INNATE LYMPHOID CELLS

The ChAT-acetylcholine pathway promotes group 2 innate lymphoid cell responses and anti-helminth immunity

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Group 2 innate lymphoid cells (ILC2s) reside in multiple tissues, including lymphoid organs and barrier surfaces, and secrete type 2 cytokines including interleukin-5 (IL-5), IL-9, and IL-13. These cells participate in multiple physiological processes including allergic inflammation, tissue repair, metabolic homeostasis, and host defense against helminth infections. Recent studies indicate that neurotransmitters and neuropeptides can play an important role in regulating ILC2 responses; however, the mechanisms that underlie these processes in vivo remain incompletely defined. Here, we identify that activated ILC2s up-regulate choline acetyltransferase (ChAT)—the enzyme responsible for the biosynthesis of acetylcholine (ACh)—after infection with the helminth parasite Nippostrongylus brasiliensis or treatment with alarmins or cytokines including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). ILC2s also express acetylcholine receptors (AChRs), and ACh administration promotes ILC2 cytokine production and elicits expulsion of helminth infection. In accordance with this, ChAT deficiency in ILC2s leads to defective ILC2 responses and impaired immunity against helminth infection. Together, these results reveal a previously unrecognized role of the ChAT-ACh pathway in promoting type 2 innate immunity to helminth infection.

INTRODUCTION

Group 2 innate lymphoid cells (ILC2s) are potent producers of type 2 cytokines, including interleukin-5 (IL-5), IL-9, and IL-13, and participate in a wide range of physiological processes, including tissue homeostasis and repair, type 2 inflammation, metabolic homeostasis, and anti-helminth immunity (1–7). In addition to activation by cytokines and environmental factors, recent studies indicate that multiple neurotransmitters and neuropeptides are key regulators of divergent ILC2 responses (8–10), highlighting the close association between the nervous system and innate immunity at barrier surfaces.

For example, a subset of cholinergic neurons expresses the neuropeptide neuromedin U (NMU), directly activates ILC2s through the NMU receptor 1 (NMUR1), induces ILC2 proliferation and type 2 cytokine production, and accelerates helminth expulsion. However, NMU-activated ILC2s can also promote allergic inflammation under certain circumstances (11–13). In contrast, adrenergic neurons express the neurotransmitter norepinephrine (NE), inhibiting ILC2 responses, type 2 inflammation, and helminth expulsion by binding to β2-adrenergic receptors (β2ARs) on ILC2s (14). In addition, another neuropeptide calcitonin gene–related peptide (CGRP) binds to its receptor CALCRL/RAMP1, which is expressed in a subpopulation of ILC2s, antagonizing ILC2 activation, proliferation, and cytokine production, and suppressing allergic inflammation and anti-helminth immunity (15–17). Noxious sensory neurons (18, 19) and a subset of cholinergic neurons (15) can produce CGRP, as well as a subset of ILC2 themselves during inflammation (15–17), creating a negative feedback loop in type 2 innate immune responses.

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Fig. 1. Up-regulated ChAT-eGFP expression in ILC2s after N. brasiliensis infection. (A to F) Representative flow cytometry plots (A and D), population frequencies (B and E), and numbers (C and F) of ChAT+ ILC2s in the lung (A to C) and mLNs (D to F) of uninfected (Uninf.) or N. brasiliensis–infected (Inf.) ChATBAC-eGFP mice analyzed on day 7 after infection, gated on total ILC2s. Representative flow cytometry plot (G) and population frequencies (H) of inflammatory (ILC2INFLAM) red and natural (ILC2NAT) blue ILC2s in the mLNs of N. brasiliensis–infected ChATBAC-eGFP mice analyzed on day 7 after infection, gated on total ILC2s (G) or ChAT+ ILC2s (H). Data are representative of two independent experiments. n = 3 mice per group. Data are means ± SEM. **P < 0.01 and ***P < 0.001.
responses. Moreover, ILC2s up-regulate the expression of tryptophan hydroxylase 1 (Tph1; the rate-limiting enzyme in serotonin biosynthesis) upon activation with IL-33 and can secrete serotonin, and conditional deletion of Tph1 in lymphocytes results in increased susceptibility to helminth infection (20). However, whether ILC2s secrete other neurotransmitters or neuropeptides and potentially form a regulatory feedback loop to control ILC2 responses is unknown.

