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FELLOWSHIP FINAL REPORT

Targeting acetylcholine receptors to enhance immunity to infection

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ABSTRACT

Group 2 innate lymphoid cell (ILC2s) responses drive type 2 immunity against helminths and are initiated by host alarmin release. Here we show that in addition to signature type 2 cytokines ILC2 also synthesise and release acetylcholine (ACh). ILC2 ACh synthesis (defined by choline acetyltransferase (ChAT) expression) following Nb or Alternaria challenge revealed pronounced ACh synthesis in ILC2 when compared to other immune cell populations. *In vivo* alarmin cytokine challenges selectively induced this ILC2 ACh responses. *Nippostrongylus brasiliensis* infection of ROR^{Cre}ChAT^{LoxP} mice (which have a targeted disruption of the ILC2 ACh response) resulted in higher intestinal helminth burdens than in control mice. This impaired control of infection associated with reduced ILC2 and CD4 IL-13 production. Adoptive transfer of ROR^{Cre}ChAT^{LoxP} ILC2s into RAG2$^{-/-}$IL-2rg$^{-/-}$ resulted in subsequent infection having a higher intestinal burden than in ChAT^{loxp} recipeints. These data identify ILC2-derived ACh as a novel axis required for optimal type 2 immunity.

1- Introduction

[Although acetylcholine (ACh) is best known as a neurotransmitter, cholinergic signalling also regulates the immune system, best described in the cholinergic anti-inflammatory pathway, in which sensory perception of inflammatory stimuli leads to a vagal reflex culminating in α7 nicotinic receptor (α7nAChR) subunit-dependent inhibition of TNF-α, IL-1β and IL-18 production by splenic macrophages (*1, 2*). Importantly, lymphocytes can synthesise and release ACh (*3-5*), and definition of these cells has been greatly facilitated by the use of reporter mice in which eGFP or tdTomato fluorescent protein is linked to expression of choline acetyltransferase (ChAT), the enzyme which synthesises ACh (*6, 7*). CD4+ T cells expressing an effector/memory (CD44+CD62L^{lo}) phenotype were identified as the source of ACh in the spleen responsible for signalling to macrophages in the cholinergic anti-inflammatory pathway (*4*), and B cells produce and release ACh in response to cholecystokinin, resulting in inhibition of neutrophil recruitment during sterile endotoxaemia (*5*). Adaptive immunity is also regulated by ACh, and optimal type 2 effector responses to the nematode parasite

Nippostrongylus brasiliensis require signalling through the M3 muscarinic receptor (mAChR) (*8*).

A role for cholinergic signalling in the pathology of asthma and Chronic Obstructive Pulmonary Disease (COPD) is well established, and anticholinergics in the form of mAChR antagonists such as tiotropium bromide are widely used (*9, 10*). These alleviate bronchoconstriction, mucus production and airway remodelling, but a facet that has not been addressed in detail is the contribution of immune cell-derived ACh to pulmonary immunity.

Group 2 innate lymphoid cells (ILC2s) play an important role in initiating type 2 immune responses, and are activated by epithelial cell-derived alarmins to release canonical effector cytokines such as IL-13 and IL-5 (*11*), which drive allergic inflammation (*12*) and immunity to helminth infection (*13*). ILC2 cells have recently been shown to be both positively and negatively regulated by neurotransmitters. Thus, release of the neuropeptide neuromedin U (NMU) by cholinergic neurons activates type 2 cytokine production by ILC2s, amplifying allergic inflammation and helminth immunity (*14-16*). In contrast, release of noradrenaline by adrenergic neurons inhibits ILC2 cytokine production and proliferation, suppressing effector functions (*17*). ILC2s expressing receptors responsive to both neurotransmitters co-localise with neurons in mucosal tissues, forming what have been termed neuroimmune cell units (NMCUs) (*18*). However, if ILC2 produce neurotransmitters in order to drive their potent downstream lymphoid and myeliod effects is unknown

In this study we demonstrate that pulmonary ILC2s upregulate their capacity to synthesise and release ACh during infection with *N. brasiliensis* and acute airway inflammation triggered by exposure to the fungus *Alternaria alternata*, and show that the cholinergic phenotype of ILC2s is induced by the alarmin cytokines IL-33 and IL-25. Disruption of the ability of ILC2s to synthesise ACh by generation of RoR^{Cre}ChAT^{loxp} transgenic mice resulted in impaired immunity to *N. brasiliensis*, and adoptive transfer experiments showed that this was associated specifically with loss of ChAT activity in ILC2s. These data indicate that production and release of ACh by ILC2s is an important factor in driving type 2 immunity in the murine lung.

