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Anti-inflammatory effects of alpha 7 nicotinic acetylcholine receptors through interacting with adenylyl cyclase 6

Running title: CHRNA7 interacts with AC6

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The authors declare that they have no conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Author contributions

C. X., S. Z., and S. H. and conceived and designed the study, analyzed the data, and wrote the manuscript. S. Z., S. H., and G. X. performed the experiments and analyzed the data. J. W., Y. S., Y. W., and R. O. analyzed the data and critically viewed. C. X., C. S., and A. D. wrote the manuscript, and critically viewed and supervised the study. All authors read and approved the final manuscript.

Abbreviations: AC, adenylyl cyclase; BALF, bronchoalveolar lavage fluid; BMDM, bone marrow-derived macrophage; cAMP, cyclic adenosine monophosphate; CHRNA7, alpha 7 nicotinic acetylcholine receptor; COPD, chronic obstructive pulmonary disease; FBS, fetal bovine serum; Fsk, forskolin; IFN-β, interferon β; MβCD, methyl-beta-cyclodextrin; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NF-κB, nuclear factor-κB; PPE, porcine pancreatic elastase; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α.

ABSTRACT

Background and purpose: Alpha 7 nicotinic acetylcholine receptors (CHRNA7) suppress inflammation through diverse pathways in immune cells, so is potentially involved in a number of inflammatory diseases. However, the detailed mechanisms underlying CHRNA7's anti-inflammatory effects remain elusive.

Experimental approach: The anti-inflammatory effects of CHRNA7 agonists in both murine macrophages (RAW 264.7) and bone marrow-derived macrophages (BMDM) stimulated with LPS were examined. The role of adenylyl cyclase 6 (AC6) in Toll-like Receptor 4 (TLR4) degradation was explored via overexpression and knockdown. A mouse model of chronic obstructive pulmonary disease was used to confirm key findings.

Results: Anti-inflammatory effects of CHRNA7 were largely dependent on AC6 activation, as knockdown of AC6 considerably abnegated the effects of CHRNA7 agonists while AC6 overexpression promoted them. We found that CHRNA7 and AC6 are co-localized in lipid rafts of macrophages and directly interact. Activation of AC6 led to the promotion of TLR4 degradation. Administration of CHRNA7 agonist PNU-282987 attenuated pathological and inflammatory end points in a mouse model of chronic obstructive pulmonary disease (COPD).

Conclusion and implications: CHRNA7 inhibits inflammation through activating AC6 and promoting degradation of TLR4. The use of CHRNA7 agonists may represent a novel therapeutic approach for treating COPD and likely other inflammatory diseases.

Key words: CHRNA7, adenylyl cyclase 6, macrophage, inflammation, COPD

Bullet point summary

What is already known

CHRNA7 suppresses inflammation through multiple pathways in immune cells;

AC6 activation shifts the endocytosis of TLR4, and contributes to the degradation of TLR4;

What this study adds

CHRNA7 interacts with AC6, while CHRNA7 agonist stimulation promotes the interaction;

CHRNA7 activation promotes AC6-mediated TLR4 degradation.

Clinical significance

We have identified AC6 as a new pathway for the anti-inflammatory effect induced by CHRNA7 activation.

Introduction

Besides its paramount role in recognizing and eliminating invading pathogens, inflammation is also indispensable for tissue repair and restoration of homeostasis following injury. However, dysregulated inflammation can lead to cancer, degenerative disorders, autoimmune and other inflammatory diseases such as diabetes and atherosclerosis (Furman et al., 2019; Wolf & Ley, 2019). The physiological regulation of the immune system encompasses comprehensive anti-inflammatory mechanisms that can be harnessed for the treatment of infectious and inflammatory disorders (Netea et al., 2017). Thus, understanding anti-inflammatory signaling could lead to novel therapeutic approaches for controlling inflammatory diseases. Macrophages play an essential role in regulating inflammation and thus are intensely studied (Glass & Natoli, 2016). When stimulated by pathogens, macrophages mount an inflammatory response to eliminate the invading microbes by increasing phagocytosis and production of pro-inflammatory cytokines such as TNF-α (Wynn et al., 2013). Macrophages then convert to an anti-inflammatory phenotype after the elimination of the pathogen in order to maintain homeostasis. Macrophages are also an active player in tissue repair processes following injury.

Neural-immune interaction, specifically the neuronal regulation of inflammation, plays a role in the pathogenesis of many diseases (Reardon et al., 2018). Immunologists and neuroscientists have largely focused on macrophages as key cells mediating the

neural influence of the immune system. Studies have shown that vagal nerve stimulation inhibits macrophage production of pro-inflammatory cytokines such as TNF-a and IL-6, while having no effect on levels of the anti-inflammatory cytokine IL-10 (Borovikova et al., 2000). This vagal anti-inflammatory pathway is reliant upon acetylcholine, the principle neurotransmitter of the vagal nerve (Tracey et al., 2002). Other studies reveal that the cholinergic anti-inflammatory pathway depends on the α7 nicotinic acetylcholine receptor (CHRNA7) (Chu et al., 2020; Wang et al., 2003). CHRNA7 suppresses inflammation via multiple pathways, including through decreasing NF-kB activity, activating JAK2/STAT3 signaling (de Jonge et al., 2005), and preventing mitochondrial DNA release and inflammasome activation (Lu et al., 2014). CHRNA7 regulates autophagy in monocytes/microglia and the CHRNA7 agonist, PNU-282987, dampens the production of cytokines and attenuates experimental autoimmune encephalomeylitis (Shao et al., 2017). These data suggest a complicated regulatory network through which CHRNA7 modulates inflammation, but the molecular mechanism through which these receptors alter functions of immune cells is unresolved.

The second messenger cAMP has multiple downstream effectors, with protein kinase A as the most prominent. cAMP also activates the Epac, a guanine-nucleotide-exchange factor, and Popeye domain-containing proteins (Rooij et al., 1998; Schindler & Brand, 2016). These effectors signal via a wide array of

downstream pathways to modulate various functions of the immune system (Raker et al., 2016). cAMP exerts potent influence on both innate and adaptive immune cells, such as macrophages, dendritic cells, as well as B and T cells alike (Arumugham & Baldari, 2017). For example, cAMP stabilizes FoxP3 expression through cAMP-response element binding factor (CREB) and is required for the generation and maintenance of regulatory T cells (Kim & Leonard, 2007). In macrophages, increased cAMP levels appear to attenuate inflammation (Raker et al., 2016).

Adenylyl cyclases (AC) produce cAMP and various agents that stimulate AC activity, such as prostaglandin E2 and forskolin (Fsk), inhibit inflammation through dampening NF-κB signaling and IFN-β at both the mRNA and protein levels (Song et al., 2007; Xu et al., 2008). There are nine transmembrane AC isoforms, each with different amino acid sequences, tissue and chromosomal distribution, and regulatory properties (Dessauer et al., 2017; Johnstone et al., 2018). We have reported that AC4 and AC6 are expressed in bone-marrow derived macrophages, but only AC6 modulates inflammatory responses. It does so by shifting the endocytosis of TLR4 from a clathrin-mediated pathway to a lipid raft-mediated pathway, thereby promoting the degradation of TLR4 in lysosomes (Cai et al., 2013).

