Title: A selective α7 nicotinic acetylcholine receptor agonist, PNU-282987, attenuates ILC2s activation and *Alternaria* induced airway inflammation

Authors:

Fang Yuan[1,3#], Lili Jiang[1#], Leon Sokulsky[2], Yuanyuan Wanyan[1], Lingli Wang[1], Xiaojie Liu[1], Lujia Zhou[1], Hock L. Tay[2], Guojun Zhang[4], Ming Yang[1, 2] * & Fuguang Li[1] *

Affiliation:

[1]Department of Immunology, College of Basic Medical Sciences, Zhengzhou University, Zhengzhou, Henan 450052, China

[2]Priority Research Centre for Healthy Lungs, School of Biomedical Sciences & Pharmacy, Faculty of Health and Hunter Medical Research Institute, University of Newcastle, Callaghan, NSW 2300, Australia

[3]Department of Medical Laboratory, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

[4]Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

Running Head: PNU-282987 inhibits ILC2s function

Address for Correspondence: Fuguang Li, Department of Immunology, College of Basic Medical Sciences, Zhengzhou University, No.100 Science Avenue, Zhengzhou, Henan, 450001, China

Tel: +86-0371-67781018

E-mail: ming.yang@newcastle.edu.au and lifuguang@zzu.edu.cn

Keywords: ILC2s, nicotinic acetylcholine receptor, PNU-282987, airway inflammation

ABSTRACT

Background: The anti-inflammatory effect of the α7nAChR agonist, PNU-282987, has previously been explored in the context of inflammatory disease. However, the impact of PNU-282987 on type 2 innate lymphoid cells (ILC2s)-mediated allergic airway inflammation has not been established. **Aims:** To determine the effects of PNU-282987 on the function of ILC2s in the context of IL-33 or *Alternaria Alternata* (AA)-induced airway inflammation. **Methods:** PNU-282987 was administered to mice that received recombinant IL-33 or AA intranasal challenge. Whole lung was collected from mice for histological analysis, and ILC2 populations were quantified in the lungs and BALF of these mice. Additionally, ILC2s were isolated from murine lung tissue and cultured *in vitro* in the presence of IL-33, IL-2 and IL-7 with or without PNU-282987, and the expression of the transcription factors GATA3, IKK, and NF-κB was determined in these cells. **Results:** PNU-282987 significantly reduced airway goblet cell hyperplasia, eosinophil infiltration, and ILC2s numbers in BALF in response to IL-33 or AA. *In vitro* IL-33 stimulation of isolated lung ILC2s showed a reduction of GATA3 and Ki67 in response to PNU-282987 treatments. When compared to the established α7nAChR agonist, GTS-21, there was a notable reduction in IKK and NF-κB phosphorylation in the PNU-282987 treated group when compared to the GTS-21 treated ILC2s. **Conclusion:** PNU-282987 inhibits ILC2s-associated airway inflammation by inhibiting ILC2s cell proliferation and the initiation of inflammatory cascades.

INTRODUCTION

Asthma is an immune disorder of the lungs associated with airway inflammation, mucus secretion and airway hyperresponsiveness (AHR), driven in part by the T-helper 2 (Th2) cytokines interleukin (IL)-4, IL-5 and IL-13.[1, 2] While $CD4^+$ Th2 cells have thought to of played an exclusive role in the production of these cytokines, innate immune cells such as M2a macrophages and type 2 innate lymphoid cells (ILC2s) have emerged as notable sources of Th2-associated interleukins.[3] The discovery of the ILC2s highlighted prior to the new decade highlighted a new class of cells that can induce inflammatory cascades in the early stages of asthma exacerbation in response to epithelial alarmin factors.[4, 5] These alarmin factors, namely IL-25, IL-33, and Thymic Stromal Lymphopoietin (TSLP), are released upon epithelial exposure to allergens or infection.[6-9] Upon exposure to ILC2s, these factors result in the activation of the ST2/IL-1RAcP receptor, upregulating IRAK and TRAF signaling cascades and subsequent activation of the transcription factors IKK, NF-κB, and MAPK.[10] The integral role for ILC2s in asthma exacerbation exceeds that for Th2 cells, as in $Rag2^{-/-}$ mice will still pathological features of allergic airway disease are introduced despite the deficient acquired immunity.[11] In $Rag2^{-/-}Il2rg^{-/-}$ mice lacking T cells, B cells, and ILCs, the levels of eosinophilic inflammation and the secretion of mucus decreased significantly, highlighting the importance of the IL-33/ILC2s axis in the development of asthma.[12] Furthermore, depletion of IL-33 will result in reduced eosinophilia and mucus secretion following aerosol allergen exposure, indicating the crucial role IL-33 has in the pathogenesis of asthma.[11]

