Type 2 Innate Lymphoid Cells Drive CD4⁺ Th2 Cell Responses

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Type 2 Innate Lymphoid Cells Drive CD4+ Th2 Cell Responses

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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 profiles: Th1 cells that produce IL-2, IFN-γ, and lymphotoxin-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 cytokine-secretion profiles: Th1 cells that produce IL-2, IFN-γ, and lymphotoxin-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 cytokine-secretion profiles: Th1 cells that produce IL-2, IFN-γ, and lymphotoxin-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.

An important question remains as to how ILC and Th cells functionally interact. Experiments showed that IL-2, a predominantly T cell–derived cytokine, can influence ILC2 cytokine production (16), suggesting that ILC and Th cells might cooperate during the immune response. However, activation of ILC generally precedes that of Th cells, and whether and how ILCs influence Th cell activation and differentiation are unknown. In the current study, we show that, although T cell–derived IL-2 drives ILC2 responses, the presence of ILC2 also influences the differentiation of naive CD4 T cells to a Th2 phenotype in a contact-dependent manner. Furthermore, a proportion of ILC2 express MHC class II molecules and can present peptide Ag to TCR-transgenic Th cells in vitro. Finally, we show that ILC2 enhance the response of Th cells to i.n. Ag, indicating an important role for ILC in both the polarization and the magnitude of the CD4+ Th cell response in vivo.

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...
Coculture and Transwell experiments

Flat-bottom tissue culture plates were coated with 1.5 μg/ml anti-CD3ε and 3 μg/ml anti-CD28 (both from BD Biosciences). ILC2 and naive 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 CellTracker 647 Dye or CFSE (eBioscience) prior to coculture, and cell division was assessed by FACS analysis of dye dilution. Where indicated, 10 ng/ml rIL-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 μg/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, FACS Calibur (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 × 10^6 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 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 naive CD44low 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 CD28, 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).

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 [3H]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+
T cells or ILC2 in coculture. Therefore, our data indicate that, in addition to the previously described effects on IL-9 production, IL-2 secreted by activated T cells directly induces ILC2 proliferation and enhances their ability to produce other type 2 cytokines.

**ILC2 induce Th2 polarization and repress Th1 responses**

We then sought to determine the effect of ILC2 on CD4+ T cell responses. ILC2 had no effect on resting naïve ST2+/− CD4+ T cells in coculture, which did not proliferate either alone or in the presence of ILC2 over the course of the 3-d culture period, as assessed by FACS analysis (Fig. 2A). When stimulated on anti-CD3/28-coated plates, CD4+ T cells diluted proliferation dye 647; however, the presence of ILC2 had minimal effect on the levels of T cell proliferation (Fig. 2B). Furthermore, FACS analysis showed that CD4+ T cells, stimulated alone for 3 d on anti-CD3/28-coated plates, produced negligible levels of type 2 cytokines upon PMA/I restimulation (Fig. 2C). Remarkably, the presence of ILC2 in coculture markedly enhanced the proportions of activated CD4+ T cells capable of producing IL-4 and, in particular, IL-5 and IL-13 upon restimulation (Fig. 2C–F). This ability of ILC2 to influence T 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 naïve CD4+ T cells in a Th1-promoting milieu. Following CD3/28 stimulation of naïve 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 naïve 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 OVA323-339 peptide in the
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 present in mLNs in mice that received ILC2 (Fig. 6E). These data suggest that cotransferred ILC2 enhance T cell migration to, and/or proliferation in, lung tissue and lymphocyte accumulation in lung-draining mLNs in response to Ag.

To determine the impact of ILC2 on Th cell polarization in vivo, mLN cells from recipient mice were cultured in the presence of OVA323–339 for 3 d, and levels of cytokines in supernatant were measured by ELISA. Peptide-induced levels of IL-2 were significantly elevated in cultures from mice receiving both T cells and ILC2 (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
T cells within gated CD4+ cells in lung and replicate samples of donor KJ-126 + DO11.10 responses to Ag in vivo. FACS dot plots show ILC2 enhance T cell re-

ulated using anti-CD3/28, blocking MHC class II did not prevent DO11.10 T cell proliferation. By contrast, when T cells were stim-

ination through Ag presentation by MHC class II, as demon-

Our data suggest that ILC2 might also directly affect T cell


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 demonstr-

ated 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

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 mLN (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.
ILC2 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 recruitment and maintenance of eosinophils (28) and alternatively activated macrophages in visceral adipose tissue (29). Therefore, cytokine production and contact-dependent functions may act in concert to allow ILC2 to regulate other innate immune cell types and T cells, respectively. Importantly, to our knowledge, our data provide the first evidence that ILC2 function not only as a first line of defense via their early production of type 2 cytokines but also are important for the induction of the adaptive immune response.

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+Lin+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.