In the present study we explored whether CHRNA7 dampens macrophage-mediated inflammation via interacting with AC6. Our data illustrate that CHRNA7 works through

AC6 activation, cAMP production, and modulation of TLR4 trafficking and degradation. Furthermore, the CHRNA7 agonist, PNU-282987, reduced inflammatory endpoints in a COPD-like mouse model. This mechanism reveals how cAMP signaling by a specific AC isoform can negatively regulate TLR4 signaling and function. Our novel findings provide a mechanistic link between neuronal activation and immunomodulation.

Materials and Methods

Reagents and antibodies

LPS from *Salmonella* Minnesota R595 was purchased from Sigma (St. Louis, MO). PNU-282987 was obtained from Sigma (St. Louis, MO). MD2-IN-1 was purchased from Selleck (Shanghai, China). TLR4 antibody (clone Sa15-21, AB_2561873) was purchased from BioLegend (San Diego, CA). All the other antibodies for FACS analysis were from BioLegend (San Diego, CA). Antibodies for β-actin (AB_2223515), AC4 (AB_2273639) and AC6 (AB_2305205) isoforms were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for CHRNA7 (AB_296838) was purchased from Abcam (Cambridge, UK). HRP-conjugated anti-mouse (AB_10015289) and anti-rabbit ((AB_2313567) secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture and transfection

RAW 264.7 cells (CVCL_0493) and HEK 293 cells (CVCL_0045) were purchased from American Type Culture Collection (ATCC, Bethesda, MD) and cultured as described (Xu et al., 2011). Briefly, RAW 264.7 cells and HEK 293 cells were cultured in DMEM medium supplemented with 10% FBS, and 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, CA). HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). RAW 264.7 cells

were transfected with TransIT-Jurket (Mirus Bio, Madison, WI) according to the manufacturer's instructions.

Bone marrow-derived macrophages (BMDMs) were obtained as described (Cai et al., 2013; Kobayashi et al., 2002). Briefly, cells were isolated by flushing the bone marrow from femurs and tibias, and then maintained in DMEM medium supplemented with 20% FBS and 30% L929 supernatant containing CSF. Six days later, adherent macrophages were dissociated and resuspended in DMEM supplemented with 10% FBS. Overexpression and knockdown of AC4 or AC6 in BMDMs were performed as previously described (Cai et al., 2013).

Animals

Female C57BL/6 mice (6 to 8 weeks) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences and were kept under specific pathogen-free conditions in the animal center of Shanghai Jiao Tong University School of Medicine (Shanghai, China). Mice were housed 5 per cage in individually ventilated cages (325 × 202 × 140 mm) with external water bottle and wire bar feeders. Food and water were provided ad libitum. Bedding consisted of wood chips and animals were kept at 22°C and 40-60% relative humidity with 12 hour light/dark cycles. All mouse experiments were approved by the Animal Welfare & Ethics Committee of the Shanghai Jiao Tong University School of Medicine. All efforts were made to minimize suffering. Animal studies are reported in compliance with the ARRIVE

guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

Measurement of AC activity

AC activities were measured in membranes of RAW 264.7 and BMDM as previously described (Bogard et al., 2011). Briefly, cells were homogenized, centrifuged at low speed, then the supernatant was transferred to a centrifuge tube and centrifuged at 5,000 g for 10 min. The pellet was suspended and added to tubes containing drug and AC assay buffer. Reactions were stopped by boiling and cAMP content was measured by enzyme immunoassay (EIA, GE Healthcare). AC activity was normalized to the amount of protein per sample as determined using a dye-binding protein assay (Bio-Rad, Hercules, CA).

Luciferase reporter assay

For luciferase reporter assay, we utilized lentivirus based on pLenti CMV V5-LUC Blast containing IFN-β or ELAM-1 and AC6 or AC4 (Xu et al., 2011). Recombinant lentivirus was used to infect cells. Twenty-four to forty-eight hours later, the cells were treated with LPS (100 ng/ml) for 6 h, then the cells were lysed, and luciferase activity was determined using reagents from Promega (Madison, WI). Relative luciferase activities were calculated as fold induction compared with matched unstimulated vector controls. This normalization of the data was performed to reduce the

interexperimental variability of raw baseline and maximal luciferase activity that is inherent in these studies due to differing levels of transfection in each experiment. The data are presented as mean ± SD of at least five independent experiments.

Immunoblotting

Immunoblotting was performed as previously described (Xu et al., 2009). Cells with or without treatment were collected and lysed in lysis buffer containing 1% NP-40. Following brief vortexing and rotation, cell lysate was separated by SDS-PAGE and transferred to PVDF membranes. These membranes were blocked with 5% fat-free milk in PBST (154 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, and 0.01% tween-20) and incubated with primary antibody and then with the appropriate HRP-conjugated secondary antibody. After subsequent washes, the immunoreactive bands were detected with ECL plus immunoblotting detection reagents (Amersham Pharmacia Biotech). All immunoblot studies were conducted in accordance with *British Journal of Pharmacology* Guidelines (Alexander et al., 2018).

RT-PCR

RT-PCR for AC isoforms was performed using the specific primer pairs. Total RNA was extracted from BMDM cells using TRIzol® reagent (Invitrogen, Carlsbad, CA), and the RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers, followed by quantitative PCR using the FastStart

Universal SYBR Green Master Kit (Roche) and an ABI PRISM 7900HT system (Applied Biosystems, Waltham, MA, USA). The reaction protocol used was 95°C 5 min, 35 cycles with 95°C 15 sec, 60°C 60 sec, and 72°C 5 min. The gene of interest expression was normalized to the reference gene, Actb, and was calculated with the $2^{-\Delta\Delta Ct}$ method.

Nondetergent isolation of lipid raft and non-lipid raft membranes

Cells were fractionated as described previously (Cai et al., 2013). Briefly, 1 ml of cellular homogenate was brought to 45% sucrose, and a discontinuous sucrose gradient was layered on top of the sample by placing 2 ml of 35% sucrose and then 1 ml of 5% sucrose. After centrifuge at 250,000g for 16 to 18 h at 4°C, the faint light-scattering band was collected from the 5 to 35% sucrose interface (lipid raft fractions), and the bottom of the gradient (45% sucrose) was collected as non-lipid raft material. Raft and non-lipid raft fractions, along with whole-cell lysate, were then analyzed by SDS-PAGE and immunoblotting.

Immunoprecipitation

For immunoprecipitation, cell lysates were incubated with the indicated antibody plus protein G sepharose in the cold room overnight. After extensive washing with lysis buffer (150 mM NaCl, 50 mM Tris, 0.5 mM EDTA, and 1% NP-40), the immune

complexes were separated by centrifugation and analyzed by immunoblotting as described above.

Endocytosis assay

For flow cytometry-based endocytosis assays, we used antibody-probed endocytosis, as previously described (Du et al., 2017; Xu et al., 2009). Cells were detached, incubated with FITC-conjugated antibodies at 4°C for 1 h, and then switched to 37°C for different periods of time for internalization. After acidic washes (0.1 M glycine and 0.1 M NaCl, pH 2.5), the cells were fixed with 3% paraformaldehyde and subsequently analyzed using a FACS Caliber flow cytometer (BD Bioscience, Mount View, CA). The percentage of internalization was calculated using the formula [mean fluorescence intensity (MFI) of the internalization at a given time point – MFI of the internalization at time zero] / MFI of the total surface molecules × 100%.

