Anti-inflammatory effects of CHRNA7 through interacting with adenylyl cyclase 6

Running title: CHRNA7 interacts with AC6

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Word count: 3872

Acknowledgements

This work was supported by National Natural Science Foundation of China 31870885, 31570905 (to C.X.), 81971181 (to A.D.), Natural Science Foundation of Shanghai Municipality 19ZR1449200 (to A.D.), Shanghai Bureau of Public Health 201840173 (to C.X.). The funders play no role in study design, data collection, data analysis, interpretation, and writing of the report.

Confliction of interests

The authors declare that they have no conflict of interest.

Author contributions

S. Z., S. H., X. Z., and C. X. conceived and designed the study, analyzed the data, and wrote the manuscript. S. Z., S. H., X. Z. 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; CHRNA7, alpha 7 nicotinic acetylcholine receptor; MβCD, methyl-beta-cyclodextrin; MFI, mean fluorescence intensity; PPE, porcine pancreatic elastase.

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 might 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.

Understanding neural-immune interplay, specifically the neuronal regulation of inflammation, has long been the research efforts of both immunologists and neuroscientists (Reardon et al., 2018). Studies show that vagal nerve stimulation inhibits pro-inflammatory cytokine production through a cholinergic anti-inflammatory pathway that depends on the α7 nicotinic acetylcholine receptor (CHRNA7) (Chu et al., 2020). CHRNA7 may suppress inflammation through 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). In monocytes/microglias, CHRNA7 regulates autophagy and the CHRNA7

agonist, PNU-282987, dampens production of cytokines such as IL-6 and IL-1β, and attenuates experimental autoimmune encephalomeylitis (Shao et al., 2017). These data suggest a complicated regulatory network through which CHRNA7 modulates inflammation, but the molecular mechanisms of how these receptors alter functions of immune cells is far from resolved.

The second messenger cAMP has multiple downstream effectors, with protein kinase A activation as the most prominent. cAMP also directly works on some guanine-nucleotide-exchange factors such as Epac. These effectors signal via a wide array of downstream pathways to modulate various biological events (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-responsive transcription factor (CREB), and is required for the generation and maintenance of Treg (Kim & Leonard, 2007). In macrophages, increased cAMP levels appear to attenuate inflammation (Raker et al., 2016).

Adenylyl cyclases (AC) produce cAMP and studies have demonstrated that adenylyl cyclase activators, such as prostaglandin E2 and forskolin, inhibit inflammation through dampening NF-κB signaling and IFN-β at both the mRNA and protein level (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, and our data demonstrate 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). TLR4 antibody (Sa15-21) was purchased from BioLegend (San Diego, CA). All the other antibodies for FACS analysis were from BioLegend (San Diego, CA). Antibodies for β-actin and AC isoforms were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for CHRNA7 was purchased from Abcam (Cambridge, UK). HRP-conjugated anti-mouse and anti-rabbit 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 and HEK 293 cells 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). 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. All efforts were made to minimize suffering.

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 an unstimulated vector control. 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 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).

RT-PCR

RT-PCR for AC isoforms was performed using the primer pairs (Supplement Table 1). 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.

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.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. The two-tailed Student's *t* test or one way ANOVA were used for statistical analyses in this study. 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 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 the cytokines, TNF-α, and IFN-β, were also dampened by pretreatment with CHRNA7 agonists (Fig. 1C). Taken together, these data confirm that CHRNA7 activation induces broad anti-inflammatory effects in macrophages.

cAMP, a ubiquitous second messenger, has been shown to suppress inflammatory signals in 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 these concentrations to measure accumulation of cAMP in macrophages. LPS alone had no effect on cAMP accumulation, but the addition of either PNU-282987 or PHA-543613 increased

levels of this second messenger (Fig. 1D). These data show that CHRNA7 agonists signal in part via increasing cAMP levels.

