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Type 2 Innate Lymphoid Cells Drive CD4+ Th2 Cell Responses

Ananda S. Mirchandani,* Anne-Gaëlle Besnard,* Edwin Yip,* Charlotte Scott,* Calum C. Bain,* Vuk Cerovic,* Robert J. Salmond,*† and Foo Y. Liew*§,†,‡

CD4+ T cells have long been grouped into distinct helper subsets on the basis of their cytokine-secretion profile. In recent years, several subsets of innate lymphoid cell have been described as key producers of these same Th-associated cytokines. However, the functional relationship between Th cells and innate lymphoid cells (ILCs) remains unclear. We show in this study that lineage-negative ST2+ICOS+CD45− type 2 ILCs and CD4+ T cells can potently stimulate each other’s function via distinct mechanisms. CD4+ T cell provision of IL-2 stimulates type 2 cytokine production by type 2 ILCs. By contrast, type 2 ILCs modulate naive T cell activation in a cell contact-dependent manner, favoring Th2 while suppressing Th1 differentiation. Furthermore, a proportion of type 2 ILCs express MHC class II and can present peptide Ag in vitro. Importantly, cotransfer experiments show that type 2 ILCs also can boost CD4+ T cell responses to Ag in vivo. The Journal of Immunology, 2014, 192: 2442–2448.

The ability of CD4+ Th cells to dictate the response to infection by secreting cytokines remains a paradigm in immunology. In this regard, Th cells can be divided into at least four distinct subsets on the basis of their cytokine-secretion profiles: Th1 cells that produce IL-2, IFN-γ, and lymphotxin-α; Th2 cells characterized by production of IL-4, IL-5, IL-9, and IL-13; Th17 cells that produce IL-17; and regulatory T cells that produce IL-10, IL-35, and TGF-β. Thus, the ability of naive T cells to differentiate into distinct Th lineages and the roles of Th subsets and individual cytokines in infection, allergy, and autoimmunity have been the subject of several decades of intense research. Interestingly, it has become clear that several distinct classes of innate lymphoid cells (ILCs) may function as an early source of these same cytokines during the immune response (reviewed in Refs. 1–4). Furthermore, a nomenclature for ILC has been proposed in which ILC1, ILC2, and ILC3 correspond to the various innate populations characterized by their production of type 1−, type 2−, and type 17−-associated cytokines, respectively (5).

ILC2 are present in the gut and airways of mice and are characterized by their lack of expression of lineage-defining cell surface markers (Lin−) and expression of CD45, IL-17R (a component of the IL-25R), the IL-33R ST2 (ICOS (CD278), and Thy1 (6–8).

Materials and Methods

Mice

ST2−/− mice (17) were a kind gift of Dr. A McKenzie (Medical Research Council, Laboratory of Molecular Biology, Cambridge, U.K.) and were backcrossed to the BALB/c genetic background. BALB/c mice were purchased from Harlan UK. DO11.10 TCR-transgenic mice were a kind gift of Prof. P. Garside (University of Glasgow). All mice were maintained in the Glasgow Biomedical Research Centre small animal unit, and procedures were performed according to United Kingdom Home Office regulations.

Cell preparation

BALB/c mice were inoculated i.n. with 1 μg rIL-33 (BioLegend) on five consecutive days. Lung tissue was digested, and ILC2 were sorted to >98% purity using a FACS cell sorter (FACS Aria; BD Biosciences) (Supplemental

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As described previously (15), ILC2 were defined as CD45^+ICOS^+ ST2^+ lymphoid cells negative for expression of lineage (CD3e, CD11c, FcεR1, CD11b, B220) markers. Naïve T cells were sorted by FACS from lymph nodes (LNs) of ST2^−/− mice as CD4^+CD44^low, whereas CD4^+ cells from LNs and spleens of DO11.10 mice were sorted using an AutoMACS (Miltenyi Biotec).