We utilized cell surface protein biotinylation to monitor TLR4 endocytosis, as previously described (Xu et al., 2009). Cells at approximately 80% confluence were treated with the indicated drugs, then 2 ml sulfo-NHS-LC-LC-biotin (1 mg), a membrane-impermeable biotinylation reagent, was added for 1 h at 4°C. Cells were then switched to 37°C for different periods of time for internalization. Cellular extracts were prepared with 200 μl of lysis buffer, and then incubated with immobilized streptavidin agarose, which was subjected to SDS-PAGE and immunoblot analysis with TLR4 antibody. For some experiments, after internalization, cell lysate was

directly subjected to SDS-PAGE and immunoblot analysis with TLR4 antibody. We then quantitatively analyzed the band densitometry using ImageJ software (NIH).

COPD-like induction

COPD-like mice model was established according to previously described (Ishii et al., 2017) with some modification. Briefly, mice were intranasally injected 1.0 units of porcine pancreatic elastase (PPE; Sigma-Aldrich, St. Louis, MO, USA) on day 0 and 25 μg of LPS (Calbiochem, Germany) on day 7, 10, and 14. The mice were administered PNU-282987 (100 mg/kg body wt) via an oral injection 2 hr prior to PPE and LPS stimulation. Only female mice were used in this study since they display greater levels of lung oxidant stress and increased activation of the TGF-β pathway in small airways (Tam et al., 2016).

BALF collection, mononuclear cell counting, and isolation of alveolar macrophages

BALF was collected as previously described (Xu et al., 2014). Briefly, the mice were euthanized, and the lungs were lavaged with 0.5 ml sterile PBS 4 times. The lavage fluid was centrifuged then collected for detection of cytokines or stored at -80 °C for later use. BALF cell pellets were suspended in PBS and alveolar macrophages were isolated as previously described. In other experiments, BALF cells were stained with antibodies AF700-CD45 (clone 30-F11), AF488-CD3 (clone 17A2), BV650-Ly-6G/Ly-6C (Gr-1, clone RB6-8C5), PE-Cy7-F4/80 (clone BM8), in FACS

buffer (PBS with 2% FBS and 1 mM EDTA) on ice for 30 min and cell counts determined using Trucount beads (BD Biosciences). All samples were examined by a FACS Canto II (BD Biosciences) and analyzed with FlowJo software V10 (Treestar).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Experimental group size of n = 5 was predetermined based on prior experience in evaluating for statistical significance. The two-tailed Student's *t* test or one-way ANOVA were used for all statistical analyses in this study. All studies were designed to generate groups of equal size using randomization. Blinded analysis was not relevant since absolute values were measured that are not sensitive to biased interpretations. Statistical analysis was undertaken only group size was at least n = 5. Each declared n was an independent observation of individual cell wells treated in separate or from different animals, not technical replicates. A *p* value less than 0.05 was considered as statistically significant.

RESULTS

CHRNA7 agonists dampen macrophage inflammatory signaling

In order to evaluate the effects of CHRNA7 activation on inflammatory responses in macrophages, we pretreated Raw 264.7 cells or primary bone marrow-derived macrophages (BMDM) with various concentrations of the CHRNA7-specific agonists PNU-282987 or PHA-543613 for 30 min then stimulated the cells with LPS. We measured NF-κB signaling and IFN-ß expression levels using luciferase activity assays. Both NF-κB and IFN-ß signaling in RAW 264.7 (Fig. 1A) and BMDMs (Fig. 1B) were decreased in a concentration-dependent manner by pretreatment with either PNU-282987 or PHA-543613. Production of TNF-α, and IFN-β by BMDMs were also dampened by pretreatment with either CHRNA7 agonist (Fig. 1C). Taken together, these data confirm that CHRNA7 activation induces broad anti-inflammatory effects in macrophages.

cAMP suppresses inflammatory signals in various immune cells, so we hypothesized that CHRNA7 agonists stimulate cAMP production. Since both PNU-282987 and PHA-543613 induced near-maximal inhibition of cytokine production by macrophages at 10 µM, we used this concentration of each agonist and measured accumulation of cAMP in BMDMs. LPS alone had no effect on cAMP levels, but the addition of either

PNU-282987 or PHA-543613 increased cAMP levels (Fig. 1D). Thus, CHRNA7 agonists increase cAMP levels in BMDMs.

Overexpression of AC6 enhances CHRNA7-mediated anti-inflammation effects

We have demonstrated that AC4 and AC6 are the predominant AC isoforms expressed in macrophages (Cai et al., 2013), so we hypothesized that one or both of these isoforms is involved in the CHRNA7-mediated stimulation of cAMP levels. To investigate this hypothesis, we overexpressed AC4 or AC6 in BMDM using recombinant lentiviruses. Immunoblot analysis confirm increased expression of each overexpressed isoform (Fig. 2A). Overexpression of AC6 resulted in a small increase in AC4 expression via an unknown mechanism. Overexpression of AC4 led to no change in the ability of PNU-282987 (10 μM) to inhibit LPS-stimulated TNF-a, IFN-ß or IL-6 (Fig. 2B and C). By contrast, overexpression of AC6 enhanced the degree to which PNU-282987 inhibited each of these anti-inflammatory measures (Fig. 2B and C). These data are consistent with the idea that AC6, but not AC4, plays an essential role in CHRNA7-mediated anti-inflammatory signaling in macrophages.

Knockdown of AC6 impairs CHRNA7-mediated anti-inflammatory effects

To confirm a role for AC6 in facilitating CHRNA7-mediated anti-inflammation, we knocked down the endogenous expression of AC isoforms in BMDM using shRNA. The expression of AC6 or AC4 was significantly reduced by the corresponding shRNA

but not by scrambled shRNA (Fig. 3A). No non-specific nor compensatory changes in either isoform was observed following knockdown. AC6 knockdown almost completely reversed the inhibitory effect of CHRNA7 on LPS-stimulated TNF-a, IFN-ß or IL-6 (Fig. 3B and C). AC4 knockdown had no significant impact on the ability of CHRNA7 to inhibit these responses (Fig. 3B and C). These data imply that the anti-inflammatory effects of CHRNA7 activation occurs through AC6 and does not involve AC4.

Agonist-activated CHRNA7 *interacts with AC6*

In the resting state, AC6 resides primarily in lipid raft-enriched domains of many cell types while AC4 is excluded from these microdomains (Johnstone et al., 2018). Even when activated by agonists, no visible redistribution of AC6 with respect to these lipid microdomains occurs (Cai et al., 2013). We hypothesized that CHRNA7 interacts with AC6 in lipid raft domains, so we characterized the distribution of CHRNA7 in lipid raft and non-raft fractions isolated from BMDMs. In both basal and agonist-activated states, CHRNA7 primarily localized in lipid-raft domains containing caveolin-1 (Fig. 4A), consistent with previous reports (Pato et al., 2008; Stetzkowski-Marden et al., 2006). We then performed immunoprecipitation to determine if this observed colocalization results in a direct interaction between CHRNA7 and AC6. In the absence of agonist treatment, there was minimal co-immunoprecipitation between CHRNA7 and AC6 no matter which protein was pulled down (Fig. 4B). Treatment with PNU-282987 (10 μM for 30 min) led to increased co-immunoprecipitation between

CHRNA7 and AC6 and this was observed when pulling down either of the proteins (Fig. 4B). These data are consistent with the idea that activation of CHRNA7 induces an interaction with AC6 in lipid raft domains of the plasma membrane.