Acetylcholine (ACh) was the first neurotransmitter discovered in 1921 (21, 22). It is synthesized from the compounds choline and acetyl coenzyme A by the enzyme choline acetyltransferase (ChAT). ACh acts on two families of receptors—nicotinic ACh receptors (nAChRs) and muscarinic ACh receptors (mAChRs), both named by their agonists—nicotine and muscarine. nAChRs are pentameric Na+ ion channels, and their activation results in Na+ influx and excitation of the neuron (23). mAChRs are G protein–coupled receptors and contain five subtypes (M1 to M5). M1, M3, and M5 receptors are generally coupled to Gq/11 proteins to perform stimulatory functions, while M2 and M4 receptors are coupled to Gi/o proteins, leading to inhibitory effects (24). ACh in the nervous system works at neuronal synapses and neuromuscular junctions, mediating communication between neurons and neural communication to the muscles. The action of ACh is terminated by acetylcholinesterase (AChE) (25). Parasitic helminths such as *Nippostrongylus brasiliensis* are known to express AChE throughout their life cycles (26, 27), suggesting that this may be a mechanism of immune evasion used by this group of pathogens. However, whether the ChAT-ACh pathway plays a role in protective immunity against helminth infection has not been defined.

In this study, we demonstrate that activated ILC2s up-regulate ChAT expression in both lymphoid organs and mucosal tissues after *N. brasiliensis* infection or after treatment with alarmins or cytokines including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). ILC2s express several AChRs, which are also regulated by IL-25 and IL-33. In addition, treatment with ACh leads to elevated ILC2-derived IL-5 and IL-13 production both in vitro and in vivo, and enhanced eosinophil and goblet cell responses and accelerated expulsion of *N. brasiliensis* in vivo. In accordance with this, conditional deletion of ChAT in ILC2s results in defective cytokine production, impaired type 2 inflammation, and delayed protective immunity to *N. brasiliensis* infection. Together, these results reveal a previously unrecognized nonneuronal source of ACh that regulates ILC2 effector function and define a critical role for the ChAT-ACh pathway in promoting innate immunity and host defense against helminth infection.

**RESULTS**

**ILC2s up-regulate ChAT expression after helminth infection**

ILC2s play a critical role in promoting innate immunity against helminth infection (1, 3–6, 28–31). To study the role of the ChAT-ACh pathway in regulating ILC2 responses after helminth infection, we first infected ChAT<sup>BAC</sup>-eGFP (enhanced green fluorescent protein) reporter mice (32) with *N. brasiliensis* and analyzed the expression...
ILC2s up-regulate ChAT expression after IL-33 treatment

The alarmin cytokine, IL-33, is constitutively expressed in the nuclei of intestinal epithelial cells, endothelial cells, and fibroblasts; as well as in mast cells, macrophages, and dendritic cells during inflammation (2, 33); and in stromal cells of white adipose tissues (34, 35). When the small intestinal barrier is breached by N. brasiliensis, IL-33 is released by stressed or damaged intestinal epithelial cells, directly acts on the IL-33 receptor complex expressed on ILC2s, and is a key activator of ILC2-dependent innate immune responses against N. brasiliensis infection (2, 36, 37). To test whether IL-33 can directly affect ChAT expression in ILC2s, we sort-purified ILC2s from the small intestinal lamina propria of ChAT(BAC)<sup>eGFP</sup>-reporter mice, cultured them in vitro with or without IL-33, and analyzed the expression of ChAT-eGFP by flow cytometry. ILC2s cultured in the presence of IL-33 exhibited significantly higher percentage of ChAT-eGFP<sup>+</sup> cells and higher ChAT-eGFP level (measured by mean fluorescence intensity (MFI)) compared with ILC2s cultured with IL-2 and IL-7 alone (Fig. 3, A to C). Consistent with the in vitro data, intraperitoneal administration of IL-33 significantly enhanced the percentages and numbers of ChAT-eGFP<sup>+</sup> ILC2s in multiple organs of ChAT(BAC)<sup>eGFP</sup>-reporter mice, including the small intestine, lung, mLNs, and colon (Fig. 3, D to I, and fig. S3, A to F). Moreover, after IL-33 treatment, elevated ChAT-eGFP<sup>+</sup> ILC2 responses were observed in the small intestinal lamina propria, lung parenchyma, mLNs, and colon of ChAT(BAC)<sup>eGFP</sup>-reporter mouse by immunofluorescence microscopy compared with untreated mice (figs. S2, A to H, and S4, A and H). Together, these results suggest that the alarmin cytokine IL-33 can up-regulate ChAT expression in ILC2s.

