Group 2 innate lymphoid cells license dendritic cells to potentiate memory T helper 2 cell responses

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Abstract

Rapid memory CD4$^+$ T helper 2 (T$_H$2) cell activation during allergic inflammation requires their recruitment into the affected tissue. Here we demonstrate that group 2 innate lymphoid cells (ILC2) play a critical role in memory T$_H$2 cell responses, with targeted ILC2 depletion profoundly impairing T$_H$2 cell localization to the lungs and skin of sensitized mice after allergen re-challenge. ILC2-derived interleukin-13 (IL-13) is critical for eliciting IRF4$^+$CD11b$^+$CD103$^-$ dendritic cells (DCs) to produce the T$_H$2 cell-attracting chemokine CCL17. Consequently, the sentinel function of DCs is contingent on ILC2s for the generation of an efficient memory T$_H$2 cell response. These results elucidate a key new innate mechanism in the regulation of the immune memory response to allergens.

Allergic airway disease affects millions of people worldwide. Although heterogeneous in etiology, a misguided acquired type-2 immune response to allergens underlies its pathology in most patients[1]. Memory T helper 2 (T$_H$2) cells are critical for antigen recall responses and subsequent type-2-cytokine-driven inflammation, although the innate immune system is also intricately involved in coordinating this process[2]. At the mucosal barrier, innate immune cells are rapidly activated by damage or microbe-associated molecular patterns to produce

cytokines, chemokines and cell-surface co-stimulatory molecules[3]. Although this inflammatory milieu enables the rapid homing, efficient activation, and survival of memory T_H2 cells, the exact mechanism is not completely understood[2, 3].

Innate lymphoid cells (ILCs) encompass a family of cells that serve as part of the innate immune system[4]. In the context of infection, ILCs function as sentinels that precede the generation of antigen-specific adaptive immune responses. Group 2 ILCs (ILC2s) are an important early cellular source of type-2 cytokines, and are activated by alarmins, including IL-25, IL-33, and TSLP. At barrier sites, ILC2s respond to helminth infection in the gut[5, 6, 7], but also to viral or allergen-induced tissue damage in the airways[8, 9, 10]. Although ILC2s influence the priming of T_H2 cells after initial allergen or helminth exposure[11, 12, 13, 14, 15], their continued role during the effector-memory T_H2 cell response following secondary antigen re-challenge is unknown.

The critical role of dendritic cells (DCs) for antigen-presentation and type-2 chemokine production during the memory T_H2 cell recall-response is well defined[2, 16]. It is also known that DCs can be stimulated by type-2 cytokines to produce the chemokines CCL17 and CCL22[17], which attract its cognate-receptor CCR4-expressing memory T_H2 cells[18, 19]. We hypothesized that as an innate source of type-2 cytokines rapidly produced locally following allergen exposure, ILC2s might help initiate the memory T_H2 cell response by creating a chemokine milieu that promotes T_H2 cell recruitment.

Here we demonstrate that the innate response mediated by both ILC2s and DCs is required for the memory T_H2 cell response in allergen-sensitized animals. We utilized iCOS-T mice[15] to ablate ILC2s prior to the initiation of the antigen-recall response while leaving intact their critical functions during T_H2 cell priming. ILC2-depleted animals failed to recruit memory T_H2 cells to the lung and skin following allergen re-challenge. We find that ILC2s act upstream of DCs, and are essential for their production of the memory T_H2 cell chemoattractant CCL17. Taken together, we demonstrate that ILC2 are critical for orchestrating an efficient localized memory T_H2 cell response in collaboration with tissue-resident DCs.

Results

Protease allergen induces a memory T_H2 cell recall response

To induce a robust memory T_H2 cell-mediated immune response, we primed and re-challenged animals intranasally with the protease-allergen papain[20], which shares similarities with parasitic protozoan clan CA peptidases, and requires its enzymatic activity to elicit innate and adaptive allergic responses[13, 21, 22] (Fig. 1a). Priming induced acute eosinophilia and increased ILC2 numbers, which largely resolved by day 15, whereas re-challenge elicited greatly amplified eosinophilic inflammation (Fig. 1b, Supplementary Figs. 1a–e). Accordingly, allergen-induced CD4+ T_H2 cells, as defined by GATA3 expression[23], promoted an amplified antigen-recall response (Fig. 1c, d, Supplementary Fig. 1f). Tetramer-traceable memory T_H2 cells were generated by the co-administration of 2W1S-peptide[24], alongside allergen. Although priming efficiently induced tetramer+ T_H2 cells, re-challenge provoked a rapid increase in lung tetramer+ T_H2 cells (Fig. 1d). Comparable inflammation

kinetics were observed with an alternative allergen, *Alternaria alternata* extract (Supplementary Fig. 2). Furthermore, the persistence of the memory T_H2 cell response was assayed by delaying the allergen re-challenge for 130 days, which yielded similar results (Fig. 1e, Supplementary Fig. 3a-d). Enzymatically active papain induced greatly amplified antigen-recall responses, and increased T_H2 cell numbers in the lung, compared to heat-inactivated papain (HP), or 2W1S-peptide alone (Fig. 1e, Supplementary Fig. 3e–i). Active papain also induced higher numbers of eosinophils and elevated amounts of the T_H2 cell chemoattractant CCL17 (Fig 1f, g). As papain protease activity is essential for ILC2 activation, these results raised the possibility that ILC2 may be important for an efficient memory T_H2 cell response to inhaled protease allergens.

ILC2 are required for memory T_H2 cell responses to allergens

To assess the role of ILC2s during the memory T_H2 cell response, without affecting their critical functions during T_H2 cell priming, we depleted ILC2s in iCOS-T mice, where diphtheria toxin (DTx) treatment temporarily ablates ILC2s, while sparing other blood lineages including CD4^{+} T cells[15] (Supplementary Fig. 4a, b). Administration of DTx before antigen re-challenge efficiently depleted ILC2s (Fig. 2a, b, Supplementary Fig. 4c). ILC2 ablation led to a dramatic reduction in T_H2 cell numbers after allergen re-challenge on day 16 (Fig. 2c). This decrease in T_H2 cell numbers was equivalent to the baseline levels observed in papain-sensitized control animals. We also observed a strong correlation between ILC2s and tissue-infiltrating T_H2 cells (Fig. 2d). The total number of lung CD4^{+} T cells after re-challenge was unaffected in ILC2-depleted animals (Fig. 2e). Importantly, by day 20 in the lungs of re-challenged mice depleted of ILC2s, there was a similar impairment in the numbers of antigen-specific memory 2W1S-tetramer^{+} T_H2 cells as compared to ILC2-replete controls (Fig. 2f). Similar results were obtained for 2W1S-tetramer^{−} T_H2 cells (Supplementary Fig. 4d). The decrease in T_H2 cell numbers was not due to impaired T cell proliferation in the ILC2-ablated mice as evident by equivalent Ki67 staining in the T cells of control mice (Fig. 2g). Additionally, we re-challenged and analyzed iCOS-T mice 130 days after priming (Supplementary Fig. 4e). ILC2 depletion resulted in significantly reduced total T_H2 cell numbers in the lungs after re-challenge (Fig. 2h), as well as reduced 2W1S-tetramer^{+} T_H2 cells (Supplementary Fig. 4f). After re-challenge we also observed an increase in lineage^{−}CD127^{+} non-ILC2 or ILC3, which did not express IL-13; this induction was absent after ILC2-depletion (Supplementary Fig. 4g-h). Finally, T_H2 cell numbers were also reduced after re-challenge in ILC2-depleted mice treated with an alternative allergen, *Alternaria alternata* (Supplementary Fig. 4i). Functionally, in addition to reduced T_H2 cell numbers, we also noted decreased IL-4 bronchoalveolar (BAL) concentrations after papain re-challenge in the absence of ILC2s (Figure 2i), and T_H2 cells are the major source of IL-4 after papain re-challenge (Supplementary Fig. 4j). Thus, ILC2 play an important and previously unappreciated role in the generation of memory T_H2 cell responses.

ILC2 activation precedes recruitment of memory T_H2 cells

We hypothesized that ILC2-produced IL-13 might be essential for the rapid secretion of DC-derived CCL17[25], thereby promoting recruitment of CCR4^{+} memory T_H2 cells to the site of allergen exposure. Indeed, prior to and immediately after allergen re-challenge, ILC2s were the major producers of IL-13 compared to T_H2 cells (Fig. 3a). A time-course analysis

following allergen challenges showed that BAL concentrations of IL-13 and CCL17 were induced rapidly and followed similar kinetics (Fig. 3b, c). The expression of these factors correlated with a rapid influx of CCR4$^+$CD4$^+$ T cells into the lung tissue after allergen re-challenge (Fig. 3d, e). To test the involvement of IL-13 in T$_H$2 cell recruitment to the lung we used neutralizing antibodies to block IL-13 during papain re-challenge, resulting in a significant reduction of T$_H$2 cells (Fig. 3f, Supplementary Fig. 5a). Notably this also mirrored the reduction in lung T$_H$2 cells observed when CCL17 was blocked (Fig. 3f). Both IL-13 and CCL17 neutralization resulted in a lower frequency of CD44$^+$CD62L^{lo}CCR4$^+$CD4$^+$ memory T cells in animals after allergen re-challenge (Fig. 3g, h). These data demonstrate that blocking IL-13 or CCL17 result in a phenocopy that suggests they contribute to the same pathway for memory T$_H$2 cell induction.