CHRNA7 activation accelerates AC6-dependent TLR4 degradation

To confirm the role of TLR4 in the CHRNA7/AC6 anti-inflammatory pathway we pretreated BMDMs with PNU-282987 alone or in combination with MD2-IN-1, a TLR4 antagonist. We then stimulated cells with LPS and measured levels of TNF-α and IFN-β. Treatment with MD2-IN-1 (10 μM) reduced the production of TNF-α and IFN-β in LPS-stimulated BMDMs (Figure 5A). With the CHRNA7 pathway blocked by knockdown of AC6, the TLR4 antagonist could still inhibit TNF-α and IFN-β (Figure 5A), supporting the idea that TLR4 operates downstream of CHRNA7/AC6 in this anti-inflammatory pathway.

Clathrin-mediated endocytosis of TLR4 is essential for activation of specific signal transduction pathways that lead to the induction of IFN-β (Kagan et al., 2008). We have shown that AC6 activation shifts endocytosis of TLR4 away from clathrin-mediated endocytosis and to a lipid raft-mediated pathway, which leads to degradation of the receptor (Cai et al., 2013). Since CHRNA7 activates AC6, we examined whether treatment of CHRNA7 agonists would induce a similar shift in the TLR4 internalization route. We pretreated BMDMs with PNU-282987 (10 μM) and

measured LPS-induced endocytosis of TLR4 using antibody-probed endocytosis. Dynasore, a specific inhibitor of dynamin that inhibits LPS-induced TLR4 endocytosis (Fig. 5B), had no effect on TLR4 endocytosis in the presence of PNU-282987 (Fig. 5C). By contrast, disruption of lipid rafts with methyl-ß-cyclodextrin (MßCD) blocked TLR4 endocytosis when cells were treated with PNU-282987. Thus, CHRNA7 activation shifts TLR4 endocytosis away from clathrin-mediated endocytosis and toward a lipid raft-dependent endocytic pathway.

To examine the role of AC6 in the CHRNA7-mediated shift of TLR4 endocytosis, we knocked down AC6 expression with shRNA. We observed a reduced influence of PNU-282987 on the shift of TLR4 endocytosis (Fig. 5B), suggesting the shifting effect of TLR4 endocytosis induced by CHRNA7 agonist is dependent on expression of AC6. To confirm these findings, we used a different method, cell surface protein biotinylation, to measure TLR4 endocytosis. The CHRNA7 agonist, PNU-282987, induced TLR4 internalization in the presence of LPS (Fig. 5D). CHRNA7-stimulated TLR4 internalization was reversed by lipid-raft disruption with MßCD and by knockdown of AC6 expression, but there was no effect when TLR4 trafficking via the clathrin pathway was blocked by dynasore. To confirm that TLR4 internalization via lipid-raft mediated endocytosis leads to degradation of the receptor, we measured total TLR4 levels in BMDM in the presence of cycloheximide, a protein synthesis inhibitor. In these conditions, pretreating with PNU-282987 led to reduced TLR4

immunoreactivty and either treatment with MβCD or knockdown of AC6 reversed the effect (Fig. 5E). These data are consistent with the idea that CHRNA7 activation stimulates AC6 and induces a shift the route of internalization of TLR4 stimulated by LPS to a lipid raft-mediated pathway that leads to degradation of TLR4.

PNU-282987 attenuates inflammation in COPD

Alveolar macrophages are central players in pathogenesis of chronic obstructive pulmonary disease (COPD). To examine the role of AC6 in CHRNA7-mediated anti-inflammatory effects, we established a COPD-like mouse model using intratracheal delivery of porcine pancreatic elastase followed by LPS (Sohn et al., 2013). The protocol timeline for the pathophysiological model is shown in Fig. 6A. In this model, we observed a thickening of terminal tracheal walls (Fig. 6B), widespread inflammatory cell infiltration in lung tissue (Fig. 6C), and sizable increases of TNF-α, IFN-β and IL-6 in BALF (Fig. 6D). Administration of PNU-282987 (3 mg/kg) attenuated the COPD-like histopathological findings (Fig. 6B), suppressed tissue invasion by inflammatory cells (Fig. 6C, and supplemental figure 1) and reduced levels of inflammatory cytokines (Fig. 6D). Some mice were inoculated *i.t.* with lentivirus containing AC6 shRNA. Immunoblot analysis of macrophages isolated from these mice after 48 hours showed that AC6 expression was reduced following this *in vivo* shRNA treatment (Fig. 6E). In mice with reduced expression of AC6, PNU-282987-mediated reductions of TNF-α, IFN-β and IL-6 in BALF were impaired

(Fig. 6D). These data extend our in vitro findings to an in vivo pathophysiological model of lung disease. Taken together, our data demonstrate that CHRNA7 suppresses inflammation through interacting with and stimulating AC6, thereby causing a shift in TLR4 endocytosis to a lipid raft-mediated pathway that leads to destruction of TLR4 receptors and a reduction in inflammatory responses.

Discussion

Diverse signaling pathways participate in CHRNA7-mediated anti-inflammatory effects. In the present study, we report a novel mechanism through which CHRNA7 regulates inflammation through stimulation of and interaction with AC6 in macrophages. We further show that AC6 is involved in the physiological effects of a CHRNA7 agonist in a COPD-like disorder in mice. These findings highlight how CHRNA7, signaling through AC6 and altering TLR4 trafficking, can modulate inflammatory diseases (Fig. 7). This mechanism may be one of the links through which the nervous system can modulate inflammation.

Muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR, respectively) can be characterized based on selective agonists. mAChR are G-protein-coupled receptors, while nAChR are a family of ligand-gated ion channels formed by homo- or heteropentameric subunits with distinct pharmacological properties (Ulloa et al., 2005). nAChR can be further classified as muscle (in neuromuscular junctions) or neuronal (in neuronal synapses) receptors. Among these receptor isoforms, only CHRNA7 is to expressed in macrophages. In these cells, several CHRNA7s form a homopentamer and possess high affinity for a-bungarotoxin and a lower affinity for nicotine. Nicotine inhibits the NF-κB pathway and controls the production of pro-inflammatory cytokines, thus has some therapeutic potential for ulcerative colitis (Ulloa, 2005). A selective CHRNA7 agonist, GTS-21, has a potential neuroprotective role by activating CHRNA7

in the cerebral cortex and hippocampus (Kitagawa et al., 2003). Although CHRNA7 had been shown to suppress inflammation in immune cells, the underlying mechanisms of these effects are the subject of debate (Báez-Pagán et al., 2015). At least three pathways have been reported by which CHRNA7 activation dampens inflammation: (1) promoting the phosphorylation (activation) of JAK2/STAT3 and inhibiting NF-κB (Yamada et al., 2018); (2) inhibiting IκB degradation and NF-κB nuclear translocation; and (3) inhibiting inflammasome activation and preventing release of mitochondrial DNA (Lu et al., 2014). Other reported mechanisms involve PI3K/Akt, Wnt, and MAPK signaling pathways (Ma et al., 2019). Our study provides experimental evidence that AC6 regulation of TLR4 receptor endocytosis and degradation is another key mechanism by which CHRNA7 can modulate inflammation. Various AC isoforms have been shown to play specific roles in modulation of immune responses (Ganea & Delgado, 2002). AC7 takes part in fine-tuning the functions of B and T cells (Duan et al., 2010). AC6 has been shown to colocalize in lipid rafts with CHRNA7, where they interact with each other (Oshikawa et al., 2003). More recently, AC6 has been suggested as a downstream adaptor of CHRNA7 signaling, but how they interact has not been explored (Tarnawski et al., 2018). The biological significance of this interaction remained unknown until this present study. AC6 appears to modulate inflammatory responses through at least two mechanisms: catalyzing the production of cAMP, which has its own immunomodulatory effects, and

accelerating degradation of TLR4 via lipid raft-mediated endocytosis (Cai et al., 2013). The molecular mechanism by which CHRNA7 activates AC6 remains to be elucidated.

