Cholinergic nervous system as therapeutic approach for the treatment of arthritis
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The α7 nicotinic acetylcholine receptor on fibroblast-like synoviocytes and in synovial tissue from rheumatoid arthritis patients

a possible role for a key neurotransmitter in synovial inflammation


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ABSTRACT

**Objective.** Recent studies have suggested an important role for neurotransmitters as modulators of inflammation. Therefore, we undertook this study to investigate the expression of the α7 subunit of the nicotinic acetylcholine receptor (α7nAChR) and its function in rheumatoid arthritis (RA).

**Methods.** The potential role of the α7nAChR in modulating proinflammatory cytokine expression in fibroblast-like synoviocytes (FLS) was identified by screening an adenoviral short hairpin RNA (Ad.shRNA) library. An α7-specific antibody was used for immunohistochemistry, and fluorescein isothiocyanate–labeled α-bungarotoxin, which binds specifically to the α7nAChR, was used for immunofluorescence. Gene expression in FLS was determined by quantitative polymerase chain reaction with primers specific for the α7nAChR. In addition, we analyzed messenger RNA (mRNA) expression of dup α7, a variant α7 transcript. Next, we studied the functional role of the α7nAChR in RA FLS by examining the effects of α7-specific agonists on the production of interleukin-6 (IL-6) and IL-8 by activated FLS.

**Results.** A screen using an Ad.shRNA library against 807 transcripts revealed that a specific α7nAChR shRNA potently modulated IL-8 and matrix metalloproteinase expression in FLS. The α7nAChR was expressed in the inflamed synovium from RA patients, predominantly in the intimal lining layer. We found α7nAChR expression on both the mRNA and protein level in cultured RA FLS. FLS also constitutively expressed dup α7 mRNA. Specific α7nAChR agonists reduced tumor necrosis factor α–induced IL-6 and IL-8 production by FLS.

**Conclusion.** The α7nAChR and its dup α7 variant are expressed in RA synovium, where they may play a critical role in regulating inflammation. Targeting the α7nAChR could provide a novel antiinflammatory approach to the treatment of RA.
Rheumatoid arthritis (RA) is an immune-mediated inflammatory disease of unknown etiology that is characterized by inflammation of the synovium leading to destruction of cartilage and bone (1). In this disease, the intimal lining layer of the inflamed synovium becomes hyperplastic and forms a condensed tissue mass, which promotes destruction of cartilage and bone. Accumulating evidence suggests that fibroblast-like synoviocytes (FLS) play a major role in the initiation and perpetuation of the chronic inflammatory process in RA synovial tissue (2-4).

In the present study we used a novel approach to identify new therapeutic targets expressed by FLS from RA patients. FLS were isolated from arthroscopic synovial biopsy samples. We performed a screen using an adenoviral short hairpin RNA (Ad.shRNA) against 807 transcripts. The aim of the screen was to identify cell surface-expressed or -secreted proteins that when down-regulated can up-regulate proinflammatory cytokine expression in order to identify secreted proteins or receptors that play a role in modulating the inflammatory response in primary human RA FLS. The screen revealed that a specific α7 nicotinic acetylcholine receptor (nAChR) shRNA showed one of the most potent effects on modulating interleukin-8 (IL-8) expression.

In recent years, it has been demonstrated that the cholinergic nervous system can regulate inflammation via its principal neurotransmitter, ACh, a concept referred to as the “cholinergic antiinflammatory pathway” (5). ACh can interact with members of the nAChR family. Most studies point towards a crucial role for the α7 subunit of the nAChR in the antiinflammatory effect of vagal nerve signaling (6). Nicotine exerts antiinflammatory effects on macrophages that can be counteracted by selective α7 antagonists (6-8). Selective α7nAChR agonists have proven effective in reducing macrophage cytokine production and inflammation in animal models of pancreatitis (9) and peritonitis (10) and experimentally induced ileus in mice (11) and have improved survival in mice challenged with lipopolysaccharide (12;13). Vagal stimulation failed to reduce serum levels of tumor necrosis factor α (TNFα) in α7nAChR-knockout mice, and splenocytes and peritoneal macrophages derived from these mice were shown to be insensitive to the cytokine-inhibiting effects of cholinergic agonists (6;14). Taken together, these studies support the notion that ACh may be important in modulating inflammation.