2- Experimental details

Animals and parasite infection. This study was approved by the Animal Welfare Ethical Review Board at Imperial College London, and was licensed by and performed under the UK Home Office Animals (Scientific Procedures) Act Personal Project Licence number 70/8193: 'Immunomodulation by helminth parasites' and in France all protocols were approved by by the CNRS institutional animal research ethical committee under the number (CLE CCO 2015-1085). Female BALB/c and C57BL6/J mice, aged 6-8 weeks old were purchased from Charles River. ChAT-eGFP^{BAC} were purchased from Jackson Laboratories and subsequently bred in-house. Mice were infected with *N. brasiliensis* by sub-cutaneous inoculation with 500 infective larvae and parasites maintained by established methods (*19*).

Murine model of allergic airway inflammation. Extracts of *Alternaria alternata* were obtained as a gift from Henry McSorley (University of Edinburgh) or purchased as lyophilised protein extract from Greer Laboratories (USA). Mice were lightly dosed with isoflurane before intranasal administration with 50 µg *A. alternata* extract with or without 20 µg active or inactive AChE in a final volume of 50 µl PBS. Mice were exposed to a single dose of *A. alternata* for 24 or 48 hrs, and where indicated received a second intranasal dose of active or inactive AChE at 24 hrs. Control animals were dosed with 50 µl PBS in the same schedule.

Tissue preparation. For isolation of bronchoalveolar cells, lungs were lavaged twice in a total of 2 ml PBS with 0.2% BSA and 2 mM EDTA. Erythrocytes were lysed, leukocytes resuspended and counted. For lung single cell suspensions, lungs were perfused via cardiac puncture with 10 ml PBS then infused with 1.5 ml PBS containing 5 mg ml^{-1} dispase II neutral protease (Sigma) via the trachea. The thymus and lung-draining lymph nodes were removed, lungs ligatured, removed into 1.5 ml digest solution, incubated at room temperature for 25 min, then for a further 30 min at 37^{0}C. Lungs were mechanically dissociated in Dulbecco's

Minimal Essential Medium (DMEM) with 25 mM HEPES and 100 U ml^{-1} DNAse I (Sigma), and incubated at room temperature for 10 min. Samples were passed through 100 µm cell strainers and erythrocytes lysed.

Flow cytometry and cell sorting. Single cell suspensions were treated with rat anti-mouse CD32/CD16 (FcBlock, BD Biosciences), washed then stained. For intracellular staining, cells were stimulated for 4 hrs at 37°C with 1 ug ml^{-1} PMA/100 ng ml^{-1} ionomycin with Brefeldin-A (GolgiPlug, BD Biosciences), fixed for 30 min at room temperature, permeabilised using the FoxP3/transcription factor staining buffer kit (eBioscience) and stained with fluorochrome-conjugated antibody cocktails (eBioscience, Miltenyi Biotec or Biolegend). For ILC2s, cells were identified as low side/forward scatter, lineage negative, CD45+CD90+ICOS+ST2+CD127+ cells. The lineage panel consisted of antibodies to CD3, CD4, CD8, B220, CD19, TER119, CD49b, FcεRI and CD11b. Eosinophils were identified as CD11b+SIGLECF+GR-1^{lo} $CD11c^{lo}$. Neutrophils were identified as CD11b+SIGLECF-GR-1^{hi} $CD11c^{lo}$. Unstained samples and fluorescence minus one controls were used as appropriate. When analyzing eGFP fluorescence from ChAT-$eGFP^{BAC}$ reporter mouse cells, wild type (C57BL6/J) cells were used as negative controls. Cells were analysed on a BD LSR Fortessa™ analyser. For FACS sorting of ILC2s, lung tissue was processed to a single cell suspension as described, the lineage negative population enriched by magnetic activated cell sorting, depleting other cells via a PE-conjugated lineage cocktail (Miltenyi Biotec), then ILC2s sorted on a BD FACS ARIA III cell sorter.