Some studies have shown that selective agonists of CHRNA7 attenuate, while CHRNA7 antagonists aggravate, ischemia/reperfusion-induced acute lung injury through suppression of the TLR4/NF-κB signaling pathway (He et al., 2016). CHRNA7 also contributes to suppression of LPS- and *E. coli*-induced acute lung injury by reducing chemokine production and transalveolar neutrophil migration (Su et al., 2010). Alveolar macrophages play an essential role in lung development and pulmonary homeostasis. Inappropriate activation of alveolar macrophages contributes to diverse pathophysiological states in the lung. In acute lung injury, macrophage production of proinflammatory cytokines recruits neutrophil migration into the lungs, amplifying inflammation and injury (Huang et al., 2019). Our present findings reveal mechanism whereby CHRNA7 signals via AC6 to reduce TLR4 receptor function. Knockdown of AC6 abrogated the effects of a CHRNA7 agonist in both in vitro and in vivo measures of inflammation. How CHRNA7 activates AC6 remains unclear, but altered intracellular Ca^{2+} concentrations could play a role (Chiono et al., 1995)(Oshikawa et al., 2003).

There are ten AC isoforms in total, with AC1-9 being transmembrane, G protein regulated isoforms and AC10 a structurally unrelated soluble form (Dessauer et al.,

2017). Transmembrane ACs display isoform-specific regulation by an array of intracellular signals such as Gβγ proteins, calcium-calmodulin and PKC (Johnstone et al., 2018). Among all the transmembrane isoforms, only AC4 and AC6 are detected at the protein level in murine bone marrow-derived macrophages (Cai et al., 2013). AC6 mainly distributes in lipid raft domain of cell membrane and is calcium-responsive, while AC4 is calcium insensitive (Halls & Cooper, 2017). A lack of AC isoform-specific inhibitors or activators exist, but forskolin, a direct activator of all ACs (except it only weakly stimulates AC9), is commonly used as a receptor-independent AC stimulus. Thus, studies of specific roles of individual AC isoforms depend on knockdown or overexpression approaches (Johnstone et al., 2018; Dessauer et al., 2017).

The anti-inflammatory effects of cAMP are diverse and, in some cases, seemingly contradictory. As a ubiquitous and versatile cellular second messenger, cAMP regulates a vast array of biological processes. Its effects are cell type specific so it is not surprising that both pro- and anti-inflammatory processes are observed across the many types of immune cells. In alveolar macrophages, cAMP signaling inhibits phagocytosis and production of pro-inflammatory mediators such as TNF-α and MIP-1α and enhances generation of anti-inflammatory cytokines such as IL-10 (Peters-Golden M, 2009). cAMP also inhibits LPS-induced expression of the genes encoding TNF-α, MIP-1α, G-CSF. Phosphodiesterases (PDE) degrade cAMP and PDE4 inhibitors are approved for treatment of COPD due to their ability to enhance

smooth muscle relaxation and reduce inflammation (Giembycz & Maurice, 2014). However, the role of PDEs is complex due to the existence of many isoforms and their potential to regulate cAMP signaling in different subcellular compartments (Schmidt et al., 2020).

COPD is a leading cause of morbidity and mortality globally. There are estimated 384 million cases worldwide based on spirometric criteria of fixed airflow limitation (Adeloye et al., 2015). The Global Burden of Disease Study 2019 ranks COPD mortality as the sixth largest risk in the general population and the third largest risk for those 75 years and older (Vos et al., 2020). About 3.2 million patients died of COPD in 2015 alone (Wang et al., 2016). Combinations of long-acting β-agonists and long-acting muscarinic antagonists are mainstay treatments for COPD, but have no considerable ability reduce disease progression. Inhaled corticosteroids are added for patients with severe COPD, frequent exacerbations or higher risks of pneumonia (Rabe et al., 2017). Corticosteroids have broad anti-inflammatory activity and may also work, in part, via stimulation of cAMP (Koziol-White C et al., 2020; Nunez et al., 2020). Chronic inflammation plays an essential role in COPD pathogenesis leading to destruction of the lung parenchyma and peripheral airways, which results in progressive and irreversible airflow limitation (Barnes et al., 2016). Disease progression is associated with increased numbers of alveolar macrophages, neutrophils and lymphocytes in the airways. Activated alveolar macrophages release

a variety of cytokines and chemokines, playing large roles in the progression of the disease (Tuder et al., 2012). Our study utilized cultured and primary macrophage cell models and a COPD-like animal model and show that CHRNA7 can reduce inflammatory responses. Our findings reveal a novel pathway that could be leveraged for treating COPD or other inflammatory diseases. However, more work needs to be done to extend our findings to other models of inflammatory disease, especially in human cells or tissues.

In summary, our study defines a novel mechanism of anti-inflammatory signaling by CHRNA7 in macrophages. Considering the key role of these cells in the pathophysiology of diverse diseases of the pulmonary and cardiovascular systems, our data highlight a potential application of targeting AC6 for treatment of a wide array inflammatory diseases.

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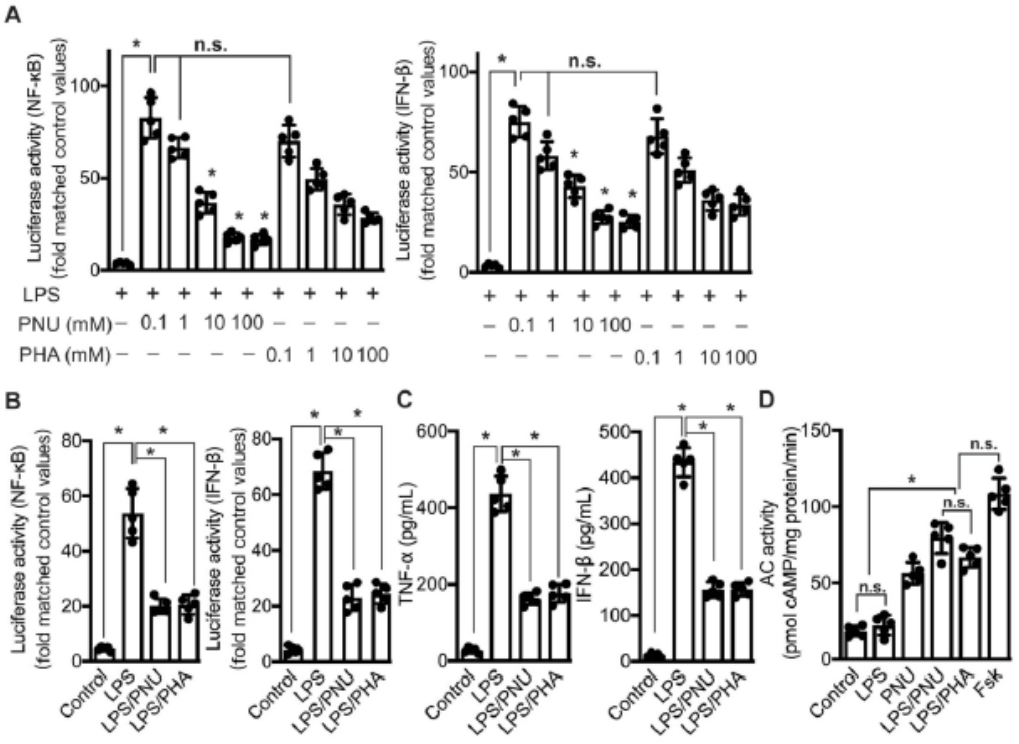