Previously, Tracey *et al.* identified a cholinergic anti-inflammatory pathway (CAP) that is mediated by the vagus nerve, a primary neurological regulator of organ function and systemic responses.[12, 13] In the context of immunology, CAP has been shown to influence inflammatory pathways through the release of the neurotransmitter acetylcholine, which can stimulate the α7 nicotinic acetylcholine receptor (α7nAChR) that is expressed on macrophages, T cells and B cells.[14-16] Animal models of inflammatory disorders such as diabetes, sepsis, cystic fibrosis, ulcerative colitis and arthritis, that are run in α7nAChR-deficient mice present with a notable reduction in disease development due to downregulated activation of macrophages, T cells and B cells.[17-21] Recently, it has been observed that ILC2s are another inflammatory cell that is influenced by acetylcholine, expressing α7nAChR at a higher expression when compared to macrophages and CD4+ve lymphocytes. [22]

Employing α7nAChR agonists, such as PNU-282987 and GTS-21, in mouse models of inflammation have identified α7nAChR as a crucial mediator of NF-κB transcription and subsequent TNF-α and IL-1β secretion.[23, 24] Although studies into the use of α7nAChR agonists in treating asthma are limited, the compound GTS-21 has recently been shown to reduce airway inflammation induced by ILC2s.[22] However, despite its classification as an α7nAChR agonist, the appropriateness of GTS-21 as an anti-inflammatory therapeutic is questionable given the high binding affinity it has for the α4β2nACh receptor. At approximately 100 times higher compared to α7AChR.[25] The use of GTS-21 in a clinical setting is a less attractive venture due to the association between α4β2nAChR activation and anxiety.[26] In

contrast, the compound PNU-282987 has a high affinity to α7nAChR with little or no effect on the α1, β1, γδ and α3β4nAChR variants, making it an ideal alternative to GST-21 due to its potency and selectivity.[27] PNU-282987 has been demonstrated to reduce acute lung injury by altering macrophage proliferation in mice [28] and has an additional role in reducing cellular damage due to oxidative stress and can rescue cells from apoptosis in response to mitochondrial inactivation.[29] It is currently unknown if PNU-282987 will reduce ILC2s induce inflammation, and if α7nAChR selectivity will affect the inflammatory signature of ILC2s-induced lung inflammation.

In this paper, we explored the effects of PNU-282987 on ILC2s and to compare the difference between PNU-282987 and GTS-21 on ILC2s using both *in vivo* models and *in vitro* stimulation of isolated ILC2s from mouse lung. Here, we found that PNU-282987 significantly reduces ILC2s-mediated airway inflammation in mice following IL-33 or *Alternaria Alternata* (AA) treatment and that this compound inhibits the proliferation and function of ILC2s *in vitro*. Compared to GTS-21, PNU-282987 had a significantly higher inhibitory effect on IKK and NF-κB both in non-treated and IL-33-treated mice. Our results outline that selectivity for the α7nAChR exhibited by the PNU-282987 compound could be exploited for therapeutic benefit in ILC2s-driven disease such as asthma and application of PNU-282987 analogs in humans should be further investigated as a novel treatment for ILC2s-induced asthma.

MATERIALS AND METHODS

Mice. Six to eight week old C57BL/6J female mice were purchased from Beijing Vitonlihua Company [license No. SCXK (Beijing) 2012 / 0001] and were raised at the SPF animal housing facility, Zhengzhou University. The experiment was approved and permitted by the Animal Ethics Committee of Zhengzhou University (Approval Number: ZZURIB20180120).

Murine model. Mice were intranasally (i.n) administered with recombinant mouse IL-33 (0.5μg/dose, R&D) in the presence or absence of PNU-282987 (20mg/kg, Abcam) or GTS-21 (20mg/kg, Abcam) over three consecutive days. For *Alternaria* experiments, mice were treated with AA (100μg/dose, Greer Labs) in the presence or absence of PNU-282987 or GTS-21 on four consecutive days. Mice were sacrificed by dislocation of the cervical spine on the second day after the last challenge.

Collection of bronchoalveolar lavage fluid (BALF) cells. Mouse tracheas were cannulated, and the lungs were lavaged three times with 0.8 ml of ice-cold PBS for the collection of BALF cells. Supernatants were collected for ELISA. Red blood cells were lysed by using hypotonic red blood cell lysis buffer, and BALF was then centrifuged to collect cellular infiltrate. Total cell numbers were quantified via haemocytometry.

Lung Histology. Lung tissue was stained with hematoxylin and eosin (for histopathology) or periodic acid-Schiff (for mucus-secreting cells). Sections were then stained with chromotrope-hematoxylin or periodic acid-Schiff (PAS). Scorings for

histopathology (inflammatory infiltrates) and PAS (mucus-producing cells) were performed according to a set of morphological criteria, as previously described.[30]