RT-PCR and qPCR. RT-PCR was carried out according to standard procedures, using the following primer pairs (RORA: 5´-GTGGAGACAAATCGTCAGGAAT-3´, 5´-GACATCCGACCAAACTTGACA-3´; RORC 5´-CCGCTGAGAGGGCTTCAC-3´, 5´-TGCAGGAGTAGGCCACATTACA-3´; GATA3: 5´-CTCGGCCATTCGTACATGGAA-3´, 5´-GGATACCTCTGCACCGTAGC-3´; TCF7 5´-CGCTGCATAACAAGCC-3´, 5´-CCAGCTCACAGTATGGG-3´; ChAT 5´-GCTGCCGTGCTGGCTTCTGA-3´, 5´-CGCTCCCACCGCTTCTGCAA-3´; beta□actin: 5´-TGGAATCCTGTGGCATCCATGAAAC-3´, 5´-TAAAACGCAGCTCAGTAACAGTCCG-3´). For qPCR, reactions were carried out using the Quantitect SYBR Green PCR kit according to manufacturer's instructions (Qiagen) in an ABI F7500 real-time PCR thermocycler. RNA (2.5 ng per reaction) was reverse transcribed to cDNA and primers used at a concentration of 0.5 µM. Ct thresholds were set to 0.2 and relative transcript levels determined by the comparative CT method ($2^{-\Delta\Delta CT}$) (*20*) using previously calculated primer efficiencies for each primer pair (ChAT: 5´-GGCCATTGTGAAGCGGTTTG-3´, 5´-GCCAGGCGGTTGTTTAGATACA-3´, and with 18S rRNA, eukaryotic elongation factor 2 and peptidyl-prolyl isomerase A as reference genes.

Detection of acetylcholine release. Cells from FACS-sorted populations were incubated at 37^0C for 30 min in 96 well round-bottomed plates (10^5 cells in 150 µl), centrifuged, supernatants removed, the AChE inhibitor BW284C51 added to a final concentration of 10 µM and samples stored at -80°C until analysis by HPLC-mass spectrometry. Control samples were spiked with 50 nM internal standard (acetylcholine -1,1,2,2,-D4 chloride, QMX laboratories).

Statistical analysis. Flow cytometry data was analysed using FlowJo software (Treestar). Graphs and students unpaired t-tests were carried out using Graphpad prism software (Graphpad). Data represent mean ± SEM unless otherwise stated. Statistical significance between groups is indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s. = non-significant difference ($p>0.05$).

3- Results and discussion

Pulmonary ILC2s synthesise and release acetylcholine during type 2 immunity

ChAT-$eGFP^{BAC}$ mice (*6*) were used to show how infection with *N. brasiliensis* influenced cholinergic phenotypes of pulmonary immune cells. At day 7 post infection (d7 p.i.), the proportion of total CD45+ cells in lung tissue

which expressed ChAT and therefore had the potential to synthesise and secrete ACh was increased compared to uninfected controls (Figure 1A). The majority of ChAT+ cells were from lymphoid rather than myeloid lineages, as previously documented in other tissues (*5*). However, in addition to appreciable expression in B cells, the most significant upregulation of ChAT early in infection was shown by ILC2s (Figures 1B, 1C). Kinetic analysis of ChAT expression by ILC2s in total lung and bronchoalveolar lavage (BAL) samples during infection showed a significant increase by d4 p.i., peaking at d7, and remained elevated in both sites at d21, 13 days after expulsion of parasites from the intestine. The proportion of ILC2s which were ChAT+ was notably much greater in BAL than in the lungs (Figure 1D).

ILC2s were isolated and expression of ChAT and transcription factors associated with ILC2 development were established by reverse transcription-polymerase chain reaction (RT-PCR). Expression of transcription factor 7 (TCF-7), retinoic acid receptor-related orphan receptor alpha (RORα), GATA3, ChAT, and the absence of RORC confirmed their identity (Figures S1A, 1B). Real-time qPCR assay showed increased ChAT expression in pulmonary ILC2s from infected mice when compared to uninfected animals (Figure 1E). HPLC-mass spectrometry further verified enhanced ILC2 synthesise and release of ACh following Nb infection (Figure 1F).