Coculture and Transwell experiments

Flat-bottom tissue culture plates were coated with 1.5 μg/ml anti-CD3e and 3 μg/ml anti-CD28 (both from BD Biosciences). ILC2 and naïve CD4^+ T cells (1 × 10^5) were cultured alone or in combination (at a 1:1 ratio) in complete RPMI 1640 (Life Technologies) for 3 d. In some experiments, ILC2 or T cells were loaded with Cell Tracker 647 Dye or CFSE (eBiology) prior to coculture, and cell division was assessed by FACS analysis of dye dilution. Where indicated, 10 ng/ml IL-12 (PeproTech), 10 μg/ml anti–IL-2 mAb (BD Pharmingen), or 20 μg/ml anti–MHC class II mAb (eBioscience) was added. In coculture experiments, 0.4-μm Transwell porous 96-well plates (Corning Life Sciences) were coated with anti-CD3/28, as described above. For restimulation, cells were cultured in RPMI 1640 containing PMA (50 ng/ml) and ionomycin (0.9 nM) (PMA/I) (both from Sigma-Aldrich). GolgiStop (6.7 μl/ml; BD Biosciences) was added for the last 4 h of the 6-h stimulation, and cells were fixed and permeabilized in Cytofix/Cytoperm (BD Biosciences). Levels of intracellular cytokines were measured on an LSR II, FACSCalibur (BD Biosciences), or Cyan flow cytometer (Beckman Coulter). Fluorescent-conjugated Abs were from eBioscience, BD Pharmingen, BioLegend, or MD Biosciences. In studies of Ag-specific responses, DO11.10 cells were cultured alone or in coculture with either ILC2 or irradiated splenocytes at a 1:1 ratio in the presence or absence of OVA_{323–339} peptide, as indicated.

DO11.10/ILC2 cotransfer experiment

DO11.10 CD4^+ T cells, with or without ILC2, were injected into the tail veins of ST2^−/− BALB/c mice. Mice were immediately inoculated i.n. with 1 μg IL-33 and 100 μg OVA. After 5 d, mice were sacrificed, and lungs and mediastinal LNs (mLNs) were removed. FACS analysis of digested lung tissue and mLN cells was performed as described above. In restimulation assays, 1.5 × 10^5 mLN cells were cultured for 3 d with OVA_{323–339} peptide at concentrations stated in the figure legends. Levels of cytokines in culture supernatants were determined by ELISA using Abs from BD Pharmingen or eBioscience, according to the manufacturer’s instructions.

Statistical analysis

Two-tailed Student t tests were performed using Prism software.

Results

**CD4^+ T cell–derived IL-2 drives ILC2 cytokine production**

We sought to determine the reciprocal effects of CD4^+ Th cells and ILC2 in coculture experiments. Lin^− CD45^+ST2^+ICOS^+ ILC2 were expanded in vivo by i.n. administration of IL-33 and sorted by flow cytometry from the lungs of BALB/c mice (Supplemental Fig. 1A, 1B). ILC2 were further characterized by staining for a panel of cell surface Ags. Thus, sorted lung ILC2 expressed high levels of CD127, CD44, CD25, and MHC class I; in addition to their lack of expression of lineage-defining markers, they did not express CD40, CD62L, or CD49b (Supplemental Fig. 1D). ILC2 were cultured in the presence or absence of naïve CD44^−/low CD4^+ T cells (1:1 ratio ILC2/T cell) from BALB/c ST2^−/− mice, allowing easy discrimination of Th cells and ILC by FACS analysis of expression of CD4 and ST2, respectively. ILC2 cultured alone for 3 d on anti-CD3/CD28–coated plates showed reduced viability and did not proliferate, as indicated by a lack of dilution of CFSE (Fig. 1A). By contrast, ILC2 cultured in the presence of CD3/CD28-stimulated CD4^+ T cells typically went through several rounds of division, as indicated by FACS analysis of CFSE dilution (Fig. 1B). Furthermore, ILC2 cultured alone produced only low levels of IL-4 and IL-5 upon PMA/I restimulation (Fig. 1C), whereas ILC2 from the CD4^+ T cell cocultures produced high levels of IL-4, IL-5 (Fig. 1D), and IL-13 (Supplemental Fig. 2). The ability of CD4^+ T cells to stimulate ILC2 responses required their TCR stimulation, but it was not diminished by separation of ILC2 and T cells by Transwell (Fig. 1E). By contrast, a neutralizing IL-2 mAb markedly impaired Th cell–dependent ILC2 responses (Fig. 1F–H).