Flow cytometry and FACS sorting of ILC2s. Lung tissue was digested with Liberase TM and DNase (Roche) for single-cell suspensions and treated with Fc block (CD16/CD32, BD Biosciences). Cells were stained in the dark with antibodies targeting KLRG1, Sca-1 and lineage markers. Lung ILC2s were defined as lineage negative (CD3e, CD45R, Ly6G, Ly6C, CD11b, CD11c, Ly-76, NK1.1, TCR-β, and TCR-γδ), KLRG1 positive, and Sca-1 positive. Lineage markers negative cells were enriched by both density gradient centrifugation and magnetic beads isolation and purified by flow cytometry for the collection of ILC2s. Intracellular staining of IL-5 or IL-13 was performed with BD Fixation and Permeabilization Solution (BD Biosciences) according to the manufacturer's instructions. The detected of IKK-P, and NF-κB p65 were carried out in accordance with the manufacturer's instructions. The single-cell suspension made from each group of lung tissue is used to detect $CD3^+$ $CD4^+$ cells and $CD3^-$ $CD19^+$ cells. FACSCanto Ⅱ Flow (BD Biosciences) and MoFlo XDP cell sorter (Beckman Coulter) were employed for flow cytometry and cell sorting. Data were analyzed with software FlowJo version 10.0. All antibodies used in these experiments are shown below:

Antibody	Color	Company
Hamster anti-mouse KLRG1	PE	BD Biosciences
Hamster anti-mouse KLRG1	PerCP-Cy™5.5	BD Biosciences
Rat anti-mouse Sca-1 (Ly-6A/E)	FITC	BD Biosciences
Rat anti-mouse Siglec-F	PE	BD Biosciences
Rat anti-mouse/anti-human IL-5	PE	BD Biosciences
Rat anti-mouse/anti-human IL-13	PE	Invitrogen

Rabbit anti-mouse Phospho-IKKα/β	PE	Cell Signaling Technology
Rabbit anti-mouse Phospho-NF-κB p65	Alexa Fluor®488	Cell Signaling Technology
Mouse anti-Ki-67	Alexa Fluor®647	BD Biosciences
Lineage Negative Cocktail	APC	BD Biosciences
Hamster anti-mouse CD3	PE	BD Biosciences
Anti-mouse CD4	FITC	Biolegend
Rat anti-mouse CD19	APC	BD Biosciences

***In vitro* stimulation of ILC2s.** Purified ILC2s were stimulated with IL-33 (50 ng/mL), IL-2 (20 ng/mL) and IL-7 (20 ng/mL) in the presence or absence of PNU-282987 (20 μM) or GTS-21 (20μM) for 24 or 72 hours.

Proliferation Assay Method. The purified ILC2s cells were counted by placental blue staining and evenly grouped. ILC2s were plated at 1.5×10^4 cells per well and were stimulated with recombinant (r)-IL-33, IL-2, or IL-7 (BD Biosciences) in the presence or absence of PNU-282987 or GTS-21. After 72 hours of culture, the number of cells in each group was counted again, and the expression of Ki67 (BD Biosciences) in each group was detected by flow cytometry.

Quantitative PCR. Total RNA was extracted from whole lung tissue, and the levels of IL-5, IL-13, GATA3, and IL-33 transcripts were quantitated by quantitative real-time RT-PCR (QuantStudioTM 5 systems, Applied Biosystems, Carlsbad, CA) in accordance with the manufacturer's protocol. The primers were as follows:

Primer	Sequence
IL-5 [31]	F-5′-TGAGACGATGAGGCTTCCTG-3′ R-5′-CCACACTTCTCTTTTTGGCGG-3′
IL-13 [31]	F-5′-CCCTCAGCCATGAAATAACT-3′ R-5′-GCGTAACAGGCCATTCTTCC-3′
GATA3 [32]	F-5′-CGAGATGGTACCGGGCACTA-3′ R-5-GACAGTTCGCGCAGGATGT-3′
IL-33 [33]	F-5′-ACTATGAGTCTCCCTGTCCTG-3′

	R-5′-ACGTCACCCCTTTGAAGC-3′

ELISA. IL-5 and IL-13 from BALF and *in vitro* culture supernatants were detected according to the manufacturer's instructions (MultiSciences Biotech, Ltd). Data collection and analysis were performed on a microplate reader (MD CMax Plus).

Statistical analysis. SPSS21.0 software was used for statistical analysis. One-way ANOVA was used to compare multiple samples. Sample size calculation reference to "sample size estimation method for comparison of Multi-independent Group Metrological data" provided by Eugenia Garden Medical website in China. The result is expressed as the mean ± standard error of the mean (SEM). When $P < 0.05$, the difference between samples is considered statistically significant.

RESULTS

Both PNU-282987 and GTS-21 inhibit IL-33-induced airway inflammation.

Here, we explored the effects of PNU-282987 and GTS-21 has in an *in* vivo model of IL-33-driven inflammation in the lung. PAS and HE staining of lung tissue revealed a significant increase of goblet cells in the airway epithelium of the IL-33-exposed group when compared to the PBS group (Fig. 1A). In response to PNU-282987 and GTS-21 treatment, there was a significant reduction in goblet cell hyperplasia and mucus production in airways and reduced the histopathological scores following IL-33 exposure (Fig. 1A). We examined the infiltration of eosinophils and ILC2s in BALF, and demonstrated that both compounds drastically decreased the migration of both cells (Fig. 1B). Despite this, populations of $CD4^+$ T cells and $CD19^+$ B cells in the three groups were unchanged between the IL-33 treated groups (Fig. 1C).

PNU-282987 and GTS-21 attenuates the production of IL-5 and IL-13 secreted by ILC2s *in vivo*.