Antigen challenge induces CCL17$^+$CD103$^-$CD11b$^+$ DCs

We next investigated the cellular source of the CCL17 produced post allergen challenge. Lung B220$^-$CD11c$^+$MHCII$^+$ DCs can be sub-divided into CD11b$^-$CD103$^+$ and CD11b$^+$CD103$^-$ populations, with the latter being implicated in allergic lung inflammation[26] (Fig. 4a). These CD11b$^+$CD103$^-$ lung DCs also express higher levels of the transcription factor IRF4, which is associated with type-2 allergic inflammation[27, 28]. An acute challenge of the lungs with papain dramatically increased the numbers of the CCL17-producing type-2-associated CD11b$^+$CD103$^-$IRF4$^+$ DC subpopulation (Fig. 4b). Gene expression analysis of CD11b$^+$CD103$^-$ and CD11b$^-$CD103$^+$ lung DC populations, as well as those of CD11b$^+$Siglec-F$^-$ and CD11b$^-$Siglec-F$^+$ lung macrophages, monocytes and activated and naive lung ILC2 was conducted (Fig. 4c). We observed that CD11b$^+$CD103$^-$ lung DCs express *Irf4* and slightly more *Ccl17* compared to CD11b$^-$CD103$^+$ lung DCs. Moreover, lung macrophages do not express higher levels of *Ccl17* compared to lung DCs. Furthermore, although naive ILC2 express *Il13* but not *Ccl17*, lung DCs express high levels of *Il4ra*, *Il13ra1,* and *Stat6*. Thus, B220$^-$CD11c$^+$MHCII$^+$CD11b$^+$CD103$^-$*Irf4*$^+$ lung DCs represent an important potential source of CCL17 in response to allergen challenge and express mRNA for IL-13 receptors, raising the possibility that they respond to ILC2-produced IL-13.

ILC2s are critical for allergen-induced CCL17 production

Mirroring their high expression of *Il13ra1*, lung DCs were also found to be IL-13Rα1-positive by flow cytometry as compared to weakly staining Siglec-F$^+$CD11c$^+$F4/80$^+$ macrophages (Fig. 5a). Moreover, IL-13-neutralized, *Il13*$^{-/-}$, and *Il13ra*$^{-/-}$ mice each showed reduced CCL17 concentrations in BAL (Fig. 5b), and reduced numbers of CCL17$^+$CD11b$^+$CD103$^-$ lung DCs (Fig. 5c) after papain stimulation compared to wildtype controls. Similarly, *Il33*$^{-/-}$ mice, which fail to activate lung ILC2s efficiently, exhibited impaired CCL17 production (Supplementary Fig. 5b). ILC2-deficient *Rora*$^{sg/sg}$-bone-marrow-transferred (BMT)[29, 30] or *Rora*$^{-/fl}$*Il7ra*-Cre mice[15] (Fig. 5c, d), and ILC2-depleted iCOS-T mice (Fig. 5e) had considerably fewer CCL17$^+$CD11b$^+$CD103$^-$ lung DCs following papain administration as compared to controls. ILC2-deficient and ILC2-depleted animals also had reduced CCL17 concentrations in the BAL after papain challenge and re-challenge respectively (Fig. 5b and f). Similar results were obtained in animals treated with the allergen *Alternaria alternata* (Supplementary Fig. 5c). Furthermore, co-cultures of lung-

tissue-derived ILC2s and DCs together with OT II-transgenic CD4^{+} T cells led to the efficient production of CCL17 as compared to ILC2s and DCs individually (Fig. 5g). Taken together, these results demonstrate a critical interplay between ILC2, IL-13 and DC-produced CCL17.

IL-13 is critical for DC-driven memory T$_H$2 cell recruitment

Lung DCs are known to serve an important sentinel function, and are critical for T$_H$2 cell induction and memory responses[2]. Indeed, the depletion of lung DCs in CD11c-DTR mice during papain re-challenge profoundly impaired T$_H$2 cell recruitment, while sparing ILC2s (Fig. 6a, b, Supplementary Fig. 6a–e). However, we now propose that ILC2s through their production of IL-13 regulate DC activation and their expression of the memory T$_H$2 cell-attracting chemokine CCL17. To investigate the importance of IL-13 acting on DCs during the memory T$_H$2 cell response, we generated CD11c-LuciDTR + WT and CD11c-LuciDTR + *Il13ra1*[−/−] mixed bone marrow chimeras (Supplementary Fig. 6f). Consequently, DTx administration before re-challenge eliminates functionally normal DCs carrying the DTR-transgene, and in the case of mixed chimeras yields animals that contain primarily wildtype or *Il13ra1*[−/−] DCs (Fig. 6c, d, Supplementary Fig. 6g). As predicted by the proposed upstream role of ILC2s and IL-13 in the response to allergen, the numbers of lung ILC2s and the concentration of IL-13 in the BAL were unaffected by the deletion of DCs or by having predominantly IL-13-unresponsive DCs (Fig. 6e, f), as were the numbers of lung macrophages (Supplementary Fig. 6h). In contrast, the concentration of CCL17 in BAL was markedly reduced, compared to controls, in the mixed bone marrow chimaeras in which IL-13Rα1 was specifically absent on DCs (Fig. 6g). Indeed, the absence of IL-13Rα1 signaling in the DC population resulted in concentrations of CCL17 falling to those observed following the ablation of DCs (Fig. 6g). Moreover, the absence of IL-13Rα1 expression by DCs also led to a reduction in the number of T$_H$2 cells in the lungs following papain re-challenge (Fig. 6h). Once again this impairment mirrored the effect of DC ablation (Fig. 6h), despite the equivalent lung DC numbers between wildtype and *Il13ra1*[−/−] mixed chimeras, (Fig. 6d). Thus, IL-13 signals are essential for the rapid activation of DCs and their production of the memory T$_H$2 cell-recruiting chemokine CCL17.

Dermal and gut ILC2s mediate expansion of CCL17^{+} DCs

CCL17 is known to be important for T$_H$2 cell infiltration into the skin[3]. By intracellular staining, we identified cutaneous CCL17[+] DCs, the majority of which expressed CD11b and IRF4 (Fig. 7a). Cutaneous IL-13-producing ILC2s have also been described[31, 32, 33], suggesting that, as in the lung, skin CCL17[+] DCs might be controlled by ILC2-derived IL-13. Lineage[−]CD127[+]GATA3[+] cutaneous ILC2 were readily detected in naive and papain-stimulated wildtype or *Il13*[−/−] animals, but were nearly absent in *Rora*[−/fl]*Il7ra*-Cre mice (Supplementary Fig. 7a). Acute papain challenge-induced eosinophilic inflammation was significantly impaired in *Il13*[−/−] or *Rora*[−/fl]*Il7ra*-Cre animals (Supplementary Fig. 7b). Moreover, the increase in the number of CCL17[+] DC after allergen challenge was impaired in the absence of ILC2s or IL-13 (Fig. 7b), mirroring our results obtained in the lung. In addition to its role in the skin and lung, CCL17 is also known to be important for T$_H$2 cell homing to the gut, where ILC2-derived IL-13 is critical for responding to helminth infections via IL-25 or IL-33[5, 6, 7]. Infection with the helminth *Nippostrongylus brasiliensis*

(*N.b.*) induces a potent type-2 response[34], and we observed increased numbers of peritoneal CCL17+CD11b+CD103−IRF4+ DCs on day 6 after infection (Figure 7c, d). The administration of IL-13 also resulted in a similar increase in CCL17+CD11b+ DCs in the peritoneum (Fig. 7e). Stimulation with IL-33 yielded increased numbers of CCL17+CD11b+ DCs in wildtype animals but not in ILC2-deficient mice (Fig. 7f). Therefore, ILC2 are important for CCL17 production from CD11b+IRF4+ DCs in the skin and peritoneum.