To test whether other alarmins or cytokines also regulate ChAT expression in ILC2s, we treated ChAT(BAC)<sup>eGFP</sup>-reporter mice with IL-25 or TSLP intraperitoneally and found that both IL-25 and TSLP significantly increased the percentages of ChAT-eGFP<sup>+</sup> ILC2s in the mLNs, with a stronger effect in IL-25-treated mice compared with TSLP-treated mice (Fig. 3J). To test whether other models of Type 2 inflammation also regulate ChAT expression in ILC2s, we infected ChAT(BAC)<sup>eGFP</sup>-reporter mice with the helminth Trichuris muris intragastrically or treated them with the allergen Alternaria intranasally and found significantly higher percentages of ChAT-eGFP<sup>+</sup> ILC2s in the cecum of T. muris-infected mice compared with uninfected mice, as well as significantly higher percentages of ChAT-eGFP<sup>+</sup> ILC2s in the lung of Alternaria-treated mice compared with naïve mice (Fig. 3K and L). These data suggest that after exposure to helminths or allergens that trigger the release of IL-25, IL-33, or TSLP, the significant increase in ChAT expression in ILC2s in multiple tissues and organs is a conserved response to multiple stimuli.

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**ILC2s express ACh receptors**

Previous studies identified the expression and function of AChR subunits in immune cell populations, such as α7-nAChR in macrophages and α9-nAChR in B cells (38, 39). To examine whether ILC2s express AChR subunits, first, we screened the expression of all 21 AChR subunits in cultured small intestinal ILC2s by reverse transcription PCR (RT-PCR). ILC2s express Chrm4 and Chrm5, which are G protein–coupled muscarinic receptors, as well as the α subunits Chrna1, Chrna5, Chrna9, and Chrna10, and the β subunits Chrb1 and Chrb2, which form pentameric nicotinic ligand-gated channels (Fig. 4, A to D, and fig. S5, A to D). We confirmed the expression of these candidate AChRs in ex vivo sorted small intestinal ILC2s and lung ILC2s by qPCR or in cultured ILC2s by Western blot (Fig. 4E and fig. S5, E and F). To test whether IL-25 or IL-33 directly regulates the expression of these candidate AChRs in ILC2s, we used qPCR and compared their expression in cultured ILC2s with or without treatment with IL-25 or IL-33 compared with ILC2s cultured with IL-2 and IL-7 alone. Both IL-25 or IL-33 stimulation promoted the expression of Chrm4 and inhibited the expression of Chrns5, Chrna2, Chrna10, and Chrb2. However, IL-25 promoted the expression of Chrna5, Chrna9, and Chrb1, whereas IL-33 inhibited the expression of these three AChRs (Fig. 4, F to M). Collectively, these data indicate that AChRs are expressed on ILC2s and their expression levels are selectively regulated by IL-25 versus IL-33.

**ACh promotes ILC2 cytokine production in vitro**

To investigate whether ACh directly activates ILC2s, we sorted small intestinal ILC2s and incubated them in media with or without ACh for 4 hours in vitro in the presence of IL-2, IL-7, IL-25, and IL-33. We found that ACh potently activated ILC2s as measured by intracellular cytokine staining of IL-5 and IL-13 (Fig. 5, A to D). To investigate whether ACh alone activates ILC2s, we sorted small intestinal ILC2s from YetCre-13 [IL-13–YFP (yellow fluorescent protein) reporter] mice (6), incubated them in media with or without ACh for 4 hours in vitro, and observed that treatment with ACh alone could significantly activate ILC2s as measured by IL-13–YFP expression (Fig. 5, E and F), although to a greater extent when IL-2, IL-7, IL-25, and IL-33 were present (Fig. 5, G and H). Moreover, both ipratropium bromide (a pan-mAChR antagonist) and d-tubocurarine (a pan-nAChR antagonist) inhibited the effects of ACh on ILC2 IL-13–YFP expression (Fig. 5, I and J), suggesting that ACh may act through both mAChRs and nAChRs expressed on ILC2s to promote cytokine production.