Next, we determined whether PNU-282987 and GTS-21 inhibit the secretion of IL-5 and IL-13 by ILC2s. RNA extracted from mouse lung tissue was used to determine the transcription levels of IL-5 and IL-13, and the protein levels of both interleukins were also determined. While the expression of IL-5 and IL-13 mRNA and protein in the IL-33 treatment group were significantly higher than in the control PBS group, PNU-282987 significantly decreased the levels of IL-5 and IL-13 transcripts in lung and protein in BALF following IL-33 administration (Fig. 2A & B). Although this

response was comparable to GTS-21 administration, levels of IL-13 mRNA in IL-33/GTS-21 group were lower than that in IL-33/PNU-282987 group. Despite this, there was no difference in the BALF protein level of IL-13 between the two groups (Fig. 2B). Furthermore, there was no significant difference between PNU-282987 and GTS-21 in inhibition of IL-5$^+$ILC2s and IL-13$^+$ILC2s induced by IL-33 (Fig. 2C). No IL-5 or IL-13-secreting ILC2s was identified in PBS, PNU-282987, or GTS-21 treatment groups (Data not shown).

PNU-282987 and GTS-21 abolish AA-mediated airway inflammation.

We then investigated the anti-inflammatory effects of PNU-282987 on ILC2s-regulated airway inflammation, in comparison to that of GTS-21, in AA challenged animals. Our experiments confirmed that the level of IL-33 mRNA in the AA treatment group was significantly higher than that in the PBS group, which is reduced to baseline levels after PNU-282987 and GTS-21 administration (Fig 3A). Furthermore, treatments with PNU-282987 or GTS-21 attenuated the AA-induced airway inflammation when we examined the histopathological changes of lung tissue similar to what was observed post-IL-33 treatment (Fig. 3B). Levels of eosinophil in BALF and ILC2s in lung tissues in the AA/PNU-282987 and AA/GTS-21 groups were significantly lower than those of AA only group (Fig. 3C), and there was no difference in the levels of CD4$^+$ T cells and CD19$^+$ B cells in lung between AA, AA/PNU-282987 and AA/GTS-21 groups (Fig. 3D).

PNU-282987 and GTS-21 inhibit the production of IL-5 and IL-13 in the lung of AA-challenged mice.

Similar to what occurred in the IL-33 model, PNU-282987 inhibited the production of IL-5 and IL-13 by ILC2s isolated from mice that were exposed to AA, both at transcription and protein levels (Fig. 4A & B). Furthermore, intracellular staining revealed that the levels of IL-5 and IL-13 producing ILC2s in the AA/PNU-282987 and AA/GTS-21 groups were profoundly reduced compared to the AA alone group (Fig. 4C), however there is no difference between PNU-282987 and GTS-21 in the inhibition of the IL-5 and IL-13 producing function of ILC2s.

PNU-282987 and GTS-21 inhibited the proliferation and functional activation of ILC2s *in vitro.*

Finally, we examined what transcription factors were altered in ILC2s by PNU-282987 when cultured under proliferation conditions and compared the activity of ILC2s-differentiating transcription factor expression to that of GTS-21 treated cultures. Fig. 5A & B showed that PNU-282987 and GTS-21 had the same effect on inhibiting the secretion of IL-5 and IL-13 by ILC2s. Levels of ILC2s from mouse lung tissue that were cultured with IL-2 + IL-7 and/or IL-33 experienced a significantly decreased proliferation of ILC2s if PNU-282987 or GTS-21 was administered to the culture (Fig. 5C). In accordance with the above results, Ki67, a nuclear non-histone that represents cell proliferation, was significantly decreased in the PNU-282987 and GTS-21 groups following IL-33 exposure (Fig. 5D) as was the

Th2-associated transcription factor GATA3 (Fig. 5E).[18] Both IKK and NF-κB (p65) have a crucial role in the proliferation and activation of ILC2s.[34] Our results show that both PNU-282987 and GTS-21 could inhibit the phosphorylation of IKK and NF-κB p65. However, the inhibitory effect of PNU-282987 on the phosphorylation of IKK and NF-κB p65 was significantly higher than that of GTS-21 24 hours after treatments (Fig. 5F & G). Seventy-two hours post-stimulation, the phosphorylation of these two transcriptional regulators were not detectable (data not shown).

DISCUSSION

Fungi are a common household allergens associated with asthma, where fungal proteases have been shown to be potent initiator of allergic inflammatory cascades.[35] Such cascades induce the release of alarmin factors (e.g., IL-33), which drive the activation of ILC2s and increase secretions of Th2 cytokines. The recent discovery of vagus nerve neuro-regulation of ILC2s through CAP and the α7nAChR has highlighted a novel pathway that could be exploited to attenuate asthma, which has previously been demonstrated with the α7nAChR agonist GTS-21.[22] Here, we demonstrated that the agonist PNU-282987 not only exhibits the same inhibitory effects of GTS-21, but also acts as a potent suppressor of IKK and NF-κB activity in ILC2s cells.