ILC2s are important for T_H2 cell responses in the skin

To examine the involvement of ILC2s on cutaneous Th2 cell responses in sensitized animals, we employed several models of allergen-induced T_H2 cell recruitment. Firstly, we sensitized animals by intranasal administration with papain on day 0 and 1, and intradermal re-challenge on day 15 followed by analysis of skin-infiltrating T_H2 cells on day 16 (Fig. 7g). Depletion of ILC2s prior to re-challenge resulted in a significant reduction in cutaneous T_H2 cell numbers, with a strong correlation between ILC2- and infiltrating T_H2 cell-numbers in the skin (Fig. 7h, i). Similarly, intradermally sensitized and re-challenged animals showed a correlation between ILC2s and T_H2 cells, and an impairment in IL-13+ T_H2 cell recruitment was detected in the absence of ILC2s (Supplementary Fig. 7c–f), whereas a single intradermal challenge with papain into non-sensitized animals did not induce significant T_H2 cell infiltration over the naive control, indicating that sensitization is required. Thus, ILC2s are important for T_H2 cell recruitment to the skin following allergen re-challenge in sensitized animals.

Discussion

We have identified here an ILC2-dependent pathway for the rapid activation of memory T_H2 cell responses in the lung following allergen re-exposure. We demonstrate that once activated, ILC2-derived IL-13 stimulates CD11b+CD103− lung DCs to produce the chemokine CCL17, promoting the recruitment of CCR4+ memory T_H2 cells. Targeted depletion of ILC2s in sensitized iCOS-T animals during re-challenge with papain (even after 130 days) resulted in significantly reduced numbers of IL-4-producing memory T_H2 cells in the lung that persisted for at least five days after challenge.

We show that ILC2s were the major cellular source of IL-13 in allergen-sensitized mice immediately prior to and after re-challenge, and that this preceded effector-memory T_H2 cell recruitment. Although tissue-resident memory T cells are found in distinct anatomical sites, where they function to alert and recruit other immune components upon re-challenge[3], there is little evidence yet for the existence of tissue-resident memory T_H2 cells in the airways. Moreover, the enzymatic activity of papain, which is critical for ILC2 activation, was required for memory T_H2 cell recruitment. Attenuated or inert allergens such as heat-inactivated papain or OVA might require substantially higher and prolonged dosing to induce inflammation, possibly due to inefficient ILC2 activation[12, 35]. Although other mechanisms for early IL-13 production have been proposed, including the mast cell-mediated recruitment of T_H2 cells or tissue-resident memory T_H2 cells, these models predate the discovery of ILC2s[3, 36]. Notably, IL-13 release following allergen re-challenge coincides with similarly rapid production of the memory T_H2 cell attracting chemokine

CCL17. This IL-13 signal is essential for CCL17 production specifically from IRF4$^+$CD11b$^+$CD103$^-$ DCs. Using ILC2-deficient *Rora$^{sg/sg}$* BMT and *Rora$^{-/fl}$Il7ra*-Cre mice we show IRF4$^+$CD11b$^+$CD103$^-$ DCs fail to produce CCL17 after papain challenge, demonstrating that ILC2s play a critical role in creating a type-2 inflammatory milieu. This is supported by co-culture experiments showing the instructive role for ILC2s for type-2 chemokine production from lung DCs. Indeed, the failure of DCs to release CCL17 after selective ILC2 depletion in sensitized animals indicates that ILC2s license DCs to promote memory T$_H$2 cell responses following allergen re-challenge.

Whereas the role of DCs for memory T$_H$2 cell responses is well established, we now also confirm the central role of IL-13 in this process by generating mixed bone marrow chimeras. Indeed, sensitized mice harbouring only *Il13ra1$^{-/-}$* DCs phenocopied DC-depleted animals in their inability to produce CCL17 and recruit memory T$_H$2 cells following re-challenge. Furthermore, the neutralization of IL-13 before allergen re-challenge in sensitized animals effectively prevented CCR4$^+$CD44^{hi}CD62^{lo} memory T$_H$2 cell recruitment to the lung to the same degree as CCL17-neutralized animals. Together these data provide strong evidence for the importance of ILC2-derived IL-13 for the efficient recruitment of memory T$_H$2 cells to the allergen challenged lung through collaboration with lung DCs.

Consequently, we also expand our understanding of DC function in type-2 immunity. As tissue-resident sentinels, DCs are among the first cells to respond to pathogens or allergens in the airways[2]. In naive animals, DCs are essential for antigen presentation to naive CD4 T cells, and subsequent T$_H$2 cell priming[16, 37, 38] following direct interaction with cytokines such as TSLP[39, 40]. Although alarmins can act directly on DCs, some scenarios of type-2 inflammation require ILC2s as a critical intermediate. For example, DC migration to the draining LNs following initial allergen sensitization relies on the activation of ILC2s[13]. Likewise, in our current study CCL17 production from DCs is contingent on IL-33-induced ILC2 activation. While consistent with the idea of DCs as essential modulators of adaptive T$_H$2 cell-mediated immunity, we reveal the central role of ILC2s as a critical upstream component of efficient memory T$_H$2 cell responses.

ILC2s in other anatomical sites may similarly influence the recruitment of memory T$_H$2 cells in sensitized animals. T$_H$2 cells are known to play an important role in atopic dermatitis[3] and anti-helminth immunity[34], and ILC2 have been reported in the skin[31, 32, 33] and gut[5, 6, 7]. Although memory T$_H$2 cell homing to the lung, skin and gut involve different mechanisms, it is believed that CCR4 and CCL17 have a shared role in promoting their recruitment[3]. We found that the skin and peritoneum contain a CCL17$^+$ DC population, which like the lung is also CD11b$^+$IRF4$^+$. In the skin, we found that *Il13$^{-/-}$* or ILC2-deficient *Rora$^{-/fl}$Il7ra*-Cre mice injected with papain intradermally contained fewer CCL17$^+$ DCs. Similarly, the number of CCL17$^+$ DCs was increased in the peritoneum upon infection with *N.b.*, or after administration of recombinant IL-13, providing evidence that ILC2s, the predominant source of IL-13 during parasitic helminth infection[5, 6, 7], may be driving this expansion. Indeed, administration of the ILC2-activating cytokine IL-33 results in increased numbers of peritoneal CCL17$^+$ DCs in wildtype, but not ILC2-deficient *Rora$^{-/fl}$Il7ra*-Cre mice. Thus, it is clear that ILC2s in other tissues share the ability to influence the induction of IRF4$^+$CCL17$^+$ DCs. Furthermore, ILC2-depletion in allergen-sensitized iCOS-T mice

markedly reduced memory T_H2 cell recruitment to the skin after re-challenge with papain allergen, indicating a role for ILC2s in memory T_H2 cell responses in additional tissues.

Our data also expand on the role of IL-13, a type-2 cytokine widely regarded for its 'effector' functions[2, 41]. We now reveal that its early production from ILC2s is essential for CCL17-driven recruitment of memory T_H2 cells. These results further support IL-13 as a target for therapeutic development[1, 42]. Other experimental drugs that neutralize epithelial derived IL-25, IL-33, and TSLP[42] are likely to influence ILC2 activation, and may have important additional indirect effects on memory T_H2 cells.

Thus, our results illustrate an ILC2-dependent mechanism by which memory T_H2 cells are recruited to the airways and skin following secondary allergen challenge. Whereas DCs are known to be essential for T_H2 responses, we now show that ILC2s situated at the epithelial barrier regulate IL-13 dependent DC expression of the memory T_H2 cell-attracting chemokine CCL17. Hence, ILC2 licensing of DCs is a critical component of the memory T_H2 cell response to allergens at barrier sites.

Online Methods

Mice

C57Bl/6 (B6), B6[Tg(TcraTcrb)425Cbn]/J (OT-II, JAX Laboratories), B6.Cg-*Tg(Itgax-DTR-OVA-eGFP)1Gjh/Crl* (CD11c-DTR), B6.Cg-*Tg(Itgax-EGFP-CRE-DTR-LUC)2Gjh/Crl* (CD11c-LuciDTR[43]), B6.*Il13ra1*[−/− 44], B6.*Rora*[sg/sg] (ILC2 deficient, but also develop neurological defects, JAX Laboratories), *Rora*^{−/fl}*Il7ra*-Cre (*Rora* deletion in *Il7ra*-expressing lymphocytes leads to ILC2-deficiency in the absence of neurological defects)[15, 45], B6.iCOS-T (a *loxP*-flanked *Diphtheria* toxin receptor (DTR) gene inserted into the *Icos* locus (expressed preferentially on T cells and ILC2) enables *Cd4*-cre-mediated excision of the DTR gene from T cells, but its retention in ILC2, enabling ILC2 depletion using *Diphtheria* toxin, whilst sparing T cells)[15] and B6.*Il13*[egfp/egfp] were maintained in the Medical Research Council (MRC) ARES animal facility, under specific-pathogen-free conditions. Mice were used at 4–8 weeks of age. All animal use was in accordance with the guidelines of the UK Home Office.