**ACh stimulates ILC2s in vivo and promotes helminth expulsion**

To investigate whether ACh regulates ILC2-mediated innate immunity against *N. brasiliensis* infection in vivo, we administered ACh to *N. brasiliensis–*infected mice and examined the ILC2 responses at 7 days after infection. Control mice infected with *N. brasiliensis* exhibited typical ILC2 responses as expected, with IL-5 and IL-13 production (Fig. 6, A to G), and subsequent eosinophil recruitment (Fig. 6, H to L) and goblet cell hyperplasia (Fig. 6, M and N). ACh-treated mice exhibited significantly heightened ILC2 responses after *N. brasiliensis* infection, with higher numbers of total ILC2s (Fig. 6A), significantly increased percentages and numbers of IL-5–producing ILC2s (Fig. 6, B to D), and significantly increased numbers of IL-13–producing ILC2s (Fig. 6, E to G) in the lung.
compared with control mice. In accordance with increased numbers of IL-5–producing ILC2s, ACh-treated mice exhibited enhanced eosinophil responses, with increased percentages and numbers of total and activated eosinophils in the lung compared with control mice (Fig. 6, M and N). Associated with enhanced ILC2 responses, ACh-treated mice exhibited significantly lower helminth burden compared with control mice (Fig. 6O). Together, these results suggest that ACh may promote anti-helminth immunity by facilitating ILC2 responses and type 2 inflammation.

### ILC2-derived ACh is indispensable for optimal ILC2 responses and helminth expulsion

To further analyze the effects of ILC2-derived ACh on anti-helminth innate immune responses, we crossed Chat<sup>flox/flox</sup> mice (40) with Il7r<sup>Cre</sup> mice (14, 41) to generate Il7r<sup>Cre</sup>/Chat<sup>flx/flox</sup> (Chat<sup>Il7RΔ/Δ</sup>) mice. On day 7 of <i>N. brasiliensis</i> infection, Chat<sup>Il7RΔ/Δ</sup> mice exhibited lower numbers of ILC2s in the lung compared with control mice (Fig. 7A), with reduced percentages and numbers of IL-5– and IL-13–producing IL-2, IL-7, IL-25, IL-33, PMA, and ionomycin, as determined by intracellular cytokine staining. (E and F) Representative flow cytometry plots (G and H) and population frequencies (H) of IL-13–YFP<sup>+</sup> ILC2s after 4-hour incubation in medium with or without ACh. (I and J) Representative population frequencies of IL-13–YFP<sup>+</sup> ILC2s after 4-hour incubation in medium with or without ACh in the presence of IL-2, IL-7, IL-25, and IL-33. Data are representative of two independent experiments. n = 3 mice per group. Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

**Fig. 5. Increased ILC2 cytokine production after ACh treatment.** (A to D) Representative flow cytometry plots (A and C) and population frequencies (B and D) of IL-5<sup>+</sup> (A and B) and IL-13<sup>+</sup> (C and D) ILC2s after 4-hour incubation in medium with or without ACh, in the presence of IL-2, IL-7, IL-25, IL-33, PMA, and ionomycin, as determined by intracellular cytokine staining. (E and F) Representative flow cytometry plots (G and H) and population frequencies (H) of IL-13–YFP<sup>+</sup> ILC2s after 4-hour incubation in medium with or without ACh. (I and J) Representative population frequencies of IL-13–YFP<sup>+</sup> ILC2s after 4-hour incubation in medium with or without ACh and indicated ACHR antagonists, in the presence of IL-2, IL-7, IL-25, and IL-33. Data are representative of two independent experiments. n = 3 mice per group. Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

**DISCUSSION**

Recent studies have shed light on the critical roles of neurotransmitters and neuropeptides in the regulation of innate and adaptive immune responses (8, 9, 42–45). However, the neurotransmitters and neuropeptides that regulate ILC2 responses as well as the cellular sources of these molecules in vivo, which may determine the potency and efficiency of their effects, remain incompletely defined. The present study demonstrates that the neurotransmitter ACh plays a critical role in regulating ILC2 responses, and identifies that ILC2s themselves are a previously unrecognized cell type that expresses ChAT and may be a source of ACh. Our results inform a model in which ILC2s express ChAT and use the ACh-AChR pathway to promote IL-5 and IL-13 production, type 2 inflammation, and anti-helminth immunity (fig. S6).