ILC2 ChAT expression also associated with activation status in pulmonary ILC2s. ChAT+ ILC2s had a significantly increased expression of the IL-33 receptor subunit ST2 compared to ChAT- cells at d4 and d7 p.i. (Figure 1G) and also for inducible T-cell costimulator (ICOS) at d7 p.i. (Figure 1H), suggesting that ChAT expression is associated with cellular activation.

Next, we tested if induction of the cholinergic phenotype in ILC2s was a general feature of type 2 immunity, by challenging mice with the fungal allergen *Alternaria alternata*, (*21*) which causes rapid onset type 2 eosinophilic airway inflammation (*22*, *23*). Successful induction of a type 2 response was confirmed by pulmonary eosinophilia (Figure 2A). *Alternaria* challenge also induced small but significant increases in ChAT expression in lymphocyte populations, including CD4+ T cells and NKT cells (Figure 2B). Importantly however, as in nematode infection, the greatest proportional increase in ChAT expression was observed in the pulmonary ILC2 population, both in total lung tissue and BAL (Figures 2C, 2D).

Pulmonary ILC2s therefore show a striking inducible cholinergic phenotype in response to which identifies ILC2 as the principle lymphocyte source of ACh during a type 2 immune responses.

The alarmins IL-25 and IL-33 are sufficient to induce the cholinergic phenotype of ILC2s

Elevated ILC2 ChAT expression was associated with early induction of type 2 immunity. The alarmins IL-25 and IL-33 are established as key initators of type 2 immunity and ILC2 activation is a central feature of their function. *Ex vivo* stimulation of CD45+ cells isolated from naïve ChAT^{BAC}-eGFP reporter mice with IL-33 but not IL-7 enhanced ILC2 ChAT expression, suggesting that activation through alarmin signalling pathways may specifically drive the ILC2 cholinergic phenotype (Figure 3A). IL-2 is also known to promote proliferation and cytokine production by ILC2s (*24*, *25*). We therefore isolated CD45+ cells from the lungs of naïve ChAT^{BAC}-eGFP reporter mice by FACS, stimulated them *in vitro* with combinations of IL-25, IL-33 and IL-2, and assayed ChAT expression on ILC2s 24 hrs later. As anticipated, stimulation with IL-25 and IL-33 both induced ChAT expression. IL-2 also induced ChAT expression on ILC2s, and had a synergistic effect on stimulation with either IL-33 or IL-25 (Figure 3C).

To explore this further, we dosed reporter mice intranasally with the alarmin cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP), and analysed ChAT expression on pulmonary ILC2s 24 hrs later. IL-25 and IL-33 both induced ChAT expression on ILC2s, although no effect was observed with TSLP (Figure 3B). Importantly, analysis of ChAT expression in other lymphocyte populations following IL-33 challenge showed potentiated ChAT expression only occurred in ILC2.

RoR□-driven disruption of ChAT expression in ILC2 impairs immunity to *N. brasiliensis*

In order to determine whether synthesis of ACh by ILC2s played a role in immunity to helminths, we generated RoR□^{Cre}ChAT^{loxp}

transgenic mice and infected them with *N. brasiliensis*. Intestinal worm burdens at day 6 post-infection (p.i.) were significantly higher in RoR^{Cre}ChAT^{loxp} mice than in ChAT^{loxp} controls, and this was associated with increased pulmonary cellularity (Figure 4). Production of IL-13 by both ILC2s and CD4+ T cells was significantly reduced in RoR^{Cre}ChAT^{loxp} mice compared to controls (Figure 4). These data suggest that expression of ChAT and synthesis of ACh by ILC2s contributes significantly to the generation of type 2 immunity and expulsion of *N. brasiliensis*.

As RoR expression is not confined to ILC2s (*26*) we further tested if synthesis of ACh specifically by ILC2s contributed to immunity by adoptive transfer of ILC2s isolated from *N. brasiliensis*-infected ChAT^{loxp} or RoR^{Cre}ChAT^{loxp} into RAG2$^{-/-}$IL-2rg$^{-/-}$ mice. Analysis at day 6 p.i. revealed that recipients of ILC2s from RoR^{Cre}ChAT^{loxp} mice had increased intestinal worm burdens when compared to recipients of ILC2s from ChAT^{loxp} donors, and this was accompanied by reduced expression of IL-13 in ILC2s (Figure 4). These data demonstrate that disruption of ChAT expression and impairment of ACh synthesis in ILC2s reduces the ability of mice to mount robust type 2 immunity and control *N. brasiliensis* infection.