Reagents

Anti-mouse antibodies, Biotin: CCL17 (R&D Systems); FITC: CD44 (1M7), B220 (RA-6B2) (Becton Dickinson, (BD)); Alexa Fluor (AF) 488: Foxp3 (FJK-16s) (eBioscience, (eBio)); PerCP-Cy5.5: CD103 (2E7) (eBio); PerCP-eFl710: Ly-6G/C (RB6-8C5), Ly-6G (1A8-Ly6G), RORγt (B2D) (eBio); APC: FcεR1a, (MAR-1) IRF4 (3E4), CCR4 (2G12) (eBio), 2W1S-tetramer (NIH); eFl660: GATA3 (TWAJ) (eBio); AF700: CD11c (4N418), CD4 (GK1.5) (eBio); APC-eFl780: CD11b (M1/70), B220 (RA3-6B2) (eBio); Brilliant Violet (BV) 421: CD62L (MEL-14) (Biolegend); eFl450: CD3ε (17A2), CD5 (53-73), CD11b (M1/70), CD11c (4N418), CD19 (1D3), F4/80 (BM8), Ly-6C/G (RB6-8C5), MHCII (M5/114.15.2), NK1.1 (PK136), Ter119 (TER119) (eBio); BV510: CD3ε (17A2), CD45 (30-F11), MHCII (M5/114.15.2) (Biolegend); eFl650NC: CD90.2 (53-2.1) (eBio); BV711: T-bet (04-46) (BD), Ly-6C (HK1.4) (eBio); BV785: CD8α (53-6.7) (Biolegend); PE:

Streptavidin (SA), SiglecF (E50-2440) (BD), IL-13 (eBio13A), 2W1S-tetramer (NIH); PE-CF594: CD25 (PC61) (BD); PE-Cy7: CD49b (DX5), Ki67 (So1A15), IL-13 (eBio13A) (eBio). Protein transport inhibitor (eBio), AnnexinV (eBio) and Foxp3 Intracellular staining kits (eBio) were used. CountBright (Life Technologies) beads were used to quantify events by flow cytometry. The following ELISAs were used: IL-13 (eBio); CCL17 (R&D Systems). We used *A.alternata* (Greer) papain (Sigma), OVA-peptide (ISQAVHAAHAEINEAGR, AnaSpec), 2W1S-peptide (EAWGALANWAVDSA, Designer Bioscience), recombinant-IL-13, IL-33, and TSLP (eBio). We used the Fortessa (BD) for flow cytometry and a Synergy (Sony) for FACS. Percoll (Sigma), Collagenase I (Life Technologies), DNase I (Roche) were used for tissue digest and processing. Flowjo X (Tree Star) was used for flow cytometry data analysis.

Primary leukocyte preparation and intracellular staining

Cell suspensions were prepared from lung by mechanical dissociation, followed by digest in 3 ml of RPMI-1640 containing collagenase I (500 U/ml) and DNase I (0.2 mg/ml) for 45 minutes at 37 °C on a shaker (220 rpm), followed by filtration through a 70 μm strainer and 30% Percoll gradient enrichment of leukocytes and red blood cell (RBC) lysis. Spleen and LN were strained through a 70 μm strainer with PBS, followed by RBC lysis. BAL cells and fluid were obtained in PBS as described[13]. Skin cells were isolated from the ears by enzymatic digestion of the separated dorsal and ventral sides in 1.5 ml RPMI-1640 containing collagenase I (750 mg/ml) and DNase I (0.3 mg/ml) for 60 minutes at 37°C on a shaker (1000 rpm), followed by filtration through a 70 μm strainer and 30% Percoll gradient enrichment of leukocytes. Peritoneal cells were collected by flushing the peritoneal cavity with 2 ml of PBS. Single-cells were re-stimulated and stained for surface and intracellular markers as described[13].

Flow cytometry

Single cells were incubated with anti-mouse CD16/32 (eBio) to block Fc receptors and then stained with eFluor 450-conjugated lineage marker mAbs (CD3, CD5, CD19, NK1.1, CD11b, FcεR1α, F4/80, Ly-6C/G, and Ter119), AF700-conjugated CD11c, APC-eFluor® 780-conjugated B220, PE-conjugated MHCII, PE.Cy7-conjugated CD4, and APC-conjugated CD90, BV510-conjugated CD45, FITC-conjugated T1/ST2, and DAPI viability dye. Lung ILC2 (DAPI⁻CD45⁺B220⁻Lineage⁻CD4⁻CD11c⁻CD90⁺ST2⁺) and DCs (DAPI⁻CD45⁺B220⁻CD11c⁺MHCII^hi) were isolated from wildtype mice, or mice treated with rmIL-33 (days -2 and - 1) to expand lung ILC2. OTII cells were purified by staining Fc-blocked spleen cells with FITC-conjugated CD4, APC-conjugated CD45, and PE-conjugated CD44 mAbs in DAPI, followed by gating of DAPI⁻CD45⁺CD4⁺CD44^hi cells. Fixable viability dye (eFl780 or eFl455UV) was used to identify dead cells for intracellular-stained samples. Cells were checked for purity after flow cytometry.

In vivo stimulation

Mice were anesthetized by isofluorane inhalation, followed by the intranasal or intradermal injection of rmIL-33 (0.5 μg), OVA (50 μg), *Alternaria alternata* extract (10 μg), papain or heat-inactivated papain (10 μg) in 10–40 μl of PBS. DTx (300–500 ng) in PBS, IL-13, IL-33 or PBS alone, was injected i.p. in a 100 μl volume as indicated. Anti-IL-13 mAb, anti-

CCL17-mAb, or IgG control (300 μg) in 200 μl of PBS were injected i.p. as indicated. For *N.b.* infections 500 live L3 larvae in a 100 μl volume were injected subcutaneously.

In vitro co-cultures

Flow sort-purified OTII (5×10^4), DCs (1×10^3), and ILC2 (5×10^3) were cultured in 200 μl of RPMI-1640, 10% FCS plus penicillin/streptomycin, 2-mercaptoethanol, IL-33 (10 ng/ml) and OVA-peptide (5 μg/ml) for 6 days at 37 °C.

Bone marrow transplantation

Recipient mice were lethally irradiated (2 doses of 4.5 Gy) followed by intravenous transplantation of 10^7 whole bone marrow cells from 4-6 week old mice. Mice were given Baytril in drinking water for 4 weeks, and used for analysis at 24-32 weeks post transplant.

Gene expression analysis

We obtained microarray data sets for the listed cell-types from data assembled by the ImmGen consortium[46] (Heng et al., 2008), which was compared to our own ILC2 microarray data[9]. Data analysis was performed with FlexArray 1.5 (Genome Quebec).

Statistics

Data were analyzed using GraphPad Prism 6 (GraphPad Software). Pearson's test was performed to determine goodness of fit, as denoted by r^2. An ANOVA, two-tailed Student's t test or Mann-Whitney test was used to determine the statistical significance between groups with p 0.05 being considered significant (*), p 0.01 = **, p 0.001 = ***, p 0.0001 = ****.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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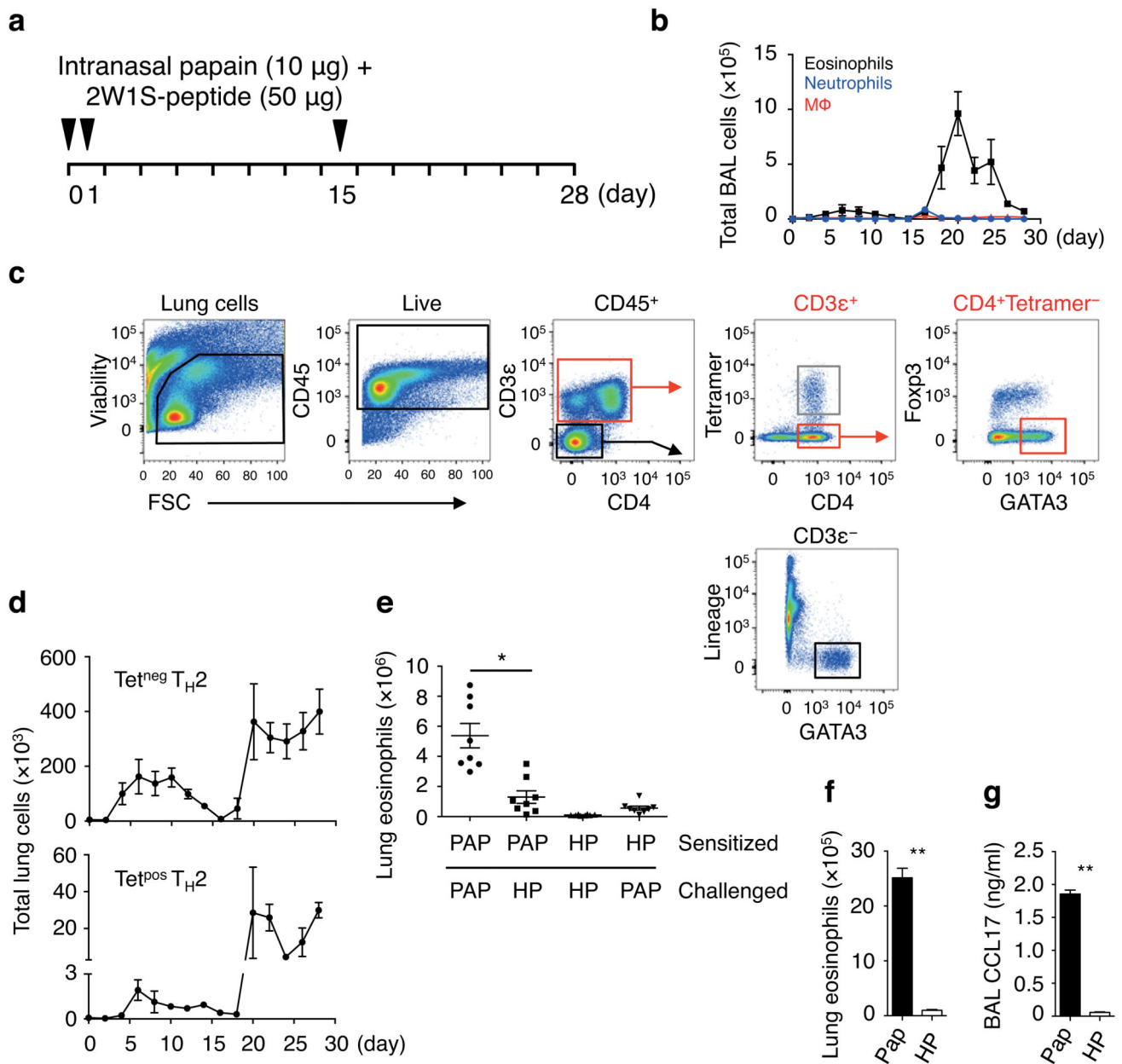
Fig. 1. Protease allergen induces a robust memory T_H2 cell-mediated recall response in sensitized mice