Figure 1. CHRNA7 agonists dampens inflammation induction in macrophages. Either RAW 264.7 (**A**) or BMDM (**B**) cells were transfected with an ELAM-1 promoter-controlled (left panel) or an IFN-β promoter-controlled luciferase-reporter gene (right panel) and pretreated with the indicated concentrations **(A)** or 10 μM **(B)** of PNU-282987 or PHA-543613 for 30 min then treated with LPS (100 ng/ml) for 6 h. Cells were lysed, and relative luciferase activities were determined. (**C**) The levels of TNF-α and IFN-β in BMDM supernatants were detected by ELISA. Data are mean ± SD of five independent experiments, *p<0.05. (**D**) Membranes were prepared from BMDM cells treated with LPS (100 ng/ml) for 4 h and then incubated with PNU-282987 or PHA-543613 (10 μM) in buffer for 30 min, or treated with forskolin (Fsk, 10 μM), a direct activator of AC, for 30 min. AC activities were monitored by determining cAMP content by EIA. Data are mean ± SD of five independent experiments.

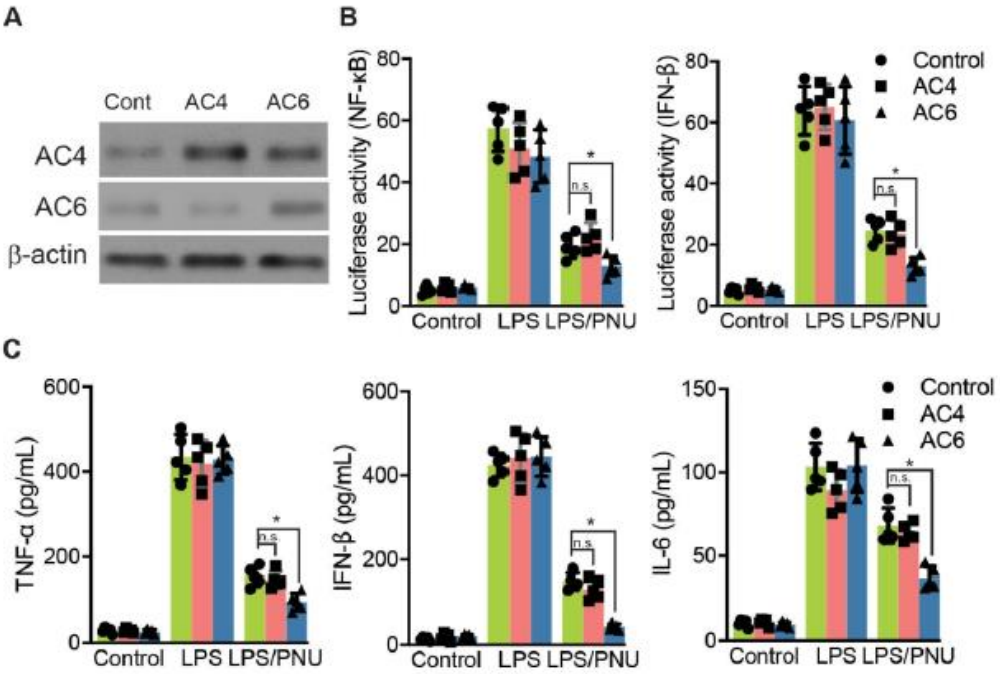

Figure 2. Overexpression of AC6 increases CHRNA7-mediated anti-inflammatory effects. (**A**) BMDMs were transfected with plasmids expressing either Myc-AC4 or Myc-AC6 and lysates subjected to SDS-PAGE and immunoblotting with anti-Myc antibody. Images shown are representative of five independent experiments. (**B**) BMDMs were infected by lentivirus expressing luciferase-reporter gene, ELAM-1 or IFN-β and AC6 or AC4 with a multiplicity of infection of 10. 24 to 48 h later, the cells were treated with LPS (100 ng/ml) for 6 h. Supernatants were collected, and the cells were lysed before measurement of luciferase activity. (**C**) TNF-α and IL-6 levels were detected in cell supernatants using ELISA. The results represent the mean ± SD of five independent experiments (B and C). (*p<0.05).

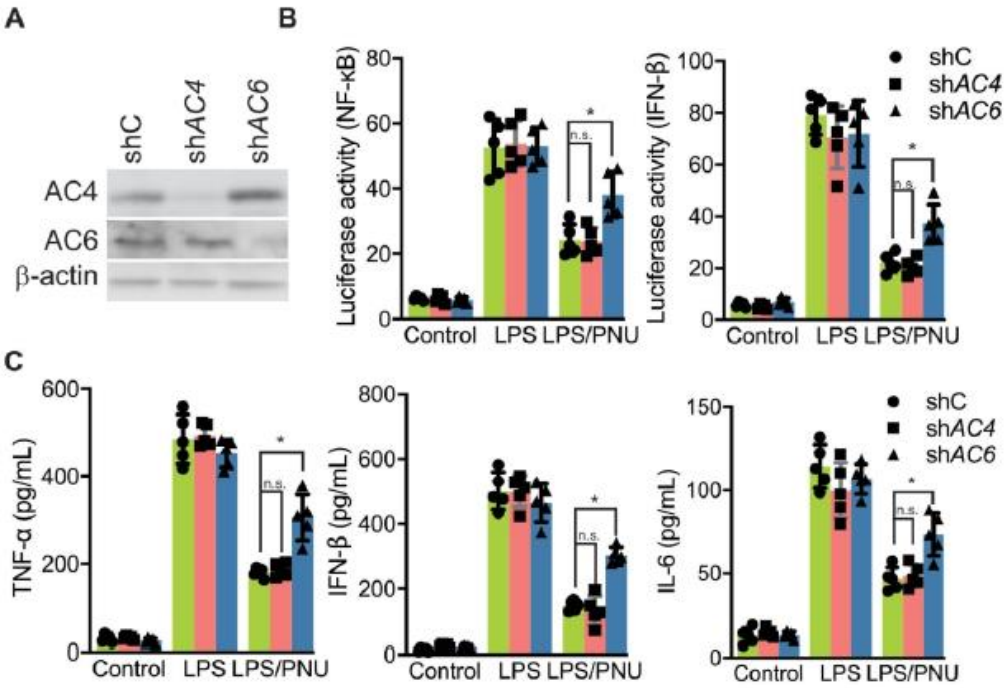

Figure 3. Knockdown of AC6 impairs CHRNA7-mediated anti-inflammatory effects. (**A**) AC6- or AC4- specific shRNA sequences were transfected into BMDMs and the expression of AC6 or AC4 was assessed via immunoblot analysis. Images shown are representative of five independent experiments. (**B**) BMDMs were infected by lentivirus expressing luciferase-reporter gene, ELAM-1 or IFN-β and AC6 or AC4 with a multiplicity of infection of 10. 24 to 48 h later, the cells were treated with LPS (100 ng/ml) for 6 h. Supernatants were collected, and the cells were lysed before measurement of luciferase activity. (**C**) TNF-α and IL-6 levels were detected in cell supernatants using ELISA. The results represent the mean ± SD of five independent experiments (B and C). (*p<0.05).