Overexpression of AC6 enhances CHRNA7-mediated anti-inflammation effects

cAMP is the product of AC, which are expressed ubiquitously. 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 this CHRNA7-mediated effects. To investigate this hypothesis, we overexpressed AC4 or AC6 in BMDM using recombinant lentiviruses (Fig. 2A). We observed no significant effects of AC4 overexpression on the ability of PNU-282987 (10 μM) to inhibit any of the inflammatory end points (Fig. 2B and C). By contrast, overexpression of AC6 enhanced the anti-inflammation effects of PNU-282987 (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 further 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 TLR4 activation and downstream release of multiple inflammatory cytokines (Fig. 3B and C). AC4 knockdown had no significant impact on CHRNA7-mediated effects (Fig. 3B and C). These data imply that anti-inflammatory effects of CHRNA7 occurs exclusively through the AC6 isoform and does not involve AC4.

CHRNA7 *regulates AC6 activation through interaction*

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 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 macrophages. In both resting and agonist-activated states, CHRNA7 primarily localized in lipid-raft domains containing caveolin-1 (Fig. 4A). Since CHRNA7 distributes in the lipid raft-enriched domains similarly to AC6 (Pato et al., 2008; Stetzkowski-Marden et al., 2006), we performed immunoprecipitation to determine if this colocalization facilitates a direct interaction between CHRNA7 and AC6. In the absence of agonist treatment, there was minimal co-immunoprecipitation between CHRNA7 and AC6 (Fig. 4B). Treatment with PNU-282987 (10 μM for 30 min) led to increased co-immunoprecipitation between CHRNA7 and AC6

(Fig. 4B). These data are consistent with the idea that activation of CHRNA7 induces an interaction of CHRNA7 with AC6 in lipid raft domains of the plasma membrane.

CHRNA7 activation accelerates AC6-dependent TLR4 degradation

Endocytosis of TLR4 is essential for activation of specific signal transduction pathways such as induction of IFN-β, which occurs when the receptor is internalized through a clathrin-mediated pathway. We have shown that AC6 activation shifts endocytosis of TLR4 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 shift the TLR4 internalization route. We pretreated BMDM 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, while disruption of lipid rafts with methyl-ß-cyclodextrin (MßCD) blocked endocytosis when PNU-282987 was present (Fig. 5A). Thus, CHRNA7 activation shifts TLR4 endocytosis toward a lipid raft-dependent endocytic pathway. To examine the role of AC6 in CHRNA7-mediated shift of TLR4 endocytosis, we knocked down AC6 expression with shRNA. We observed a reduced influence of CHRNA7 agonist 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 degradation in the presence of LPS (Fig. 5C). CHRNA7-stimulated TLR4 degradation was reversed by the lipid-raft disruptor, MßCD, or if AC6 expression was knocked down, but there was no effect when TLR4 trafficking via the clathrin pathway was blocked by dynasore. 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 lipid raft-mediated pathway that leads to degradation of TLR4.

PNU-282987 attenuates inflammation in COPD

Alveolar macrophages are central players during 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). In our model, we observed a thickening of terminal tracheal walls (Fig. 6A), widespread inflammatory cell infiltration in lung tissue (Fig. 6B), and sizable increases in TNF-α, IFN-β and IL-6 in BALF (Fig. 6C). Administration of PNU-282987 (3 mg/kg weight) attenuated the COPD-like histopathological findings (Fig. 6A), suppressed tissue invasion by inflammatory cells (Fig. 6B) and reduced levels of inflammatory cytokines (Fig.

6C). Some mice were inoculated *i.t.* with lentivirus containing AC6 shRNA. Immunoblot analysis of macrophages isolated from these mice showed that AC6 expression was reduced following this *in vivo* shRNA treatment (Fig. 6D). In mice with reduced expression of AC6, PNU-282987-mediated reductions of TNF-α, IFN-β and IL-6 in BALF were impaired (Fig. 6C). 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 interaction with AC6 in macrophages. We further show that AC6 is involved in the therapeutic 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. This mechanism may be one of the links through which the nervous system can modulate inflammation.