(**a–d**) Mice were sensitized and re-challenged with papain + 2W1S-peptide as indicated (**a**) followed by analysis every 2 days for: broncho-alveolar lavage (BAL) eosinophils, neutrophils or alveolar macrophages (MΦ) as described in Supplementary Fig. 1a (**b**). Lung ILC2 (Live CD45+CD3ε−CD4− Lineage−GATA3+) and 2W1S-tetramer+/− T_H2 cells (Live CD45+CD3ε+CD4+ Foxp3−GATA3+) were detected by flow cytometry (**c**), and quantified (**d**).

(**e**) Sensitized mice were re-challenged on day 132, and total lung eosinophils were measured on day 135.

(**f–g**) Papain + 2W1S sensitized mice were re-challenged with 2W1S + papain (PAP) or heat-inactivated papain (HP) on day 15, followed by an analysis of lung eosinophils (**e**) or CCL17 levels in the BAL (**f**) on day 16.

Data are representative of at least two independent experiments per group, each containing at least three animals. Individual points indicate individual animals in (**e**). Mean values ± SEM are indicated in b, d–g. p 0.001 = *, p 0.0001 = **.
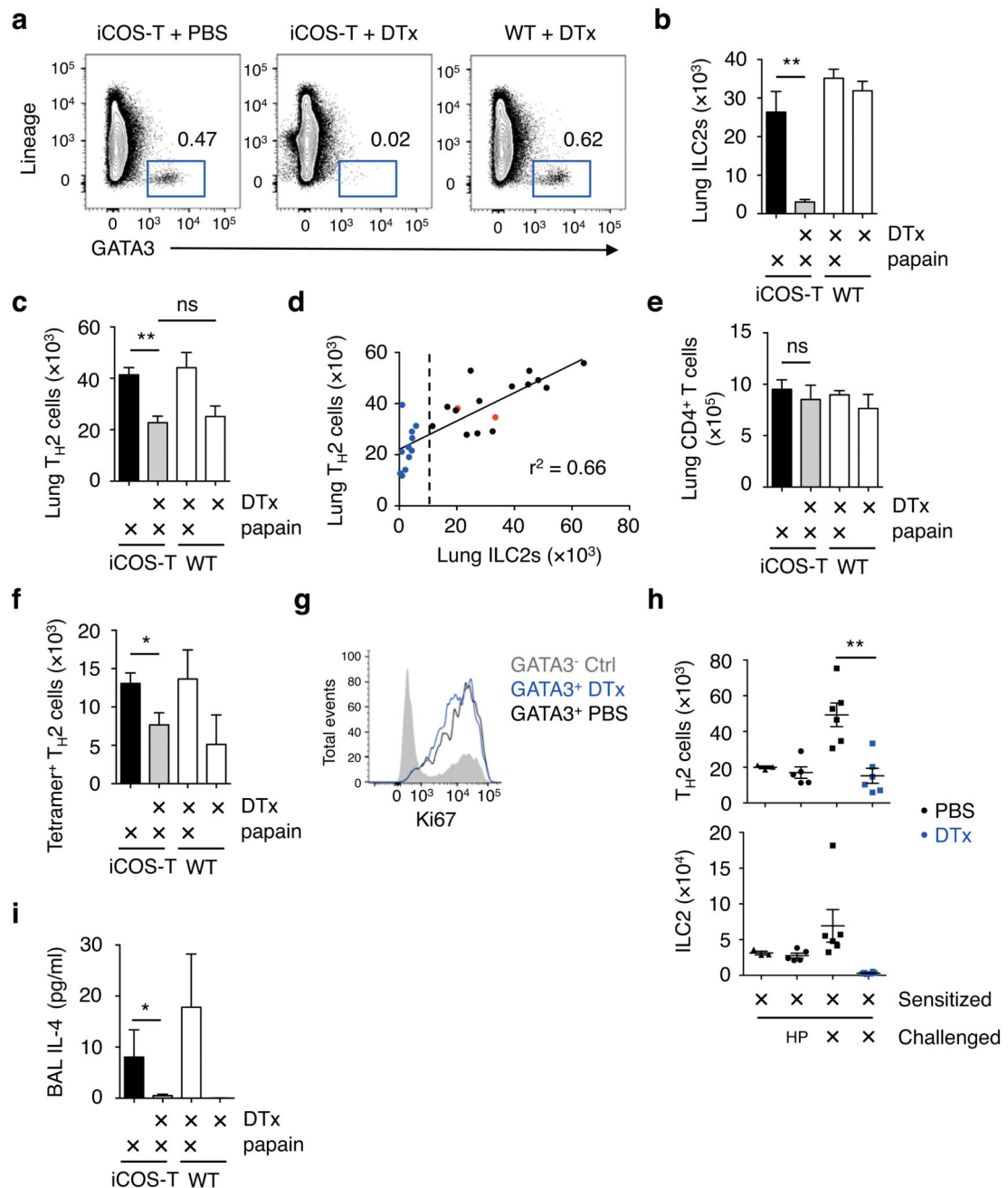
Fig. 2. ILC2 are required for memory T_H2 cell response to allergens

(**a–e**) Wildtype (WT) or ILC2-depleted iCOS-T mice were sensitized, re-challenged, and treated with DTx as indicated (Supplementary Fig. 4a). Lung ILC2s were detected by flow cytometry as in Fig. 1c (**a**) and quantified (**b**). Total lung T_H2 cells in ILC2 non-depleted (black), depleted (gray), or WT control mice on day 16 were detected as in Fig. 1c and calculated (**c**). Total ILC2s and T_H2 cells in the lungs of iCOS-T mice treated with PBS (black) or DTx (blue) on day 16 were quantified and plotted (**d**). Two iCOS-T mice failed

ILC2-depletion (red), as indicated by increased ILC2 numbers over the threshold of average naive lung ILC2s (dotted line). Total numbers of lung CD4$^+$ T cells were quantified (**e**). (**f–g**) WT or iCOS-T mice were treated as indicated (Supplementary Fig. 4b) and total numbers of tetramer$^+$ T$_H$2 cells in the lungs were quantified (**f**). Intracellular Ki67 expression was measured in lung CD4$^+$ T cell populations (**g**).

(**h**) Sensitized iCOS-T mice were re-challenged on day 132 as indicated, followed by quantification of lung ILC2 and T$_H$2 cells on day 135. PBS (black) or DTx (blue) was administered (Supplementary Fig. 4e).

(**i**) BAL IL-4 concentrations were measured on day 16 in WT or iCOS-T animals treated as indicated (Supplementary Fig. 4a).