Using both recombinant IL-33 and AA in mouse models to induce airway inflammation, we have demonstrated that PNU-282987 reduces eosinophil and ILC2s numbers in BALF and in lung tissue and decreases goblet cell hyperplasia in the airway. In both IL-33 and AA exposed mice, PNU-282987 and GTS-21 not only reduced the proliferation of ILC2s, but also inhibited the ability of ILC2s to secrete IL-5 and IL-13 *in vivo*, suggesting that both PNU-282987 and GTS-21 reduce Th2 cytokines by acting on ILC2s. Notably, these two cytokines are involved in eosinophil recruitment, AHR, and mucus production.[36] We also observed a marked reduction in IL-33 expression in response to PNU-282987. It has been reported that airway epithelial cells express α7nAChR and are affected by exogenous stimuli such as

nicotine.[37, 38] Combined with our results, we suspect that PNU-282987 not only directly acts on ILC2s, but also indirectly inhibits the proliferation and functional activation of ILC2s by binding to α7nAChR expressed in airway epithelial cells to inhibit the release of IL-33 from airway epithelial cells.

Acetylcholine is a major parasympathetic neurotransmitter that has previously been shown to effectively inhibit the release of the inflammatory cytokines, such as TNFα, from peripheral macrophages stimulated by LPS *in vitro*.[13] Stimulation of efferent vagus nerve can inhibit systemic inflammation, as demonstrated in a study that the level of TNFα in serum and liver decreased significantly in Lewis rats with endotoxemia after cervical vagotomy by electric stimulation.[13] Furthermore, the release of norepinephrine by vagus nerve stimulates memory T cells to secrete acetylcholine for the negative regulation of inflammation on α7nAChR positive cells.[39, 40] Our finding that ILC2s-driven airway inflammation is negatively regulated by α7nAChR is supported by the observation that ILC2s activity is reduced by memory T-cell release of acetylcholine.[41] ILC2s express β_2-adrenergic receptors (that respond to norepinephrine), which, when activated, will agonistically suppress ILC2s proliferation and secretion of Th2 cytokines.[42] While this study only focused on exploiting α7nAChR-agonism to regulate ILC2s inflammation, the effects of norepinephrine on the inflammatory cascades explored above should be further investigated to determine if any overlap exists between the two neuro-inhibitory pathways.

The expression of the cell proliferation marker, Ki67, and the transcription factors, GATA3, which are essential for cell proliferation and the production of IL-5 and IL-13, was down-regulated in IL-33/PNU-282987 group when compared to IL-33 group. This was not surprising, as α7nAChR-mediated pathways have been associated with JAK2-STAT3 and MyD88-IKK-NF-κB inflammatory pathways.[43, 44] STAT3 activated by α7nAChR is a negative regulator of inflammatory response, and in α7nAChR/IKK/NF-κB signaling axis, α7nAChR further inhibits the nuclear translocation of NF- κB by inhibiting the phosphorylation of upstream signal IKK.[45] As Dowling *et al.* observed, nicotine alone can hinder the NF- κB signal pathway by binding to α7nAChR, supporting the notion that the anti-inflammatory mechanism of α7nAChR may be related by IKK and NF- κB transcriptional regulators.[46, 47]

α7nAChR interaction with I-κB kinase (IKK) and NF-κB as I-κB kinase (IKK) induces NF- κB nuclear activation in isolated ILC2s,and that PNU-282987 inhibited the expression of IKK and p65 at a significantly higher degree when compared with GTS-21. For the different inhibitory effects of PNU-282987 and GTS-21 on IKK and NF-κB, it is likely that binding ability of GTS-21 to other nicotine receptors results in reduced specificity and receptor activation for GTS-21, a feature not observed with PNU-282987.[48] While the effectiveness of PNU-282987 and GTS-21 in inhibiting the function of ILC2s was comparable to each other, the former one had a stronger inhibitory effect on IKK and NF-κB, which play a vital role in the growth and development of ILC2s.[34] Through these experiments, we have demonstrated that

PNU-282987 inhibits airway inflammation associated with asthma by attenuating the proliferation and function of ILC2s.

In accordance with our data, the viability of ILC2s in culture and *in vivo* was successfully inhibited by PNU-282987 through GATA3, Ki67, and IKK/NF-κB. Although this mechanism needs to be explored further, it is clear that our results show that PNU-282987 is useful in the treatment of ILC2s-mediated airway inflammation induced by IL-33 and AA and employing analogs of PNU-282987 in asthmatic patients could alleviate airway inflammation caused by ILC2s. The importance of cholinergic anti-inflammatory pathway and of α7nAChR as a pharmacological target for the treatment of inflammatory diseases needs further investigation. More importantly, the notable inhibitory effect PNU-282987 has on IKK and NF-κB should warrant further attention both in the context of PNU-282987 function and ILC2s proliferation.

FOOTNOTES:

Acknowledgments

We thank Dr. Zheng Huaixin at Zhengzhou University for the revision of this article.

Disclosure Statement

The authors have no conflicts of interest to declare.

Financial Support: This work was supported by Henan Provincial Higher Education Key Research Project Grant (18A310030).