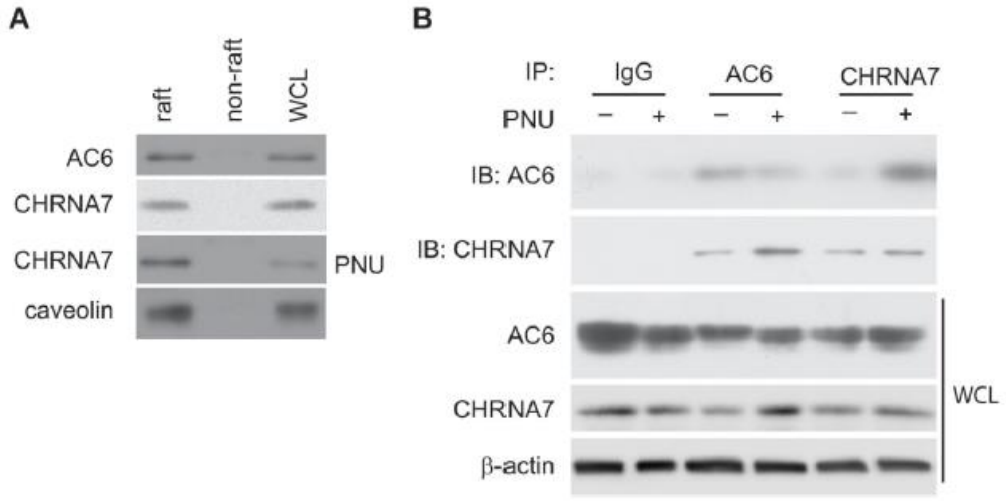

Figure 4. CHRNA7 regulates AC6 activation through interaction. (**A**) BMDMs cells were treated with PNU-282987 (10 μM) for 30 min, and then lysed and fractionated by sucrose gradient centrifugation. The light-to-heavy fractions were designated as fractions 1-9. Fractions were analyzed by Immunoblotting with AC6 or CHRNA7 antibodies. Images shown are representative of five experiments. (**B**) BMDMs cells were treated with PNU-282987 (10 μM) for 30 min, lysed and then AC6 (left panel) or CHRNA7 (right panel) were immunoprecipitated. The precipitates were subjected to immunoblotting with the indicated antibodies and the bands were visualized with an ECL chemiluminescence kit. WCL = whole cell lysate. All images shown are representative of five experiments.

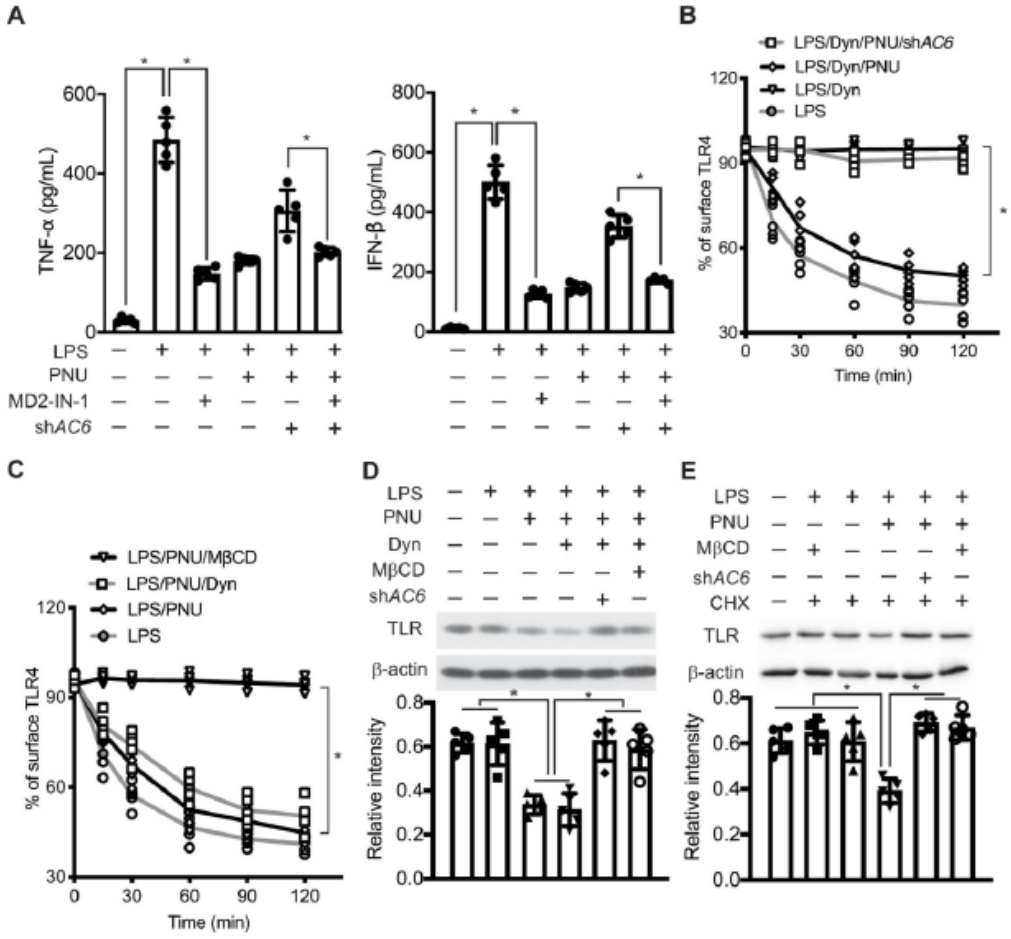

Figure 5. CHRNA7 agonist treatment accelerates AC6-dependent TLR4 degradation. (**A**) BMDM with or without AC6 knockdown were pretreated with MD2-IN-1 (10 μM) for 2 h alone or in combination with PNU-282987 (10 μM) for 30 min, then treated with LPS (100 ng/ml) for 6 h. Supernatants were collected, and TNF-α and IFN-β levels were detected in cell supernatants using ELISA. (**B**) BMDM were pretreated with PNU-282987 (10 μM) for 30 min, with or without the presence of 5 μM MβCD or Dynasore (50 μM), then stained with FITC-TLR4 and processed as described in Materials and Methods. The internalization rates were calculated with the formula described in Materials and Methods. Data are presented as the mean ± SD of