Data are representative of at least two independent experiments per group, each containing at least three animals, and one experiment containing 3–6 animals (**h**). Individual points indicate individual animals in d and h. Mean values ± SEM are indicated in b, c, e, f, h, and i, Pearson's test was performed in (**d**). ns = not significant, p 0.05 = *, p 0.01 = **.
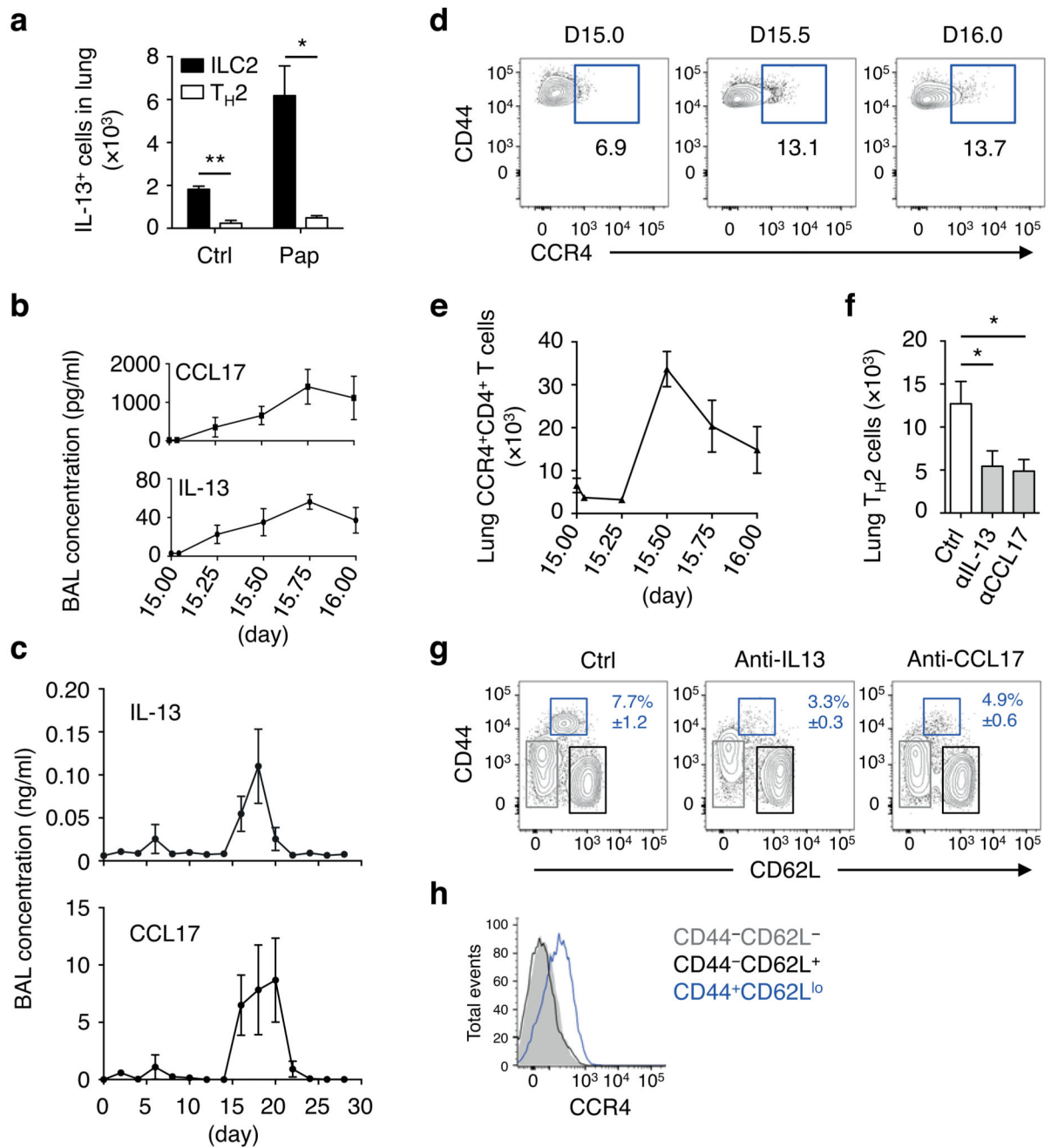
Fig. 3. ILC2 activation occurs early after allergen challenge, and precedes IL-13 and CCL17-mediated recruitment of memory T_H2 cells

(**a**) Sensitized mice (Fig. 1a) were analyzed before challenge (Ctrl) or 6 hours after re-challenge (Pap). IL-13+ lung ILC2s and T_H2 cells (identified similar to Fig. 1c) were quantified.

(**b–c**) Sensitized mice (Fig. 1a) were analyzed for IL-13 and CCL17 levels in the BAL immediately after re-challenge (**b**), or over 28 days (**c**).

(**d–e**). Lung CD44^{hi}CD62L$^-$CD4$^+$ T cells were gated and analyzed for the CCR4$^+$ percentage (**d**) and total numbers of CCR4$^+$CD44^{hi}CD62L$^-$CD4$^+$ T cells (**e**). (**f–h**) Sensitized mice were treated with neutralizing antibodies prior to challenge (Supplementary Fig. 4e). Total lung T_H2 cells (identified similar to Fig. 1c) were quantified on day 16 (**f**). The percentage of lung CD44^{hi}CD62L^{lo} (blue) among total gated CD4$^+$ T cells was identified (**g**). CCR4 expression was analyzed on lung CD4$^+$ T cell populations in control treated mice (**h**).

Data are representative of at least two independent experiments per group or at least three animals. Mean values ± SEM are indicated in a–c, e–g. p 0.05 =*, p 0.01 = **.
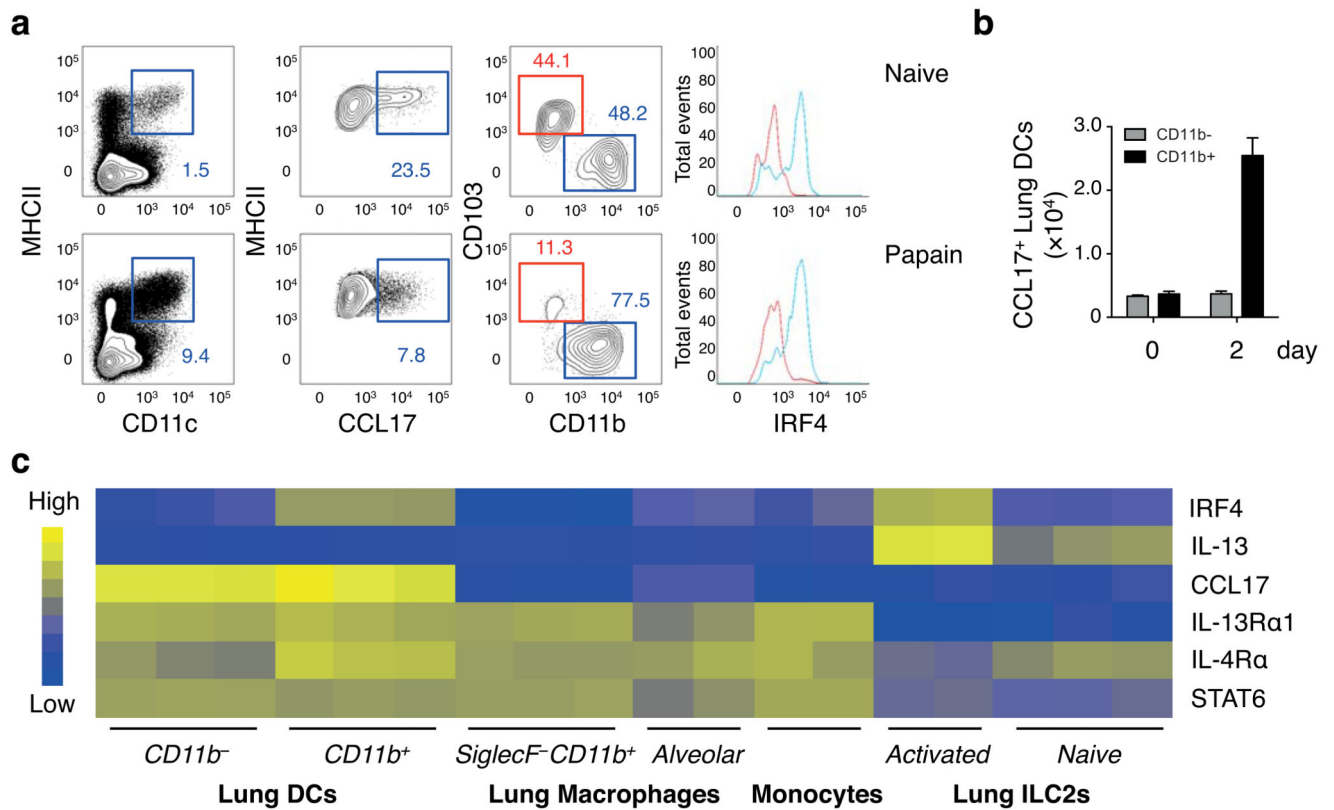
Fig. 4. CCL17+CD103−CD11b+ DCs expand after antigen challenge

(**a–b**) Mice were treated on days 0 and 1 with papain followed by analysis on day 2 for intracellular CCL17 in lung B220−CD11c+MHCII+ DCs (**a**). CD11b+CD103− (blue) and CD11b− CD103+ (red) CCL17+ DC populations were analyzed for IRF4 expression and quantified (**b**).