Author Contributions

Performed experiments: FY, LLJ, YYWY, WLL, HLT. Analyzed and interpreted data: FY, XJL, LJZ, FGL, MY. Conceived and designed experiments: FGL, MY. Wrote and edited manuscript: FY, LS, GJZ, FGL, MY.

Author notes

# Fang Yuan and LiLi Jiang equally contributed to this work. * Ming Yang and Fuguang Li equally contributed to this work.

REFERENCES

1 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med.* 1996; **183**:195-201.

2 Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science.* 1998; **282**:2258-61.

3 Kim HY, DeKruyff RH, Umetsu DT. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat Immunol.* 2010; **11**:577-84.

4 Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, Furusawa J, Ohtani M, Fujii H, Koyasu S. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature.* 2010; **463**:540-4.

5 Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, Bucks C, Kane CM, Fallon PG, Pannell R, Jolin HE, McKenzie AN. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* 2010; **464**:1367-70.

6 Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity.* 2001; **15**:985-95.

7 Kim BS, Siracusa MC, Saenz SA, Noti M, Monticelli LA, Sonnenberg GF, Hepworth MR, Van Voorhees AS, Comeau MR, Artis D. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med.* 2013; **5**:170ra16.

8 Oboki K, Ohno T, Kajiwara N, Arae K, Morita H, Ishii A, Nambu A, Abe T, Kiyonari H, Matsumoto K, Sudo K, Okumura K, Saito H, Nakae S. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A.* 2010; **107**:18581-6.

9 Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, Huang LC, Johnson D, Scanlon ST, McKenzie AN, Fallon PG, Ogg GS. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med.* 2013; **210**:2939-50.

10 O'Neill LA. The role of MyD88-like adapters in Toll-like receptor signal transduction. *Biochem Soc Trans.* 2003; **31**:643-7.

11 Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity.* 2012; **36**:451-63.

12 Tracey KJ. The inflammatory reflex. *Nature.* 2002; **420**:853-9.

13 Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature.* 2000; **405**:458-62.

14 Nizri E, Hamra-Amitay Y, Sicsic C, Lavon I, Brenner T. Anti-inflammatory properties of cholinergic up-regulation: A new role for acetylcholinesterase inhibitors. *Neuropharmacology.* 2006; **50**:540-7.

15 Skok MV, Grailhe R, Agenes F, Changeux JP. The role of nicotinic receptors in B-lymphocyte development and activation. *Life Sci.* 2007; **80**:2334-6.

16 Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature.* 2003; **421**:384-8.

17 Chung YC, Ko HW, Bok E, Park ES, Huh SH, Nam JH, Jin BK. The role of neuroinflammation on the pathogenesis of Parkinson's disease. *BMB Rep.* 2010; **43**:225-32.

18 Ghia JE, Blennerhassett P, Collins SM. Impaired parasympathetic function increases susceptibility to inflammatory bowel disease in a mouse model of depression. *J Clin Invest.* 2008; **118**:2209-18.

19 Greene CM, Ramsay H, Wells RJ, O'Neill SJ, McElvaney NG. Inhibition of Toll-like receptor 2-mediated interleukin-8 production in Cystic Fibrosis airway epithelial cells via the alpha7-nicotinic acetylcholine receptor. *Mediators Inflamm.* 2010; **2010**:423241.

20 Marrero MB, Lucas R, Salet C, Hauser TA, Mazurov A, Lippiello PM, Bencherif M. An alpha7 nicotinic acetylcholine receptor-selective agonist reduces weight gain and metabolic changes in a mouse model of diabetes. *J Pharmacol Exp Ther.* 2010; **332**:173-80.

21 Parrish WR, Rosas-Ballina M, Gallowitsch-Puerta M, Ochani M, Ochani K, Yang LH, Hudson L, Lin X, Patel N, Johnson SM, Chavan S, Goldstein RS, Czura CJ, Miller EJ, Al-Abed Y, Tracey KJ, Pavlov VA. Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling. *Mol Med.* 2008; **14**:567-74.

22 Galle-Treger L, Suzuki Y, Patel N, Sankaranarayanan I, Aron JL, Maazi H, Chen L, Akbari O. Nicotinic acetylcholine receptor agonist attenuates ILC2-dependent airway hyperreactivity. *Nat Commun.* 2016; **7**:13202.

23 Li F, Chen Z, Pan Q, Fu S, Lin F, Ren H, Han H, Billiar TR, Sun F, Li Q. The protective effect of PNU-282987, a selective alpha7 nicotinic acetylcholine receptor agonist, on the hepatic ischemia-reperfusion injury is associated with the inhibition of high-mobility group box 1 protein expression and nuclear factor kappaB activation in mice. *Shock.* 2013; **39**:197-203.

24 Yue Y, Liu R, Cheng W, Hu Y, Li J, Pan X, Peng J, Zhang P. GTS-21 attenuates lipopolysaccharide-induced inflammatory cytokine production in vitro by modulating the Akt and NF-kappaB signaling pathway through the alpha7 nicotinic acetylcholine receptor. *Int Immunopharmacol.* 2015; **29**:504-12.

25 Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu Rev Pharmacol Toxicol.* 2007; **47**:699-729.