Different neurotransmitters or neuropeptides and their receptors lead to divergent outcomes in ILC2s. For example, the neuropeptide NMU signals through the G<sub>q/11</sub>-coupled receptor NMUR1, which leads to calcium influx and NFAT<sup>T</sup> (nuclear factor of activated T cells) signaling, and potently drives ILC2 activation and helminth expulsion (11–13). NE and CGRP signal through their G<sub>q</sub>-coupled receptors β<sub>2</sub>AR and CALCRL/RAMP1, respectively, which lead to adenosine 3',5'-monophosphate (cAMP) signaling, and inhibit ILC2 activation and anti-helminth immunity (14–17). In the case of AChRs, the mAChR M4 is coupled to G<sub>q/11</sub> proteins, and M5 is coupled to G<sub>q/11</sub> proteins (24). mAChRs are pentameric Na<sup>+</sup> ion channels.
ACh administration in vivo are direct effects of ACh on ILC2s, rather than indirect effects subsequent to ACh effects on other cells. Here, we focused on the intestinal phase of N. brasiliensis infection. Given that N. brasiliensis migrates from the epidermis via blood vessels to the lung, then via the trachea to the intestine, and is eventually expelled from the intestine, further studies on the role of ILC2 ChAT-ACh pathway in other phases of N. brasiliensis infection are needed, as well as in naïve mice and in other models of helminth infection and allergic inflammation.

Although further analysis is required to define how the ACh-AChR pathway is regulated in ILC2s, our data indicate that the ChAT-ACh pathway is induced by the alarmin IL-33 and is likely dependent on the IL-33R–MyD88 signaling pathway (47, 48). In the nervous system, ACh is stored in vesicles and released after the neuron receives specific neurotransmitter signals. Similar mechanisms were found in immune cells—ChAT+ CD4+ T cells release ACh after stimulation with NE, and ChAT+ B cells release ACh after stimulation with cholecystokinin (47, 48). ILC2s respond to several neurotransmitters including NE. On the basis of these findings, further investigation on the regulation of ACh release from ILC2s is needed.

(23). The diverse combination of different α and β subunits assembling heteromeric nAChRs, and with several α subunits including α9 and α10 subunits assembling homomeric nAChRs (46), leads to numerous nAChRs that vary in their properties and functions. Given that ILC2s express at least two mAChRs plus at least six nAChR subunits, using new AChR conditional knockout mice to determine which specific receptor subunit pairs of the AChRs play a role in the activation of ILC2s in the context of N. brasiliensis infection or exposure to other stimuli will be critical in future studies. Generation of these new genetic tools will also aid in the analysis of a potential ILC2-ACh autocrine loop and directly test whether the observed effects of ACh administration in vivo are direct effects of ACh on ILC2s, rather than indirect effects subsequent to ACh effects on other cells. Here, we focused on the intestinal phase of N. brasiliensis infection. Given that N. brasiliensis migrates from the epidermis via blood vessels to the lung, then via the trachea to the intestine, and is eventually expelled from the intestine, further

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**Fig. 6. Enhanced ILC2 responses and accelerated expulsion of N. brasiliensis after ACh treatment.** (A to G) Numbers of total ILC2s (A), representative flow cytometry plots (B and E), population frequencies (C and F), and numbers (D and G) of IL-5+ (B to D) and IL-13+ (E to G) ILC2s in the lungs of PBS- or ACh-treated mice analyzed on day 7 of N. brasiliensis infection. (H to L) Representative flow cytometry plots (H), population frequencies (I and K), and numbers (J and L) of eosinophils (I and J) and Siglec Fhigh–activated eosinophils (K and L) in the lungs of PBS- or ACh-treated mice analyzed on day 7 of N. brasiliensis infection. Given that N. brasiliensis affects on other cells. Here, we focused on the intestinal phase of N. brasiliensis infection. Given that N. brasiliensis migrates from the epidermis via blood vessels to the lung, then via the trachea to the intestine, and is eventually expelled from the intestine, further
Pharmacological AChR agonists are already being tested in clinical trials for neurological diseases as well as excessive tumor necrosis factor–α (TNF-α) and IL-1β production mediated by macrophage responses (49–54). Given that the ChAT-ACh pathway influences optimal ILC2 responses in both lymphoid tissues and at mucosal barriers in the context of allergen- or helminth-induced type 2 inflammation, ACh and AChR signaling pathways could be potential therapeutic targets to manipulate ILC2 functions in multiple inflammatory and infectious diseases.

