in the presence of IL-9 were determined by one way ANOVA with Dunnett's post-test. *P < 0.05, ** P < 0.01.

Supplementary Figure 6



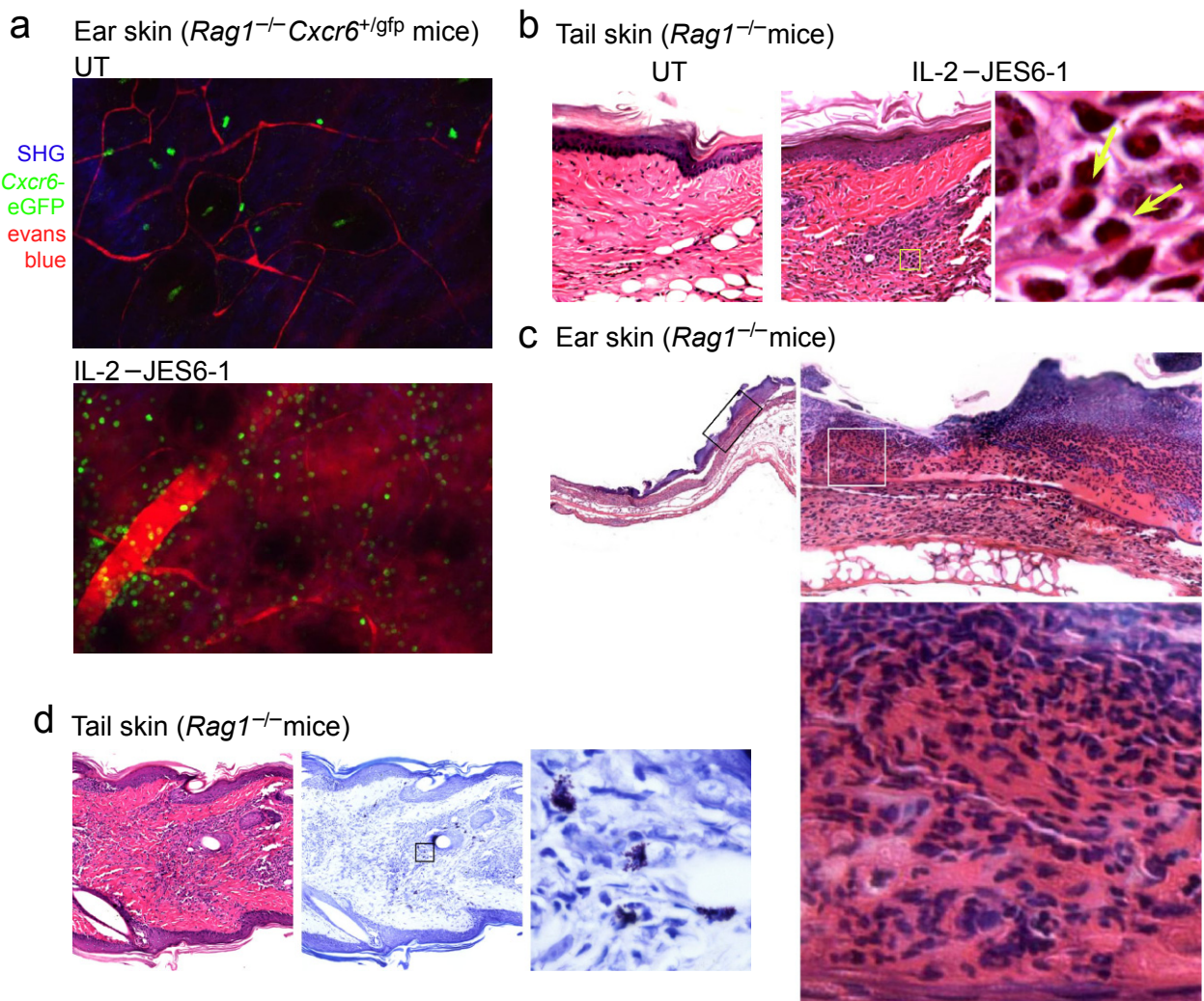
Supplementary Figure 6. ILC in the spleen and skin of mice treated with anti-CD25 and anti-CD90. (a) Left: Representative contour plots of CD45⁺CD11b⁻ splenocytes from untreated *Rag1*^{-/-} mice and mice treated with either anti-CD25 (clone PC61) or anti-CD90.2 (clone 30H12). Mice were treated with 200 µg antibody per day for two days prior to assessment. CD90-expressing cells were detected using clone 53-2.1. Red boxes indicate CD90^{hi}CD2⁻ ILC. Right: percentage of CD90^{hi}CD2⁻ ILC (of total CD45⁺ cells) in the spleens of antibody-treated mice. (b) Left: Representative contour plots of CD45⁺CD11b⁻ skin cells from untreated *Rag1*^{-/-} mice and mice treated with either anti-CD25 or anti-CD90 as described in (a). Red boxes indicate CD90^{hi}CD2⁻ dILC2. Right: Percentage of CD90^{hi}CD2⁻ dILC2 (of total CD45⁺ cells) in the skin of antibody-treated mice. Data in (a) and (b) are mean ± s.e.m. from 2 independent experiments. Symbols represent individual mice.

Supplementary Figure 7



Supplementary Figure 7. Comparative phenotype of NK cells, NKp46⁺ ILC and ILC2 in *Rag1*^{-/-} mice. (a) Representative dotplots depicting *Cxcr6*-eGFP and CD25 expression by NK1.1⁺ NK cells, NKp46⁺ ILC2 and KLRG1⁺ ILC2 (ref. 38) isolated from the lamina propria of *Rag1*^{-/-} *Cxcr6*^{+/gfp} mice. Data are representative of two independent experiments (*n* = 2). (b) Summary of cell surface expression (flow cytometry) of indicated proteins by NK cells, NKp46⁺ ILC and ILC2 from spleen, lung, mesentery, skin and lamina propria of *Rag1*^{-/-} mice. -, not detected; +, positive; hi, high expression; lo, low expression; +/-, expression by some but not all cells. Data are summarized from 3 independent experiments (*n* $\geq$ 2 per marker).

Supplementary Figure 8



Supplementary Figure 8. IL-2-induced skin inflammation. (a) Representative images of IL-2-JES6-1 treated and untreated *Rag1*^{-/-} *Cxcr6*^{+gfp} mouse skin by intravital multiphoton microscopy. Each image is a z-projection through a volume of 88 μ m within the dermis, obtained 30 min after injection of Evans blue (red). Extracellular matrix in the dermis was detected by SHG signals (blue). dILC2 shown in green. (b) Tail sections obtained from untreated and IL-2-treated *Rag1*^{-/-} mice, stained with hematoxylin and eosin. Yellow arrows indicate eosinophils. (c) Ear section obtained from an IL-2-treated *Rag1*^{-/-} mouse, cut through a spontaneous lesion. Enlarged regions indicated the thickened epidermis with attached crusts and leukocyte infiltrate. (d) Tail sections obtained from an IL-2-treated *Rag1*^{-/-} mouse, stained with hematoxylin and eosin (left) or toluidine blue (middle and right). Enlarged region indicates rounded, hypodense mast cells. Data in (a), (b), (c) and (d) are representative of two independent experiments ($n = 4$).