4- Conclusion

ILC2s are recognised to be pivotal in translating epithelial cell cytokine production into robust type 2 immune responses. Here we show here that in addition to IL-13 and IL-5 production of the neurotransmitter ACh by ILC2s is a requirement for type 2-driven immunity to *N. brasiliensis*.

The alarmin cytokines IL-25 and IL-33 were demonstrated to upregulate ChAT expression on ILC2s both in vivo and in vitro (Figure 3). Initially, we hypothesised that IL-33 might regulate the ILC2 cholinergic phenotype, as expression of ChAT by B cells is induced by MyD88-dependent Toll-like receptor signalling (*5*), and members of the IL-1 family such as IL-33 also signal through this adapter protein (*27*). However, the observation that IL-25 was also a major regulator of ChAT expression demonstrated that MyD88-dependent signalling is not essential for this in ILC2s. TSLP did not induce ChAT expression in ILC2s when administered in vivo at the same dose. Although TSLP has been reported to influence ILC2 activation (*28*), most studies identify IL-25 and IL-33 as the major inducers of ILC2 responses in the lung and gut (*29, 30*). IL-2 was also shown to induce ChAT expression either by itself or in combination with IL-25 and IL-33 (Figure 3). IL-2 is known to be a critical regulator of ILC2s in the lung and dermis, in both instances driving cell survival and proliferation, and augmenting type 2 cytokine production (*31, 32*).

We have previously demonstrated that cholinergic signalling through the M3 mAChR is required for maximal activation and cytokine expression in CD4+ T cells (*8*), so it is possible that ILC2s are an important source of the ACh required for optimal T cell responsiveness and anti-parasite immunity.

Reduced expression of IL-13 by ILC2s in RoR^{Cre}ChAT^{loxp} mice suggests that ACh may also play an autocrine role in ILC2 activation. A recent report indicated that an 7-selective nicotinic receptor agonist reduced ILC2 effector function and airway hyperreactivity (*33*), although in contrast, muscarinic receptor antagonists such as tiotropium are well documented to ameliorate allergen-induced airway inflammation and remodelling (*34, 35*), which may be accompanied by reduced type 2 cytokine production (*36*). Signalling through different AChRs can therefore result in quite different outcomes.

Finally, numerous species of parasitic nematode secrete AChEs, postulated to promote parasite persistence via inhibition of cholinergic signalling (*37*). ILC2s link innate and adaptive immunity, driving type 2 immune responses which play a critical role in allergic inflammation (*12*) and immunity to nematode infection (*13*). Our observation that ILC2s are cholinergic, and that production of ACh by these cells is an important factor in driving type 2 responses provides a rationale for secretion of AChE by these parasites, as it may help to dampen type 2 immunity to a level which allows establishment and persistence of parasites.

5- Perspectives of future collaborations with the host laboratory

My fellowship at CNRS-Orleans has been extremely productive. The report here is a summary of one body of work. In addition we have submitted another study which is currently under review in Immunity.

The current project is ongoing and we have recieved funding for this work from the BBSRC (UK), National Research Foundation (South Africa), Department of Science and Technology-DST (South Africa) and the Royal Society (UK) to continue this work.

Funding from DST and Royal Society are specifically for maintaining this collaboration. Since completing the felowship I have returned to Orleans once to carryout laboratory work and post-grad/doctoral exchanges are planned for the end of 2018 and beginning of 2019.

6- Articles published in the framework of the fellowship

To date the following publications have CNRS/Le Studium as my research affiliation:

1. **Rolot M, Dougall AM, Chetty A, Javaux J, Chen T, Xiao X, Machiels B, Selkirk M, Hokke C, Denis O, Brombacher F, Vanderplasschen A, Gillet L, Horsnell WG, Dewals BG.** Helminth-induced IL-4 expands virtual memory $CD8^+$ T cells for early control of gammaherpesvirus infection. *Nature Communications.* 2018. In Press
2. **Dzhivhuho G, Humby SA, Ndlovu H, Horsnell WGC, Brombacher F, Williamson A-L, Chege GK.** Chronic schistosomiasis suppresses HIV-specific responses to DNA-MVA and MVA-gp140 Env vaccine regimens despite antihelminthic treatment and increases helminth-associated pathology in a mouse model. *PLoS Pathogens.* 2018. In Press.
3. **Nyangahu DD, Lennard KS, Brown BP, Darby MG, Wendoh JM, Havyarimana E, Smith P, Butcher J, Stintzi A, Mulder N, Horsnell WGC[#], Jaspan H[#].** Disruption of maternal gut microbiota during gestation alters offspring microbiota and immunity. *Microbiome.* 2018. Jul 7;6(1):124.
4. **Jacobs B-A, Chetty A, Horsnell WGC, Schäfer G, Prince S, Smith KA.** Hookworm exposure decreases human papillomavirus uptake and cervical cancer migration through systemic regulation of epithelial-mesenchymal transition marker expression. *Scientific Reports.* 2018. In Press
5. **Logan E, Luabeya AKK, Mulenga H, Mrdjen D, Ontong C, Cunningham A, Tameris M, McShane H, Scriba TJ, Horsnell WGC[#], Hatherill M[#].** Elevated IgG Responses in Infants are Associated with Reduced Prevalence of *Mycobacterium tuberculosis* Infection. *Frontiers in Immunology.* 2018. Volume 9, Article 1529
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FIGURE LEGENDS

Figure 1. Pulmonary ILC2s acquire a cholinergic phenotype associated with an enhanced activation state during infection with *Nippostrongylus brasiliensis*. A) Proportion of CD45+ leucocytes expressing ChAT-eGFP in the lungs of naïve ChAT^{BAC}-eGFP mice (black bars) or animals infected with *N. brasiliensis* (*Nb*) at d7 p.i. (clear bars). B) Representative flow cytometry plots of ChAT-eGFP expression by ILC2s in wild type C57BL/6J mice infected with *Nb* (eGFP gating control), naïve ChAT^{BAC}-eGFP animals or *Nb*-infected ChAT^{BAC}-eGFP animals at d7 p.i. C) Proportion of parental cell populations expressing ChAT-eGFP in the lungs of naïve ChAT^{BAC}-eGFP mice (black bars) or animals infected with *Nb* at d4 p.i. (clear bars). D) Dynamics of ChAT expression by ILC2s in the lungs and BAL throughout infection with *Nb*. E) Levels of ChAT transcripts assayed by qPCR in FACS-sorted ChAT+ and ChAT- pulmonary ILC2s from *Nb*-infected ChAT^{BAC}-eGFP animals, normalised to 18s rRNA expression and relative to expression from ILC2s from naïve controls. F) Quantification of basal acetylcholine (ACh) release from FACS-sorted pulmonary ILC2s from C57BL6/J naïve and *Nb*-infected animals. G) Mean fluorescence intensity (MFI) of ST2 expressed by ChAT-eGFPnegative (neg, black bars) and ChAT-eGFPpositive (+, clear bars) ILC2s in the lungs throughout infection with *Nb*. H) MFI of ICOS expressed by ChAT-eGFP- (black bars) and ChAT-eGFP+ (clear bars) ILC2s in the lungs throughout infection with *Nb*. n = 4 to 5

mice/group. Data are representative of 3 individual experiments.

Figure 2. Pulmonary ILC2s acquire a cholinergic phenotype following exposure to *Alternaria alternata*. A) Intranasal administration of *Alternaria alternata* extract stimulates pulmonary eosinophilia. B) Proportion of parental cell populations expressing ChAT-eGFP in the lungs of ChAT^{BAC}-eGFP mice 24 hrs following intranasal dosing with PBS (vehicle control, black bars) or *A. alternata* allergenic extract (clear bars). C) Representative flow cytometry plots of ChAT-eGFP expression by ILC2s in lungs of wild type C57BL/6J mice exposed to *A. alternata* extract (eGFP gating control), or ChAT^{BAC}-eGFP mice dosed with PBS or *A. alternata* allergenic extract. D) ILC2 responses in the lungs of ChAT^{BAC}-eGFP mice 24 hrs following intranasal dosing with PBS (black bars) or *A. alternata* extract (clear bars) including (from left to right): proportion (%) of ChAT-eGFP+ ILC2s, total number (#) of ILC2s in the lung, and total number of ChAT-eGFP+ ILC2s. n = 4 to 5 mice/group. Data are representative of 3 individual experiments.