26 Yohn NL, Turner JR, Blendy JA. Activation of alpha4beta2*/alpha6beta2* nicotinic receptors alleviates anxiety during nicotine withdrawal without upregulating nicotinic receptors. *J Pharmacol Exp Ther.* 2014; **349**:348-54.

27 Bodnar AL, Cortes-Burgos LA, Cook KK, Dinh DM, Groppi VE, Hajos M, Higdon NR, Hoffmann WE, Hurst RS, Myers JK, Rogers BN, Wall TM, Wolfe ML, Wong E. Discovery and structure-activity relationship of quinuclidine benzamides as agonists of alpha7 nicotinic acetylcholine receptors. *J Med Chem.* 2005; **48**:905-8.

28 Pinheiro NM, Santana FP, Almeida RR, Guerreiro M, Martins MA, Caperuto LC, Camara NO, Wensing LA, Prado VF, Tiberio IF, Prado MA, Prado CM. Acute lung injury is reduced by the alpha7nAChR agonist PNU-282987 through changes in the macrophage profile. *FASEB J.* 2017; **31**:320-32.

29 Parada E, Egea J, Romero A, del Barrio L, Garcia AG, Lopez MG. Poststress treatment with PNU282987 can rescue SH-SY5Y cells undergoing apoptosis via alpha7 nicotinic receptors linked to a Jak2/Akt/HO-1 signaling pathway. *Free Radic Biol Med.* 2010; **49**:1815-21.

30 Nguyen TH, Maltby S, Tay HL, Eyers F, Foster PS, Yang M. Identification of IFN-gamma and IL-27 as Critical Regulators of Respiratory Syncytial Virus-Induced Exacerbation of Allergic Airways Disease in a Mouse Model. *J Immunol.* 2018; **200**:237-47.

31 Csoka B, Nemeth ZH, Duerr CU, Fritz JH, Pacher P, Hasko G. Adenosine receptors differentially regulate type 2 cytokine production by IL-33-activated bone marrow cells, ILC2s, and macrophages.

FASEB J. 2018; **32**:829-37.

32 Fang TC, Yashiro-Ohtani Y, Del Bianco C, Knoblock DM, Blacklow SC, Pear WS. Notch directly regulates Gata3 expression during T helper 2 cell differentiation. *Immunity.* 2007; **27**:100-10.

33 Qian G, Jiang W, Zou B, Feng J, Cheng X, Gu J, Chu T, Niu C, He R, Chu Y, Lu M. LPS inactivation by a host lipase allows lung epithelial cell sensitization for allergic asthma. *J Exp Med.* 2018; **215**:2397-412.

34 Ather JL, Foley KL, Suratt BT, Boyson JE, Poynter ME. Airway epithelial NF-kappaB activation promotes the ability to overcome inhalational antigen tolerance. *Clin Exp Allergy.* 2015; **45**:1245-58.

35 Porter P, Susarla SC, Polikepahad S, Qian Y, Hampton J, Kiss A, Vaidya S, Sur S, Ongeri V, Yang T, Delclos GL, Abramson S, Kheradmand F, Corry DB. Link between allergic asthma and airway mucosal infection suggested by proteinase-secreting household fungi. *Mucosal Immunol.* 2009; **2**:504-17.

36 Yang M, Hogan SP, Mahalingam S, Pope SM, Zimmermann N, Fulkerson P, Dent LA, Young IG, Matthaei KI, Rothenberg ME, Foster PS. Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperreactivity. *J Allergy Clin Immunol.* 2003; **112**:935-43.

37 Nastrucci C, Russo P. alpha7 nAChR in airway respiratory epithelial cells. *Curr Drug Targets.* 2012; **13**:666-70.

38 Su X, Lee JW, Matthay ZA, Mednick G, Uchida T, Fang X, Gupta N, Matthay MA. Activation of the alpha7 nAChR reduces acid-induced acute lung injury in mice and rats. *Am J Respir Cell Mol Biol.* 2007; **37**:186-92.

39 Maldifassi MC, Martin-Sanchez C, Atienza G, Cedillo JL, Arnalich F, Bordas A, Zafra F, Gimenez C, Extremera M, Renart J, Montiel C. Interaction of the alpha7-nicotinic subunit with its human-specific duplicated dupalpha7 isoform in mammalian cells: Relevance in human inflammatory responses. *J Biol Chem.* 2018; **293**:13874-88.

40 Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, Tusche MW, Pavlov VA, Andersson U, Chavan S, Mak TW, Tracey KJ. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science.* 2011; **334**:98-101.

41 Zhou Y, Wang W, Zhao C, Wang Y, Wu H, Sun X, Guan Y, Zhang Y. Prostaglandin E2 Inhibits Group 2 Innate Lymphoid Cell Activation and Allergic Airway Inflammation Through E-Prostanoid 4-Cyclic Adenosine Monophosphate Signaling. *Front Immunol.* 2018; **9**:501.

42 Moriyama S, Brestoff JR, Flamar AL, Moeller JB, Klose CSN, Rankin LC, Yudanin NA, Monticelli LA, Putzel GG, Rodewald HR, Artis D. beta2-adrenergic receptor-mediated negative regulation of group 2 innate lymphoid cell responses. *Science.* 2018; **359**:1056-61.