Although CHRNA7 had been shown to suppress inflammation in immune cells, the underlying mechanisms are the subject of debate (Báez-Pagán et al., 2015). Our study provides experimental evidence that AC6 is one signaling hub. 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). The biological significance if this latter observation remained unknown until this present study. AC6 appears to adjust inflammation 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, IR-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 have been shown to play an essential role in lung development and pulmonary homeostasis. Pathologically, inappropriate activation of alveolar macrophages contributes to diverse pulmonary disorders. In acute lung injury, macrophage production of proinflammatory cytokines recruits neutrophil migration into the lungs, leading to amplified inflammation and injury (Huang et al., 2019). Our present data reveal a novel mechanism whereby CHRNA7 signals via AC6 to reduce TLR4 receptor function. Knockdown of AC6 abnegated the effects of a CHRNA7 agonist in both in vitro and in vivo models, confirming the central roles of CHRNA7 and AC6 in mediating anti-inflammatory effects. Activation of AC could be induced by the association and activation of G protein, or by local elevated intracellular Ca^{2+} concentration (Chiono et al., 1995). However, how CHRNA7 activation contributes to AC6 activity exactly remains to be clarified, even though it has been suggested that CHRNA7 may regulate the AC activity through Ca^{2+}

(Oshikawa et al., 2003). Additionally, more work also needs to be done to extend our findings to other models of inflammatory disease especially those using 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 LEGENDS

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 PNU-282987 or PHA-543613 for 30 min in the concentration of indicated (**A**) or 10 μM (**B**), then treated with LPS (100 ng/ml) for 6 h. Cells were lysed, and relative luciferase activities were determined. (**C**) Supernatants from BMDM were used to detect the levels of cytokines TNF-α and IFN-β by ELISA. Data are mean ± SD of five independent experiments, *p<0.05. (**D**) Membranes were prepared from BMDM cells and then incubated with PNU-282987 or PHA-543613 (10 μM) in buffer 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 strengthen CHRNA7-mediated anti-inflammation effects. (**A**) BMDMs were transfected with Myc-AC4 or Myc-AC6 plasmid, and then lysed to perform SDS-PAGE and immunoblotted with anti-Myc antibody. Images shown are representative of five independent experiments. (**B**) BMDMs were infected by lentivirus containing luciferase-reporter gene, and 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**) Suppression of AC6 or AC4 expression by shRNA in BMDMs. Specific shRNA sequences were transfected into BMDMs and the efficiency of shRNA in inhibition of AC6 or AC4 expression was determined using immunoblotting. The images shown are representative of five independent experiments. (**B**) BMDMs were infected by lentivirus containing luciferase-reporter gene, and 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 in

Immunoblotting using AC6 or CHRNA7 antibodies. Images shown are representative of five experiments. (**B**) BMDMs cells were treated with PNU-282987 (10 μM) for 30 min, and then lysed and were immunoprecipitated with IgG, AC6 (left panel) or CHRNA7 (right panel) antibodies. The precipitates were subjected to blotting with the indicated antibodies, and the bands were visualized with an ECL chemiluminescence kit. All images shown are representative of five experiments.

Figure 5 CHRNA7 agonists treatment accelerates AC6-dependent TLR4 degradation. (**A**) BMDM were pretreated with PNU-282987 (10 μM) for 30 min, with or without the presence of 5 μM MβCD, Dynasore (50 μM) as indicated, then stained with FITC-TLR4 and processed as described in Materials and Methods. The internalization rates were calculated with the formula mentioned. Data are presented as the mean ± SD of five experiments (*$p < 0.05$) (**B**) BMDM with AC6 knockdown were pretreated as indicated and subject to internalization 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**) 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 TLR4 antibody. Some cells were left to internalize for 2 h, and cell lysate was directly subjected to SDS-PAGE and immunoblot analysis with TLR4 antibody. The upper panel represents densitometry 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 Knock down of AC6 abolishes suppressive effects on TLR4 signaling. COPD-like mice induced with PPE/LPS were infected with lentivirus containing shRNA motif against AC6 *i.t.*. In some COPD-like mice, PNU-282987 was administrated. Twenty-four hours later, the mice were sacrificed, and the lungs were removed and subjected to HE staining after paraffin-embedded sectioning **(A)**; BALF were also prepared and the leukocytes were collected for cell counts and identification with FACS (**B**), and inflammatory cytokines were detected with ELISA **(C).** Alveolar macrophages were also isolated to detect the expression of AC6 (**D**). All quantified data were calculated from 5 independent experiments, and presented as mean ± SD. (*$p < 0.05$)