(**c**) Microarray datasets from CD11b−CD103+ (CD11b−) and CD11b+CD103− (CD11b+) lung CD11c+MHCIIhi DCs, Siglec-F−CD11b+ and Siglec-F+CD11b− (Alveolar) CD11c+MHCIIintSSChi lung macrophages, CD3−B220−MHCII−Ly-6c+CD115+ monocytes, and activated and naive lung ILC2s[9] were analyzed for relative gene expression.

Data are representative of at least two independent experiments per group, containing at least three animals or cultures. Mean values ± SEM are indicated in b.
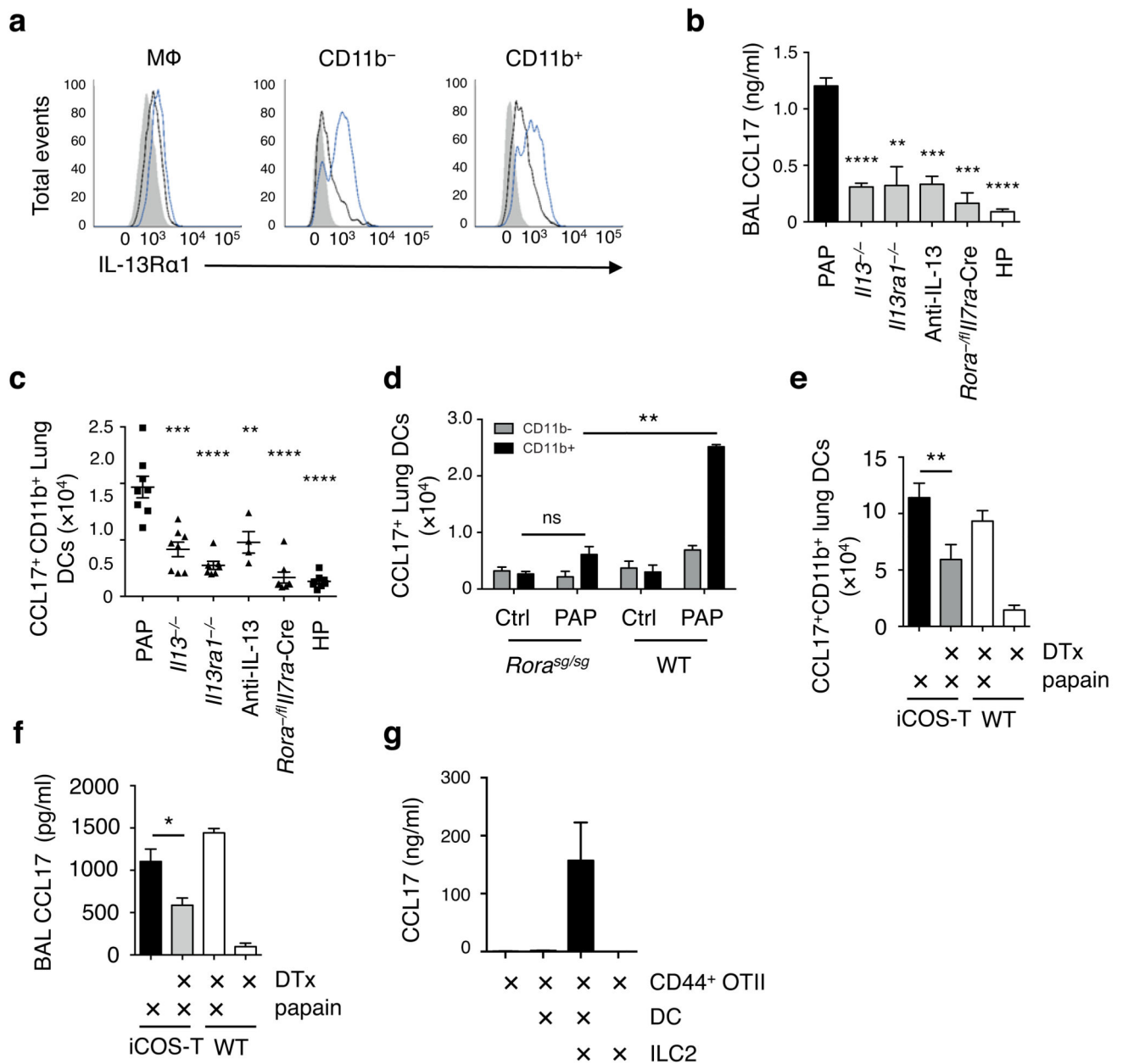
Fig. 5. ILC2s and IL-13 are critical for CCL17+ DC proliferation and CCL17 production after allergen exposure

(**a**) IL-13Rα1 expression was measured by flow cytometry on Siglec-F+CD11c+F4/80+ lung macrophages, B220−MHCIIhiCD11c+CD11b− and CD11b+ DCs. Gray shaded area indicates Fluorescence minus one (FMO) staining control, black line indicates *IL13ra1*−/− animals and blue line indicates WT animals.

(**b–c**) WT, WT + anti-IL-13 or transgenic mice were treated with papain or heat-inactivated papain (HP) on days 0 and 1, followed by analysis of BAL CCL17 concentrations (**b**) or CCL17+CD11b+CD103− DCs (**c**) on day 2. Groups were compared to WT treated with papain (PAP).

(**d**) CCL17$^+$ DC populations were quantified in the lungs of naive (Ctrl) or papain-treated (PAP) WT BMT and *Rora$^{sg/sg}$* BMT mice on day 2.

(**e–f**) CCL17$^+$CD11b$^+$CD103$^-$ DCs were quantified in the lungs (**e**) and CCL17 concentrations were measured in the BAL (**f**) of WT and iCOS-T mice treated as indicated (Supplementary Fig. 4a).

(**g**) Co-culture supernatants of antigen-experienced OTII-transgenic CD44$^+$CD4$^+$ T cells, plus WT DCs and/or ILC2s were analyzed for CCL17.

Data are representative of at least two independent experiments per group, containing at least three animals or cultures. Individual points indicate individual animals in (**c**). Mean values ± SEM are indicated in b–g. p $\leq$ 0.05 =*, p $\leq$ 0.01 = **, p $\leq$ 0.001 = ***, p $\leq$ 0.0001 = ****.
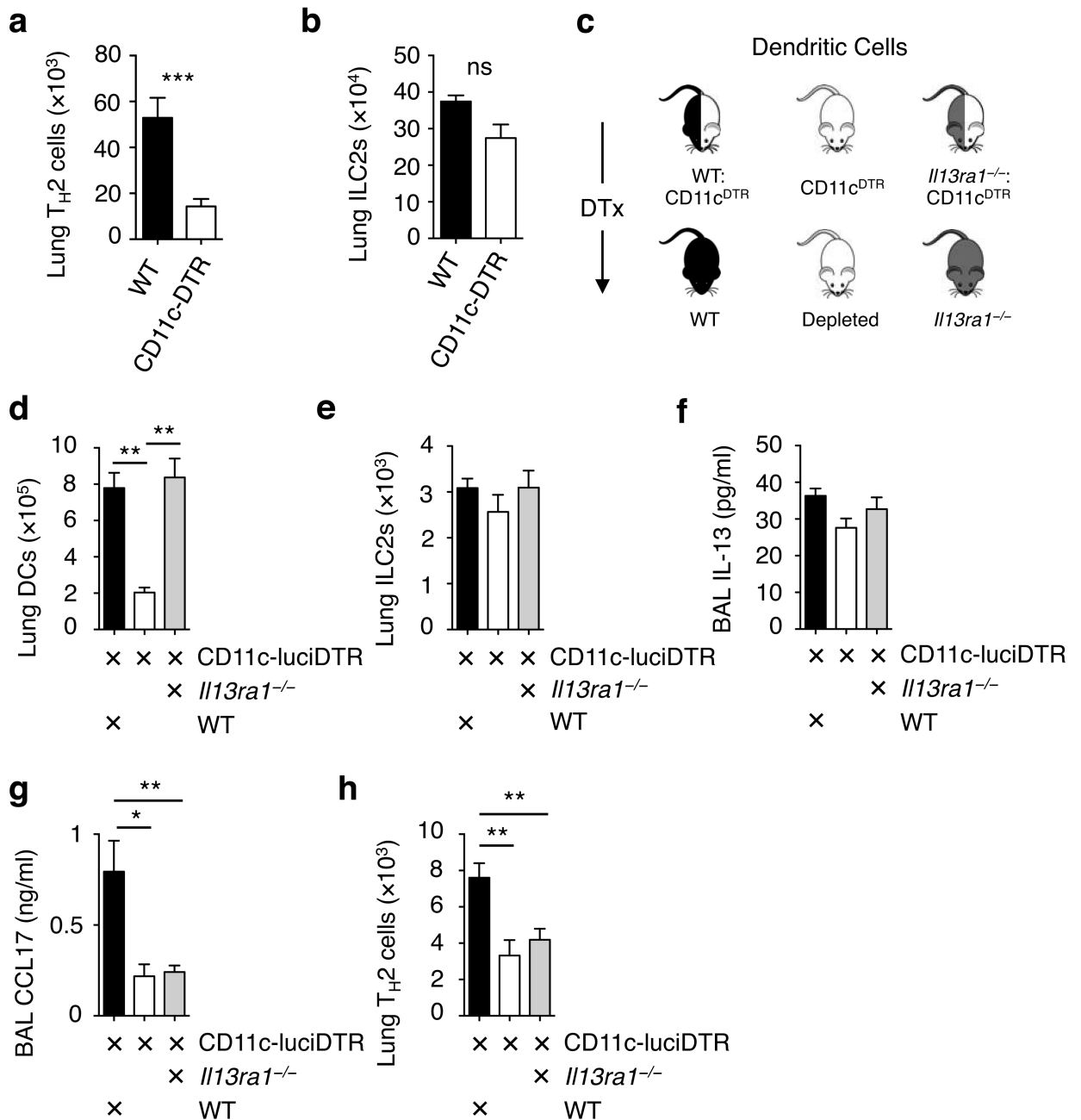
Fig. 6. IL-13 is critical for DC-mediated memory T$_H$2 cell recruitment after antigen-re-challenge
(**a–b**) CD11c-DTR mice were treated as indicated (Supplementary Fig. 6a), followed by the quantification of lung T$_H$2 cells (**a**) or ILC2s (**b**) using staining similar to Fig. 1c. (**c–h**) Bone marrow-chimeric mice were sensitized and challenged as described (Supplementary Fig. 6f, g) and selectively depleted of CD11c-LuciDTR DCs (**c**). The number of DCs (**d**), ILC2s (**e**) or T$_H$2 cells (**h**) in lung tissue was quantified following re-challenge. Concentrations of IL-13 (**f**) and CCL17 (**g**) in the BAL were measured.