To assess direct effects of IL-2, ILC2 were cultured alone with recombinant cytokine. The addition of IL-2 potently induced ILC2 proliferation, as assessed by ^[3]H thymidine incorporation (Supplemental Fig. 3A). Furthermore, levels of IL-5 and IL-13 in culture supernatants were markedly elevated following IL-2 stimulation of ILC2 (Supplemental Fig. 3B). Previous data from Stockinger and colleagues (16) suggested that IL-2 was important to drive ILC2 IL-9 production. IL-9 production is transient, and we did not detect production of this cytokine by either CD4^+
Thus proportions of IL-4+ and IL-5+ CD4+ T cells were enhanced even when cells were cultured in the presence of Th1-driving IL-12. ILC2 to enhance T cell type 2 cytokine production was retained, cells were separated in Transwell (Fig. 3C). Finally, the ability of ILC2 on Th1 cell differentiation was abrogated when addition of exogenous IL-12 (Fig. 3B, 3C). Furthermore, the inhibitory action of ILC2 on Th1 cell differentiation did not require their stimulation via addition of exogenous IL-33 (data not shown). Importantly, separation by Transwell completely abrogated the Th2-stimulating effects of ILC2 on CD4+ T cell differentiation, indicating that a contact-dependent mechanism was involved (Fig. 2D–F).

Classically, the induction of Th1 or Th2 responses is regarded as mutually exclusive with Th1-associated cytokines and transcription factors known to antagonize Th2 responses and vice versa (18). Therefore, it was important to determine the effects of ILC2 on naive CD4+ T cells in a Th1-promoting milieu. Following CD3/28 stimulation of naive CD4+ T cells for 3 d in the absence of ILC2 or exogenous cytokines, 3–5% of cells produced IFN-γ (Fig. 3A). Addition of IL-12 to CD4+ T cell cultures increased proportions of IFN-γ-producing cells ~10-fold. Interestingly, the proportions of IFN-γ+ Th cells were reduced by 50–70% when CD4+ T cells were cultured in the presence of ILC2, irrespective of the addition of exogenous IL-12 (Fig. 3B, 3C). Furthermore, the inhibitory effect of ILC2 on Th1 cell differentiation was abrogated when cells were separated in Transwell (Fig. 3C). Finally, the ability of ILC2 to enhance T cell type 2 cytokine production was retained, even when cells were cultured in the presence of Th1-driving IL-12. Thus proportions of IL-4+ and IL-5+ CD4+ T cells were enhanced to an equivalent level in cocultures, irrespective of the addition of IL-12 (Fig. 3D). Together, these data indicate that ILC2 enhance the differentiation of naive CD4+ T cells to a Th2 phenotype while inhibiting Th1 differentiation in a contact-dependent manner.

ILC2 can act as APCs

In recent years, the identity of the APCs required for induction of Th2 responses has been the subject of intense investigation. Although conventional DCs are likely to be essential for induction of type 2 responses in vivo (19, 20), an APC function of basophils was reported to be involved in the induction of Th2 responses during helminth infection (21) and in allergy (22). Previously, it was shown that ILC2 expressed transcripts for MHC class II, and a proportion of these cells expressed these molecules on their cell surface (7). FACS analysis confirmed that a proportion of lung ILC2 expressed high levels of MHC class II molecules (Fig. 4A). This raised the possibility that ILC2 might also act as APCs. ILC2 were FACs sorted to >98% purity (Supplemental Fig. 1B), with care taken to remove conventional APC populations by the inclusion of CD11c, CD11b, and B220 mAbs in the Lin- sorting strategy. CD4+ T cells from DO11.10 TCR-transgenic mice also were sorted to >98% purity (Supplemental Fig. 1C), labeled with Cell Proliferation 647 dye, and cultured for 3 d with whole OVA or cognate OVA233-339 peptide in the