Figure 3. The cholinergic phenotype of pulmonary ILC2s is induced by IL-25 and IL-33 and is augmented by IL-2. A) ILC2 responses in the lungs of ChAT^{BAC}-eGFP mice 24 hrs following intranasal dosing with PBS (black bars), IL-33 (clear bars), IL-25 (checked bars) or thymic stromal lymphopoietin (TSLP, striped bars) including (from left to right): proportion of ChAT-eGFP+ ILC2s, total number of ChAT-eGFP+ ILC2s, and MFI of eGFP expressed by ILC2s. B) Proportion of ChAT-eGFP+ pulmonary ILC2s and associated ILC2 eGFP MFI from FACS-sorted cells cultured *in vitro* for 24 hrs with medium only (control), IL-33, IL-25 and IL-2 in the combinations indicated. n = 3-5 animals per group. Data are representative of 2 individual experiments.

Figure 4. RoR□-driven disruption of ChAT expression impairs immunity to *N. brasiliensis*. A: Recovery of adult worms from the intestines of ChAT^{loxp} and RoR□^{Cre}ChAT^{loxp} mice at day 6 p.i. with *N. brasiliensis*.. B: Number (#) of IL-13-producing ILC2s (Lin-CD45+ICOS+) in the lungs. C: Number of CD4 T cells (CD3+CD4+) and frequency producing IL-13. Black circles and bars: ChAT^{loxp}, white circles and bars: RoR□^{Cre}ChAT^{loxp}, n = 5 animals per group. Data are representative of 3 individual experiments. D. **Impaired ability of ILC2s from RoR□^{Cre}ChAT^{loxp} mice to mediate control of *N. brasiliensis* infection following adoptive transfer.** ILC2s (Lin-CD45+ICOS+) were isolated from the lungs of day 6 infected ChAT^{loxp} and RoR□^{Cre}ChAT^{loxp} mice, 2 x 10^4 cells transferred intranasally into RAG2$^{-/-}$IL-2rg$^{-/-}$ mice and recipients infected with 500 *N. brasiliensis* 24 hrs post transfer. E. Recovery of adult worms at day 6 p.i. F: Proportion of lung ILC2s producing IL-13. Black circles and bars: ILC2s from ChAT^{loxp} donor mice; white circles and bars: ILC2s from RoR□^{Cre}ChAT^{loxp} donor mice. n= 7 recipient mice/group.