MATERIALS AND METHODS

Study design

The study aimed to identify the role of the ChAT-ACh pathway in regulating ILC2 responses to protect against helminth infection. Experiments included WT mice and transgenic strains. All mice used were between 6 and 16 weeks old, and age- and sex-matched littermates were used for each experiment. Most of the experiments consisted of enumeration of population frequencies and cell numbers by flow cytometry. We did not use randomization to assign animals to experimental groups. No animals were excluded from the analyses. The investigators were not blinded. The sample size was estimated from preliminary experiments or from reports in the literature. Experimental replication is described in the figure legends.

Mice

C57BL/6 (Jax 664), ChAT<sup>BAC</sup>-eGFP (Jax 7902), ChAT<sup>BAC</sup>Il13<sup>tm1(YFP/cre)Lky</sup> and YetCre-13 (Il7r<sup>gml1(TgJ)/YogJL</sup>; Jax 17353) mice were purchased from the Jackson Laboratory and bred at Weill Cornell Medicine. Il7r-Cre mice (41) were bred at Weill Cornell Medicine. All mice used were between 6 and 16 weeks old, and age- and sex-matched littermates were used for each experiment. All mice were maintained under specific pathogen-free conditions and were used in accordance with the Institutional Animal Care and Use Committee guidelines at Weill Cornell Medicine.

Helminth infection, Alternaria treatment, and IL-25, IL-33, and TSLP in vivo treatment

For N. brasiliensis infection, third-stage larvae (L3) were purified with a Baermann apparatus. After washing three times in phosphate-buffered saline (PBS), living worms were counted, and 500 purified worms in 250 μl of PBS were injected subcutaneously. Tissues were collected on day 7 after infection. Collection and enumeration of L5 adult worms was performed as previously described (55).

For T. muris infection, 200 embryonated eggs were administered by oral gavage. Ceca were collected on day 3 after infection. For Alternaria treatment, 10 μg of Alternaria alternata extract (Stallergenes Greer) in 40 μl of PBS was intranasally administered for three consecutive days and analyzed 1 day later.

For IL-25 treatment, 250 ng of recombinant IL-25 (R&D Systems) was injected intraperitoneally in a volume of 100 μl daily for three consecutive days and analyzed 1 day later. For IL-33 treatment, 500 ng of recombinant IL-33 (R&D Systems) was injected intraperitoneally in a volume of 100 μl daily for three consecutive days and analyzed 1 day later. For TSLP treatment, 10 μg of recombinant TSLP (Amgen) was injected intraperitoneally in a volume of 100 μl daily for four consecutive days and analyzed 1 day later.

Isolation of cells from intestinal lamina propria, mLNs, and lung

Small intestine or colon was removed, cleaned from remaining fat tissue, and washed in ice-cold PBS (Sigma-Aldrich). Peyer’s patches were identified and eliminated. Intestinal tissue was opened longitudinally...
and washed in ice-cold PBS. Dissociation of epithelial cells was performed by incubation on a shaker at 37°C in Hanks’ balanced salt solution (Sigma-Aldrich) containing 1% fetal bovine serum (FBS) and 5 mM EDTA (Thermo Fisher Scientific) two times for 15 min. After each step, samples were vortexed and the epithelial fraction was filtered through a 100-μm filter. Afterward, remaining tissue was chopped into small pieces and enzymatic digestion was performed using dispase (0.4 U/ml; Thermo Fisher Scientific), collagenase II (1 mg/ml; Sigma-Aldrich), and DNase III (1 mg/ml; Worthington), and deoxyribonuclease I (DNaseI) (20 μg/ml) for 1 hour at 37°C.