FIGURE 2. ILC2 induce CD4+ T cell Th2 cytokine production in a contact-dependent manner. FACS graphs show dilution of Cell Proliferation 647 dye by gated CD4+ T cells cultured alone or with ILC2 (at a 1:1 ratio) in the absence of TCR stimulation (A) or on anti-CD3/28–coated plates (B). (C) FACS dot plots represent levels of intracellular cytokine expression by gated CD4+ T cells upon PMA/I restimulation following culture alone or in the presence of ILC2 for 3 d. Proportions of IL-4+ (D), IL-5+ (E), and IL-13+ (F) CD4+ T cells following 3 d of culture on anti-CD3/28–coated plates alone (CD4) or with ILC2 in coculture (Co-cult) or in Transwell (TW). Data represent mean values of three replicate samples, and bars represent SD, from one of seven (IL-4, IL-5) or two (IL-13) repeated experiments. ***p < 0.001.
presence or absence of ILC2. DO11.10 T cells incubated with peptide in the absence of ILC2 failed to divide (Fig. 4B), whereas CD3/28 stimulation induced high levels of T cell proliferation, as shown by dilution of Cell Proliferation dye (Fig. 4C). Interestingly, peptide-pulsed, but not whole-OVA–pulsed, ILC2 stimulated DO11.10 T cell proliferation (Fig. 4D, 4E). Additional experiments compared the ability of ILC2 and irradiated splenocytes to induce Ag-specific T cell responses. Levels of DO11.10 T cell proliferation induced by peptide-loaded splenocytes were higher than those stimulated by ILC2 (Supplemental Fig. 4A). By contrast, when DO11.10 T cells were restimulated with PMA/I, a higher proportion of T cells from cultures in which ILC2 had been used as APCs was capable of producing IL-4, and with a trend for higher IL-5 production (Fig. 4F, 4G, Supplemental Fig. 4B). These data indicate that ILC2 can present peptide-Ag to induce CD4+ T cell proliferation and that, in contrast to conventional APCs present in spleen, ILC2 preferentially drive a Th2-type response.

**MHC class II expression is critical for Ag presentation but not Th2-driving effects of ILC2**

We next assessed the role of MHC class II expression in the ability of ILC2 to induce T cell responses. When added to ILC2/DO11.10 T cell cocultures, blocking MHC class II mAb, but not isotype-control mAb, completely abrogated T cell proliferation (Fig. 5A). These data indicate that, akin to conventional APCs, ILC2 can induce T cell proliferation via MHC class II–mediated peptide presentation. We then addressed the role of MHC class II in the Th2-polarizing effects of ILC2. Because TCR stimulation is critical for the ability of naive T cells to differentiate to effector Th cells, and blocking MHC class II mAb prevents peptide–TCR engagement, leading to DO11.10 cell activation, for these experiments we returned to using polyclonal anti-CD3/CD28 stimulation. As before, higher proportions of polyclonal CD4+ T cells produced Th2 cytokines following CD3/28 stimulation in the presence of ILC2 compared with control cultures (Fig. 5B). Importantly,
addition of blocking MHC class II mAb to these cultures did not reverse the ability of ILC2 to drive Th2 cell differentiation. Therefore, and in contrast to their APC function, the ability of ILC2 to enhance Th2 differentiation by polyclonally activated CD4+ T cells does not require MHC class II expression.

**ILC2 enhance CD4+ T cell responses in vivo**

Finally, the role of ILC2 in stimulating T cell responses in vivo was assessed using a cotransfer model. DO11.10 CD4+ T cells were transferred i.v. to ST2+ mice in the presence or absence of ILC2. Mice were inoculated i.n. with OVA and IL-33 to stimulate both transferred cell populations. ST2+ mice were used as recipients to preclude effects of endogenous ILC2 and additional IL-33–responsive cell populations. Mice were sacrificed 5 d after cell transfer, and cell populations in the lungs and lung-draining mLNs were analyzed. By day 5, transferred ST2+ ILC2 were found only in lung tissues (data not shown). Importantly, the proportion of DO11.10 T cells was 18-fold higher in the lung tissues of recipient mice that received both T cells and ILC2 compared with those given T cells alone (Fig. 6A, 6B). This also was reflected in more total cells and DO11.10 cells recovered from lung tissue in mice receiving both DO11.10 T cells and ILC2 but not DO11.10 cells alone (Fig. 6F). Strikingly, peptide restimulation induced high levels of IL-13 in mLN cultures from mice that received both DO11.10 cells and ILC2 but not DO11.10 cells alone (Fig. 6F). Surprisingly, levels of peptide-induced IFN-γ also were marginally enhanced at high peptide concentration in cultures from the DO11.10/ILC2 cotransfer (Fig. 6E). Taken together, these data indicate that ILC2 enhance T cell responses to Ag in vivo.