Data are representative of at least two independent experiments per group, containing at least three animals (a–b), or at least five animals per group (c–h). Mean values ± SEM are indicated in a–b and d–h. ns = not significant, p 0.05 =*, p 0.01 = **, p 0.001 = ***.
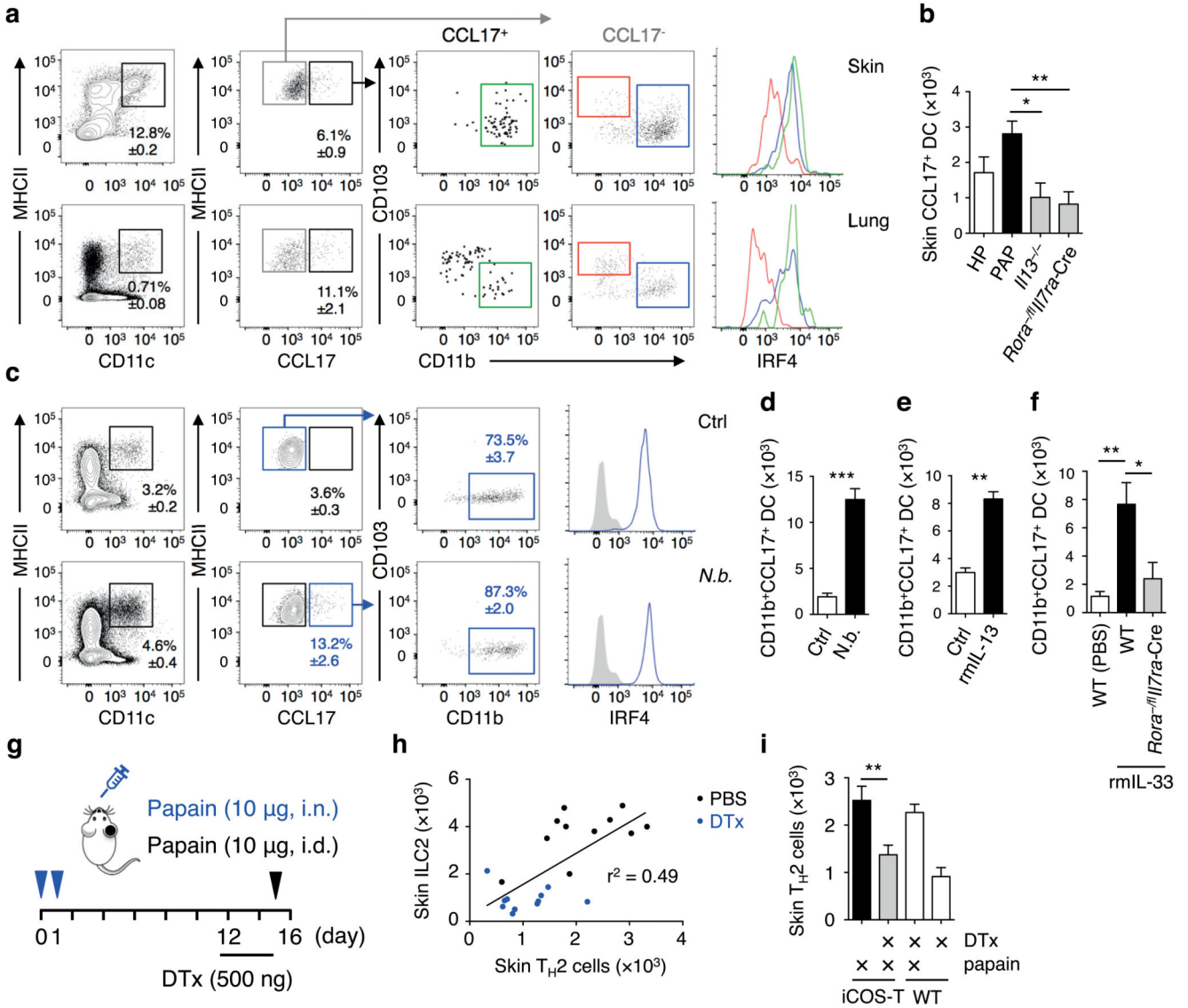
Fig. 7. ILC2 are important for skin and peritoneal CCL17+ DC expansion and memory T_H2 cell recruitment to the skin

(**a**) Naive mouse live CD45+B220−CD11c+MHCII+ DCs were analyzed for CCL17, CD11b, CD103 and IRF4 expression in the lung and skin. IRF4 expression of colored-matched gated populations is indicated in the histogram.

(**b**) WT, $Il13^{-/-}$ or $Rora^{-/fl}Il7ra$-Cre mice were injected intradermally into the ear with 10 μg of papain or HP (WT mice) on days 0 and 1, followed by quantification of CCL17+ DCs per ear on day 2.

(**c–d**) *Nippostrongylus brasiliensis* (*N.b.*) infected or uninfected (Ctrl) WT mice were analyzed on day 6 post infection for CCL17 expression in peritoneal live CD45+B220−CD11c+MHCII+ DCs as indicated. CCL17+ DCs were subsequently analyzed for CD11b, CD103 and IRF4 expression; the gray histogram depicts the FMO control for IRF4 staining (**c**). The total number of peritoneal CCL17+CD11b+CD103− DCs was quantified (**d**).

(**e**) IL-13 or PBS (Ctrl) was injected intraperitoneally on day 0 in WT mice followed by quantification of peritoneal CCL17$^+$CD11b$^+$CD103$^-$ DCs on day 1.

(**f**) IL-33 was administered intraperitoneally to WT and *Rora$^{-/fl}$Il7ra*-Cre mice, or PBS to WT mice (Ctrl) on day 0, followed by quantification of peritoneal CCL17$^+$CD11b$^+$CD103$^-$ DCs on day 1.

(**g**) WT or iCOS-T mice were sensitized intranasally on days 0 and 1 with papain (10 μg), followed by intradermal re-challenge on day 15.

(**h**) iCOS-T mice, treated as in g, were administered with DTx (blue) or PBS (black), followed by quantification of ILC2 (Live CD45$^+$lineage$^-$CD127$^+$GATA3$^+$) and T$_H$2 cells (Live CD45$^+$ CD3$^+$CD4$^+$Foxp3$^-$GATA3$^+$) in the right ear on day 16.

(**i**) iCOS-T and WT mice were sensitized as in g, and treated with DTx and re-challenged intradermally as indicated. The total number of T$_H$2 cells was quantified in the right ears. Data are representative of at least two independent experiments per group, containing at least three animals. Individual points indicate individual animals in (**h**). Mean values ± SEM are indicated in b, d–f and i, Pearson's test was performed in (**h**). p ≤ 0.05 =*, p ≤ 0.01 = **, p ≤ 0.001 = ***.