In vitro cell culture
Sort-purified small intestinal ILC2s were incubated in complete RPMI 1640 medium at 37°C and 5% CO₂ for 3 days in the presence of IL-7R (100 ng/ml), IL-25 (100 ng/ml), and IL-33 (100 ng/ml) were added if indicated. For intracellular cytokine staining, the culture was supplemented with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), ionomycin (1 μg/ml), and brefeldin A (10 μg/ml) if indicated.

**Flow cytometry and cell sorting**
Mouse single-cell suspensions were incubated on ice with conjugated antibodies in PBS. Dead cells were routinely excluded with Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific). Lineage-positive cells were excluded by staining for CD3e (145-2C11), CD5 (53-7.3), FceRI (Mar-1), F4/80 (BM8), NK1.1 (PK136), B220 (RA3-6B2), CD11b (M1/70), and CD11c (N418). For surface staining, CD45 (30-F11), CD127 (A7R34), CD90.2 (53-2.1), IL-33R (ST2, RMST2-2), KLRG1 (2F1), Siglec F (E50-2440), CCR6 (29-2L17), NKp46 (29A1.4), Ly6G (1A8), CD8α (53-6.7), CD49b (DX5), CD19 (1D3), CD4 (GK1.5), and CD25 (PC61.5) were used. Intracellular staining with IL-5 (TRFK5) and IL-13 (eBio13A) antibodies was carried out by using the BD Cytofix/Cytoperm and Perm/Wash solutions. All antibodies used in flow cytometry were purchased from eBioscience, BioLegend, or BD Biosciences. Flow cytometry experiments were acquired using a custom configuration Fortessa flow cytometer and the FACS Diva software (BD Biosciences) and analyzed with FlowJo software (TreeStar). Cell sorting experiments were performed using a custom configuration FACS Aria cell sorter (BD Biosciences).

**In vitro stimulation**
Sort-purified small intestinal ILC2s were incubated in complete RPMI 1640 medium containing 10% FBS, 50 μM 2-mercaptopethanol, 1 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml) for 4 hours at 37°C and 5% CO₂ ACh chloride (0.4 mg/ml; Sigma-Aldrich), ipratropium bromide (6 mg/ml) or d-tubocurarine (2 mg/ml), IL-2 (20 ng/ml), IL-7 (20 ng/ml), IL-25 (100 ng/ml), and IL-33 (100 ng/ml) were added if indicated. For intracellular cytokine staining, the culture was supplemented with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), ionomycin (1 μg/ml), and brefeldin A (10 μg/ml).
Immunofluorescence microscopy
Tissues were harvested in standard fashion and fixed in 4% paraformaldehyde in PBS for 2 hours at 4°C before washing three times in PBS. Tissues were then dehydrated overnight at 4°C in 30% sucrose dissolved in PBS. Dehydrated tissues were cryoprotected in optimal cutting temperature (OCT) medium (Tissue-Tek) and stored at −80°C until sectioning at a thickness of 10 μm using a cryo (Leica Instruments) and immobilization on Superfrost Plus slides (VWR). Immobilized tissues were then stored at −20°C until immunostaining. Slides were allowed to come to room temperature, excess OCT medium was washed off in PBS, and tissue sections were then blocked in PBS with 5% normal goat serum, 5% normal donkey serum (both from Jackson ImmunoResearch), and 0.1% Triton X-100 (Sigma-Aldrich) for 30 min. Tissue sections were then stained with the following primary antibodies diluted in blocking buffer overnight at 4°C: anti-GFP (Abcam, catalog no. 6556), anti-C3D 1:50 (BioLegend, clone 17A2), and anti-KLRG1 1:50 (eBioscience, clone 2F1). Sections were washed three times with PBS and then incubated with secondary antibodies [anti-rabbit immunoglobulin G (IgG)–Alexa488, anti-hamster IgG-Alexa555, and anti-rat IgG-Alexa647; Invitrogen] diluted 1:500 in blocking buffer overnight at 4°C: anti-GFP 1:200 (Abcam, catalog no. 6556), anti-CD3 (Sigma-Aldrich) for 30 min. Tissue sections were then stained with periodic acid–Schiff (PAS) combined with Alcian blue by IDEXX BioResearch. Images were acquired using a Nikon Eclipse microscope (Nikon). The number of goblet cells in villus was calculated from the number of goblet cells in more than 10 villi using a Nikon FV1000 laser-scanning confocal microscope.