**Discussion**

The data presented in this article describe a novel and marked ability of ILC2 to influence CD4+ T cell differentiation and activation both in vitro and in vivo. Importantly, the presence of ILC2 appears to particularly favor the induction of the corresponding CD4+ T cell Th2 phenotype. Furthermore, CD4+ T cells can reciprocally enhance ILC2 proliferation and cytokine production via the secretion of IL-2.

Transwell experiments indicate that the effects of ILC2 on naive CD4+ T cell differentiation require cell–cell contact or at least close proximity. This effect is apparently independent of the cytokine milieu, because the driving of Th2 cells by ILC2 in the present system is not affected by the presence of IL-12 and does not require the addition of IL-4, the canonical cytokine for the polarization of Th2. However, the polarization of Th2 cells by ILC2 requires the concomitant activation of TCR, either in the form of polyclonal (anti-CD3/CD28) stimulation or specific Ag (OVA peptide). The suppression of Th1 polarization by ILC2 in vitro is intriguing. However, the suppression of Th1 is less evident in vivo. This suggests that the suppression of Th1 polarization could be
FIGURE 5. MHC class II is required for Ag presentation by ILC2. (A) The bar graph shows proportions of divided DO11.10 T cells, as assessed by FACS analysis, following 3 d of culture either alone in complete medium (CM), with or without OVA-peptide (pep), or in the presence of ILC2 with the addition of blocking MHC class II mAb (α-MHC) or isotype control (iso). (B) Intracellular FACS analysis of IL-4, IL-5, and IL-13 production by polyclonal BALB/c CD4+ T cells restimulated with PMA/I, following coculture with ILC2, with or without blocking MHC class II mAb, on anti-CD3/28–coated plates. Values represent means of three replicate samples ± SD from one of three repeated experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

due to the ratio of Th2/Th1 in the culture system rather than a direct suppressive effect of ILC2 on Th1.

Our data suggest that ILC2 might also directly affect T cell activation through Ag presentation by MHC class II, as demonstrated by the ability of ILC2 to present peptide and, thereby, induce DO11.10 T cell proliferation. Importantly, blocking MHC class II interactions completely prevented the ability of ILC2 to stimulate DO11.10 T cell proliferation. By contrast, when T cells were stimulated using anti-CD3/28, blocking MHC class II did not prevent the ability of ILC2 to promote Th2 differentiation. Together, these data indicate that ILC2 might influence CD4+ T cell responses by at least two distinct mechanisms: MHC class II–dependent Ag presentation, and, in the presence of additional TCR stimuli, ILC2 can drive Th2 differentiation via an MHC class II–independent contact-dependent mechanism. Although the precise molecular mechanism of the enhanced Th2 polarization by ILC2 is unknown, experiments showed that blocking Abs to ICAM-1 (eBioscience) and OX40 (R&D Systems) did not impact ILC2-dependent Th2 differentiation (data not shown), suggesting that other cell surface proteins are involved in these processes. The nature of the Th2-promoting contact-dependent ILC2–T cell interaction remains to be explored.

Recent data suggested that ILC3 also may modulate T cell responses in the gut (23). This effect also appeared to involve an MHC class II–dependent mechanism; however, in contrast to the data reported in this article, ILC3 appeared to limit, rather than promote, Th17 cell activation (24). Thus, different classes of ILC can interact with and directly modulate the activation of T cells in vivo. It will be interesting to assess whether ILC3 might also enhance T cell activation under some circumstances, as is the case for ILC2 described in this article.

In addition to an effect of ILC2 on T cell activation, our data show that IL-2 production by activated T cells can drive ILC2 responses. Thus, IL-2 induces ILC2 proliferation and enhances their production of IL-5 and IL-13. Furthermore, while this manuscript was under revision, it was reported that injection of RAG−/− mice with IL-2–IL-2 mAb complexes stimulated ILC2 proliferation in vivo (25). Previously, a stimulatory function of IL-2 in the induction of ILC2 IL-9 production was reported (16). The temporal activation of ILC2 and CD4+ T cells is likely to be different during in vivo immune responses. For example, ILC2 are activated within a matter of hours following i.n. challenge of mice with the allergen Alternaria alternata (12), whereas T cell responses typically follow several days later (26). It is possible that early activation of ILC2 may contribute to the later development of a CD4+ Th2 response, whereas T cell production of IL-2 may reinforce this response in a feedback loop by further activating ILC2.