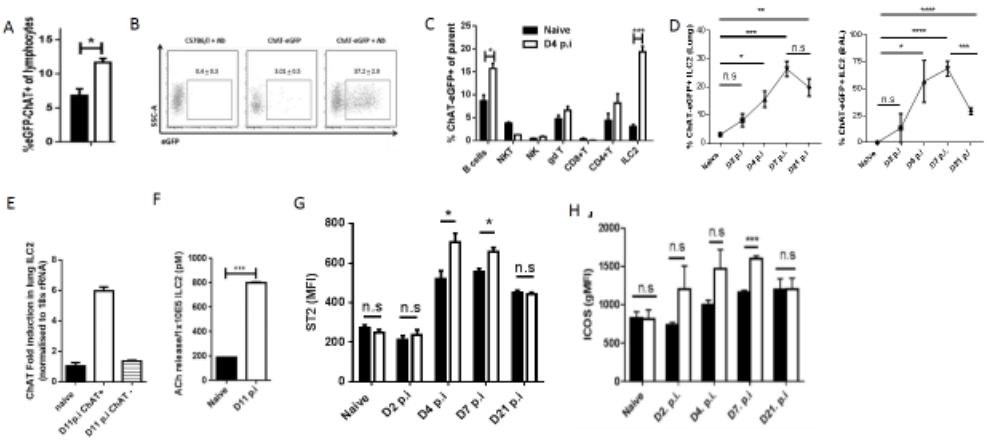

Figure 1

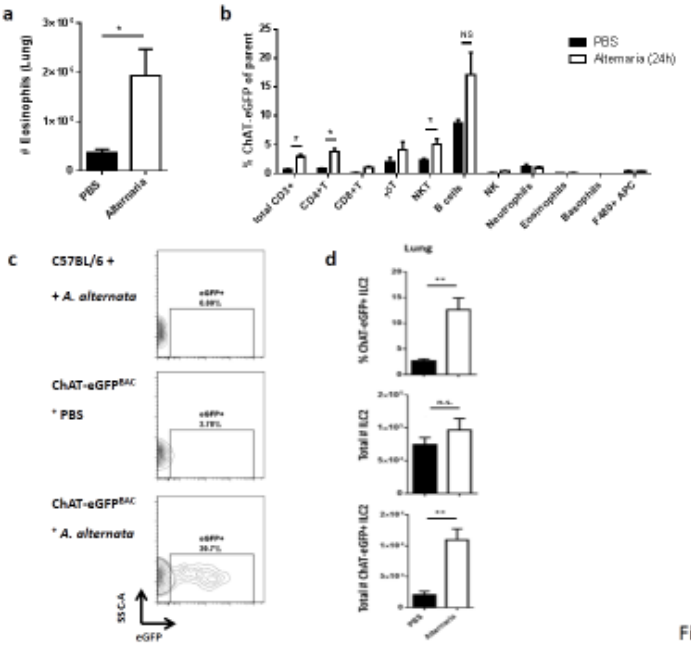

Figure 2

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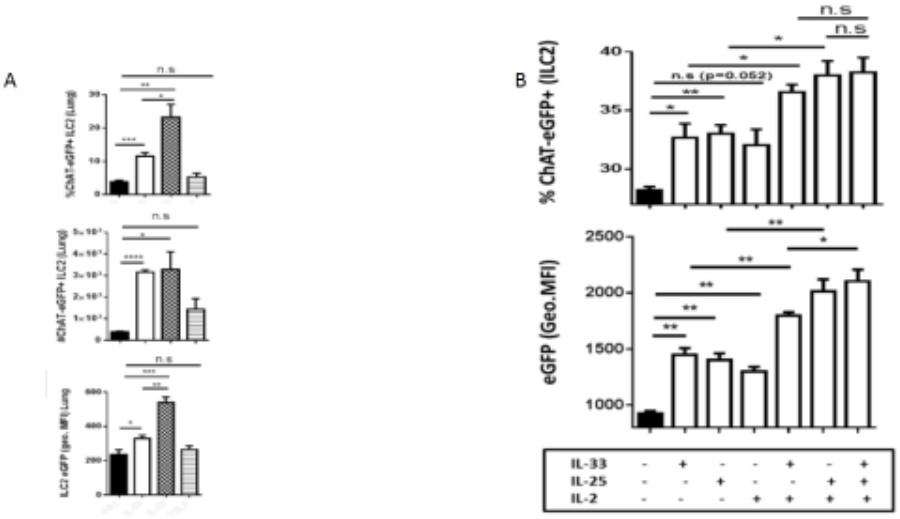
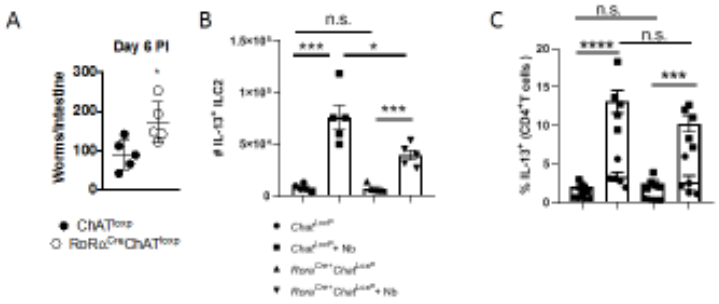
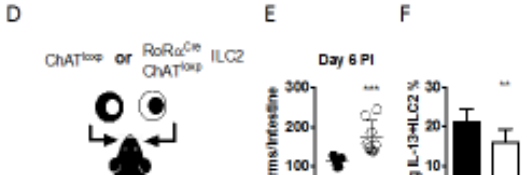

A
B
% ChAT-eGFP+ (ILC2)
eGFP (Geo.MFI)
n.s
n.s (p=0.052)
IL-33
IL-25
IL-2

Figure 3


A
B
C
Day 6 PI
Worms/Intestine
ChAT^loxp
RoRα^CreChAT^loxp
n.s.
n.s.


D
E
F
ChAT^loxp or RoRα^Cre ChAT^loxp ILC2
Day 6 PI