RT-PCR and qPCR
Cells were lysed in RLT buffer (Qiagen). RNA was extracted via RNeasy mini kit (Qiagen), as per the manufacturer’s instructions. Complementary DNA (cDNA) was prepared using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Brain and muscle cDNA were used as positive controls for RT-PCR. The primer sequences used for RT-PCR are listed in table S1. Quantitative PCR was performed on cDNA using SYBR Green chemistry (Applied Biosystems) and PrimePCR SYBR Green Assay primers (Bio-Rad). Reactions were run on an RT-PCR system (ABI 7500; Applied Biosystems). Samples were normalized to the expression of Hprt1.

Administration of ACh in vivo
ACh chloride (Sigma-Aldrich) of 10 mg/kg body weight or the vehicle PBS was injected subcutaneously in a volume of 100 μl twice daily, 10 hours apart, during days 2 to 7 of experiment. 10 hours apart, during days 2 to 7 of experiment.

Histology
Proximal intestinal tissues were fixed with 4% paraformaldehyde (bioWORLD) and embedded in paraffin, and 5-μm sections were stained with periodic acid–Schiff (PAS) combined with Alcian blue by IDEXX BioResearch. Images were acquired using a Nikon Eclipse Ti microscope (Nikon). The number of goblet cells in villus was calculated from the number of goblet cells in more than 10 villi per mouse.

Immunoblotting
ILC2s were sort-purified from small intestine and cultured in vitro for 3 days with IL-2 (20 ng/ml), IL-7 (20 ng/ml), and IL-25 (100 ng/ml). Total cell lysate proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with tris-buffered saline containing 5% bovine serum albumin and 0.1% Tween 20. To detect target molecules by immunoblotting, anti-CHRM4 (Abcam, 77956) and anti-CHRM5 (Abcam, 41171) were used. Appropriate horseradish peroxidase– conjugated secondary reagents were from Jackson ImmunoResearch Laboratories.

RNA-seq library preparation
RNA-seq libraries were prepared and sequenced by the Epigenomics Core at Weill Cornell Medicine on an Illumina NextSeq500, producing 50–base pair single-end reads. Sequenced reads were demultiplexed using CASAVA v1.8.2, and adapters were trimmed using FLEXBAR v2.4 (56).

RNA-seq analysis
Sequenced reads were aligned to the mouse genome GRCm38/mm10 using STAR v2.3.0 (57). Reads counts at the gene level were calculated using Rsubread (58). Normalization for library size and differential expression analysis were performed using DESeq2 v1.18 (59).

Statistical analysis
P value of dataset was determined by unpaired two-sided Student’s t test with 95% confidence interval. Normal distribution was assumed. If equal variances between two groups could not be assumed, Welch’s correction was performed. All statistical tests were performed with GraphPad Prism. Error bars depict the SEM.

Note added in proof: A similar study by Roberts et al. reports a critical role for ILC2-derived ACh in promoting type 2 inflammation (60).

REFERENCES AND NOTES


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The ChAT-acetylcholine pathway promotes group 2 innate lymphoid cell responses and anti-helminth immunity
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Cholinergic ILC2s support helminth clearance
The activity of group 2 innate lymphoid cells (ILC2s) is modulated by the action of neuropeptides and small-molecule neurotransmitters. To investigate whether ILC2s are a nonneuronal source of the neurotransmitter acetylcholine (ACh) that could influence potential neuro-immune cross-talk, Chu et al. used a green fluorescent protein (GFP) reporter mouse to analyze ILC2 expression of the ACh-synthesizing enzyme choline acetyltransferase (ChAT) following infection with helminth parasites or exposure to allergens or proinflammatory cytokines. ILC2 expression of the ChAT-GFP reporter was strongly induced in vivo after helminth infection and after exposure to Alternaria or injection of the cytokines IL-33 and IL-25. ACh stimulation of ILC2s increased their production of type 2 cytokines and enhanced helminth expulsion from the small intestine. The cholinergic potential of ILC2s revealed in this study provides further evidence of the complex interplay between the nervous system and innate lymphocytes.