IL-33 has been established as a major driver of Th2 responses in vitro and in vivo (27). The precise mechanism involved is not clear, because naïve T cells express little or no detectable IL-33R. Our results provide direct evidence that IL-33 might contribute to Th2 polarization in vivo via the induction of ILC2 that express a high density of IL-33R. It should be noted that, although IL-33 is critical for expansion of ILC2 in vivo, and was used to generate

FIGURE 6. ILC2 enhance T cell responses to Ag in vivo. FACS dot plots show proportions of donor KJ-126* DO11.10 T cells within gated CD4+ cells in lung homogenates of recipient mice that received DO11.10 cells alone (A) or DO11.10 cells + ILC2 (B). Total numbers of cells in lung tissue (C) and DO11.10 cells in lung tissue (D) and mLNs (E) of recipient mice that received DO11.10 cells alone or DO11.10 cells + ILC2. Values represent mean ± SD (n = 4). (F) Levels of IL-2, IL-13, and IFN-γ in culture supernatants following restimulation of mLN cells from recipient mice with complete media (CM) or OVA-peptide. Values represent mean ± SD (n = 3). Data are representative of three repeated experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
ILCs in the current work, the addition of exogenous IL-33 was not required for the Th2-promoting effects of ILC2 in the in vitro coculture experiments.

Data presented in this article suggest that ILC2 may have roles in modulating immune responses, in addition to their well-described function as key producers of cytokines. Recent evidence showed that IL-5 and IL-13 production by ILC2 is important for the re-activation of macrophages in visceral adipose tissue (29). Therefore, IL-5 and IL-13 production by ILC2 is important for the re-modulating immune responses, in addition to their well-described function in the current work, the addition of exogenous IL-33 was not required for the Th2-promoting effects of ILC2 in the in vitro coculture experiments.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. (A) Gating strategy for FACS sorting of lung ILC2. Live, singlet CD45+Lineage-ICOS+ST2+ ILC2 from digested lung tissue were sorted to purity of >98% (B), as indicated. Typical purity of CD4+ DO11.10 T cells used in the study (C). Gray fill = pre-sorted cells, black line = post-sort T cells. (D) Expression of cell surface markers by FACS-sorted lung CD45+Lineage-ICOS+ST2+ ILC2. Filled gray histograms represent isotype control Ab, black lines represent level of staining with the specific mAbs.
Supplementary Figure 2. Co-culture with activated T cells enhances ILC2 IL-13 production. The histogram represents levels of intracellular IL-13 expression by ILC2 after PMA/I restimulation following 3 d culture alone (gray fill) or in the presence of CD3/28-stimulated CD4+ T cells (bold line). Data are representative of 3 repeated experiments.
Supplementary Figure 3. IL-2 directly induces proliferation and enhances Type 2 cytokine production of ILC2 in vitro. (A) Proliferation of ILC2 was assessed by \(^3\)H-thymidine incorporation during the last 16 h of a 72h culture period either in complete medium alone (CM) or with 20 ng/ml IL-2. (B) Levels of IL-5 and IL-13 in culture supernatant of ILC2 cells cultured in the presence or absence of IL-2 were measured by ELISA. Values represent means ± SD (n=3) and data represent 1 of 3 repeated experiments. *** p<0.001.
Supplementary Figure 4. Comparison of ILC2 and irradiated splenocytes as APC. (A) Levels of proliferation of DO11.10 T cells were assessed by dilution of AF647 proliferation dye following 3d culture in the presence or absence of ILC2 or spleen cells ± 5 μM pOva. (B) FACS analysis of levels of intracellular IL-4 and IL-5 following PMA/ionomycin restimulation of DO11.10 T cells cultured for 3d with ILC or splenocytes. Data are representative of 3 replicate cultures within one of two repeated experiments.