five experiments (*p < 0.05) (**C**) BMDM with AC6 knockdown were pretreated as indicated and subject to internalization as described in Materials and Methods. The internalization rates were calculated with the formula described in Materials and Methods. Data are presented as the mean ± SD of five experiments (*p < 0.05) (**D**) After the indicated treatment, BMDMs were treated with 1 mg ice-cold sulfo-NHS-LC-LC-biotin for 1 h at 4°C, and then switched to 37°C for 30 min to induce internalization. Cellular extracts were prepared with 200 μl of lysis buffer, and then incubated with immobilized streptavidin agarose, which was subjected to SDS-PAGE and immunoblot analysis with antibody for TLR4. Some cells were treated for 2 h, and cell lysate was directly subjected to SDS-PAGE and immunoblot analysis with TLR4 antibody. The upper panel represents densitometric quantitation of total TLR over β-actin using ImageJ presented as the mean ± SD of five experiments (*p < 0.05). The lower panel is a representative image of these five experiments. (**E**) BMDM with or without AC6 knockdown were pretreated with PNU-282987 (10 μM) for 30 min, with or without treatment with 5 μM MβCD, then treated with LPS (100 ng/ml) in the presence of cycloheximide (CHX, 10 μg/ml) for 6 h. The cells were lysed, subjected to SDS-PAGE and subjected to immunoblot analysis using a TLR4 antibody. The upper panel represents densitometric quantitation of total TLR over β-actin using ImageJ presented as the mean ± SD of five experiments (*p < 0.05). The lower panel is a representative image of these five experiments.

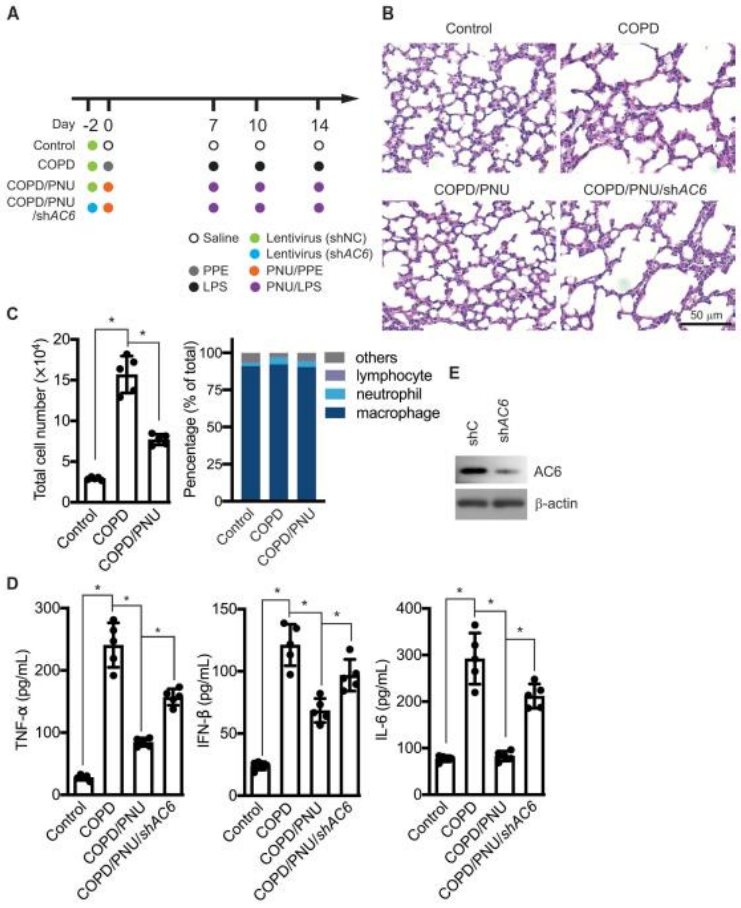

Figure 6. Knockdown of AC6 abrogates CHRNA7-mediated inhibition of lung damage in a mouse model of COPD. (**A**) COPD-like disease was induced in mice induced using PPE/LPS. Lentivirus containing shRNA against AC6 was administered *i.t.*. In some mice, PNU-282987 was administrated 2 h before treatment with PPE and LPS. **(B)** Mice were sacrificed, and the lungs were removed and subjected to H&E staining after paraffin-embedded sectioning. (**C**) BALF were also prepared and polymononuclear cells ($CD45^+$) were collected for cell counting and identification with FACS as either neutrophil (N, $Ly6C/Ly6G^+$), macrophage (M, $F4/80^+$), lymphocyte (L, $CD3^+$), or others. **(D)** Inflammatory cytokines were detected with ELISA**.** (**E**) Alveolar macrophages were isolated to and the level expression of AC6 analyzed via immunoblot analysis. All quantified data were calculated from 5 independent experiments, and presented as mean ± SD. (*$p < 0.05$)

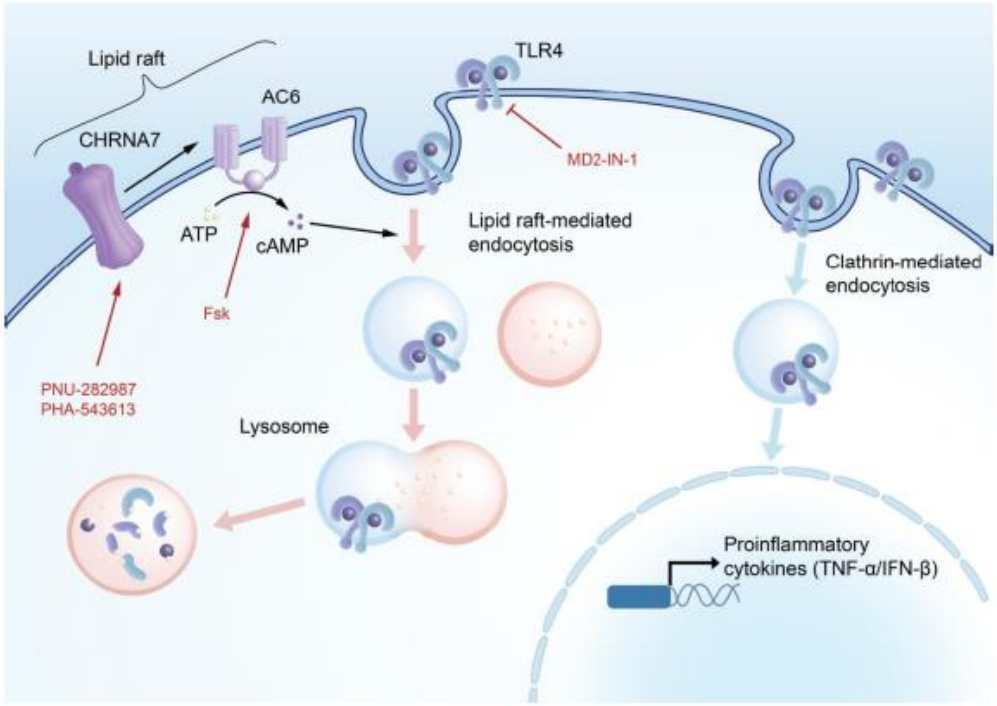

Figure 7. Schematic diagram of the mechanisms of anti-inflammatory effect by CHRNA7 via AC6. CHRNA7 stimulation leads to AC6 activation, which shifts TLR4 endocytosis to lipid raft-mediated pathway that leads to receptor degradation and reduced inflammatory signaling (left part), while clathrin-mediated endocytosis leads to production of proinflammatory cytokines, such as TNF-α, IFN-β (right part). PNU-282987 and PHA-543613, agonists of CHRNA7; Fsk (forskolin), agonist of AC; MD2-IN-1, antagonist of TLR4.