43 Chen K, Sun Y, Diao Y, Ji L, Song D, Zhang T. alpha7 nicotinic acetylcholine receptor agonist inhibits the damage of rat hippocampal neurons by TLR4/Myd88/NFkappaB signaling pathway during cardiopulmonary bypass. *Mol Med Rep.* 2017; **16**:4770-6.

44 de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, Berthoud HR, Uematsu S, Akira S, van den Wijngaard RM, Boeckxstaens GE. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol.* 2005; **6**:844-51.

45 Yoshikawa H, Kurokawa M, Ozaki N, Nara K, Atou K, Takada E, Kamochi H, Suzuki N. Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine

receptor alpha7. *Clin Exp Immunol.* 2006; **146**:116-23.

46 Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol.* 1996; **14**:649-83.

47 Dowling O, Rochelson B, Way K, Al-Abed Y, Metz CN. Nicotine inhibits cytokine production by placenta cells via NFkappaB: potential role in pregnancy-induced hypertension. *Mol Med.* 2007; **13**:576-83.

48 Kem WR. Alzheimer's drug design based upon an invertebrate toxin (anabaseine) which is a potent nicotinic receptor agonist. *Invert Neurosci.* 1997; **3**:251-9.

FIGURE LEGENDS

Figure 1: Compared with GTS-21, PNU-282987 has the same inhibitory effect on ILC2s-mediated airway inflammation. C57BL/6J mice intranasally challenged with recombinant mouse IL-33 or PBS and also received PNU-282987/GTS-21 or PBS on days 1-3. Assessment of lung tissue and analysis of bronchoalveolar lavage fluid (BALF) (A) Periodic acid-Schiff reagent lung section (×200, Scale bars at 100μm). Scorings for histopathology and PAS were assessed. (B) Total number of eosinophils and ILC2s in BALF determined by flow cytometry. (C) Total number of $CD4^+$ T cells and $CD19^+$ B cells determined by flow cytometry in whole lung. Data are representative of at least four independent experiments and presented as means ± s.e.m (n=4; $^{**}P < 0.01$; $^{***}P < 0.001$).

Figure 2: **PNU-282987 and GTS-21 attenuate the production of type 2 cytokines secreted by ILC2s *in vivo*.** (A, B) mRNA levels of IL-5 and IL-13 of mouse lung tissue and the protein levels of IL-5 and IL-13 in BALF were measured by qPCR and ELISA respectively. (C) Percentage of $IL\text{-}5^+$ILC2s and $IL\text{-}13^+$ILC2s in whole lung determined by flow cytometry. Data are representative of at least four independent experiments and presented as means ± s.e.m (n=6; $^{**}P < 0.01$; $^{***}P < 0.001$).

Figure 3: PNU-282987 and GTS-21 inhibit Alternaria-mediated airway inflammation. C57/BL6J mice intranasally received an extract of Alternaria (AA), AA with or without PNU-282987/GTS-21 on days 1-4. (A) Gene expression of IL-33 in lung tissue. (B) PAS staining (×200, Scale bars at 100μm). Scoring for histopathology and PAS was assessed by light microscopy. (C) Total number of EOS

and ILC2s in BALF. (D) Total number of $CD4^+$ T cells and $CD19^+$ B cells determined by flow cytometry in whole lung. Data are representative of at least four independent experiments and presented as means ± s.e.m (n=4; **P* < 0.05; ***P* < 0.01; ****P* < 0.001).

Figure 4: PNU-282987 and GTS-21 inhibit the function of ILC2s in Alternaria-mediated Airway inflammation. (A, B) The mRNA levels of IL-5 and IL-13 of lung tissue and the protein levels of IL-5 and IL-13 in BALF were measured by qPCR and ELISA. (C) Percentage of $IL\text{-}5^+$ILC2s and $IL\text{-}13^+$ILC2s was determined by flow cytometry. Data are representative of at least four independent experiments and presented as means ± s.e.m (n=6; **P* < 0.05; ***P* < 0.01; ****P* < 0.001).

Figure 5: **PNU-282987 and GTS-21 inhibit the proliferation and functional activation of ILC2s *in vitro*.** The levels of IL-5 (A) and IL-13 (B) from cultured ILC2s were measured by ELISA and qPCR. Lin^- cells from C57/BL6J female mice were first separated by magnetic beads, and ILC2s were isolated by FACS. (C) Cell count of ILC2s and (D) Percentage of $Ki67^+$cells in isolated lung ILC2s, stimulated with IL-33 in the absence and presence of PNU-282987/GTS-21 for 72 hours. (E) Mean fluorescence intensity and the mRNA levels of GATA3 in isolated lung ILC2s were analyzed by flow cytometry and qPCR. MFI of phosphorylated IKKα/β (F) and NF-κB p65 (G) with or without PNU-282987/GTS-21 for 24 hours. Data are representative of at least four independent experiments and presented as means ± s.e.m (n=5; **P* < 0.05; ***P* < 0.01; ****P* < 0.001).