The α7nAChR is expressed by various immune cells, including monocytes (15), macrophages (5;16), T and B lymphocytes (17;18), and dendritic cells (19). In addition to this classical α7nAChR, the human genome contains an alternative α7 transcript designated dupa7 or “cholinergic receptor family with sequence similarity to 7A” (CHRFAM7A) (20). In this transcript variant, exons 5-10 of the gene have been duplicated in a “tail-to-head” orientation, and this partially duplicated gene is combined with 4 novel exons (A-D) to form a new gene. It has been reported that in human brain (21) as well as leucocytes (22), this gene is transcribed as a 45-kd protein. It remains unclear whether dupa7 is appropriately processed to form a functional receptor,
because in cells expressing dupα7, no binding of α-bungarotoxin and no nicotine-induced electrical current could be demonstrated (22), possibly explained by the fact that dupα7 lacks the binding sites for nicotine and α-bungarotoxin. Instead, this dupα7 may have a regulatory role, and α7nAChR containing dupα7 may provide new pharmacological properties that differ from those of the classical α7nAChR.

It is generally thought that the joint is not innervated by the vagus nerve. Still, it has been shown that electrical and pharmacological stimulation of the vagus nerve results in decreased carrageenan-induced paw inflammation in rats (23). Consistent with these results, we recently demonstrated that manipulation of the vagus nerve has an effect on arthritis activity in the collagen-induced arthritis (CIA) model of RA (24). In addition, treatment with α7nAChR-specific agonists reduced joint inflammation in this model. The beneficial effect of stimulation of the cholinergic antiinflammatory pathway on the inflamed synovium in the absence of vagal nerve innervation of the synovial tissue could theoretically be explained by 2 mechanisms that are not mutually exclusive. First, there may be an effect of the vagus nerve on sites other than the synovium, most likely the spleen, with indirect effects on the synovium (14), although there is no neuroanatomic evidence for vagal nerve supply to any immune organ (25). Second, ACh might be produced in the synovium by cells other than nerve cells (26-28). For this mechanism to be relevant to synovial inflammation, one would expect expression of the α7nAChR in the synovium; however, data are as yet not available.

Therefore, after performing a screen using an Ad.shRNA library against 807 transcripts, we investigated the expression of α7nAChR and dupα7nAChR in synovial tissue and FLS from RA patients. Moreover, we performed functional studies by evaluating the effects of α7-specific agonists on the production of key proinflammatory cytokines and chemokines by activated RA FLS.

PATIENTS AND METHODS

Patients and tissue samples
Synovial biopsy samples were obtained from the actively inflamed knee or ankle joints of RA patients who underwent small-needle arthroscopy as previously described (29). The RA patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA (30). All patients gave informed consent, and the study protocol was approved by the Medical Ethics Committee of the Academic Medical Center/University of Amsterdam. Tissue samples used for immunohistochemistry or immunofluorescence were snap-frozen together en bloc in TissueTek OCT (Miles Diagnostic, Elkhart, IN) by immersion in liquid nitrogen and stored until sectioned for staining. Five-micrometer sections were cut with a cryostat and mounted on glass slides (Star Frost; Knittelgläser, Braunschweig, Germany). Slides were sealed and stored at -80°C until use.
**Cell culture**

RA FLS were isolated from the patients’ synovial biopsy samples by enzymatic digestion and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum (FCS). Experiments were conducted using FLS from passage 4-9 (31). For immunocytochemical staining experiments, FLS were cultured on chamber slides (Nunc, Rochester, NY) in a final concentration of 10^4 cells per well. For functional analysis, primary FLS from 8 different RA patients were cultured in medium containing 1% FCS for 24 hours prior to preincubation with nicotine or the α7nAChR-specific agonist AR-R17779 (kindly provided by Critical Therapeutics, Lexington, MA) for 15 minutes at the indicated concentrations, followed by stimulation with TNFα (10 ng/ml; R&D Systems, Abingdon, UK) for 4 hours. Thereafter, supernatant was collected and assayed for the presence of IL-6 and IL-8 using a cytometric beads array multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations.

**Screening of the Ad.shRNA library**

RA FLS were cultured in DMEM, and 1 day after cell seeding in 384-well plates (750 cells/well), a library of adenoviral shRNAs (32) was added to an average multiplicity of infection of 30,000 particles per cell (33). Five days after adenoviral transduction, the medium was replaced with 0.01 ng/ml TNFα in M199 medium. After 48 hours, supernatants were collected and analyzed for IL-8 secretion using a specific enzyme-linked immunosorbent assay (Meso Scale Discovery, Gaithersburg, MD). IL-8 concentrations were calculated in reference to a standard curve.

**Immunohistochemistry and immunocytochemistry**

Cryosections of synovial biopsy samples and FLS cultured on chamber slides were fixed for 20 minutes in acetone. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in 0.1% sodium azide in phosphate buffered saline (PBS). Expression of α7nAChR was studied by staining the sections overnight at 4°C with a rabbit polyclonal antibody against α7 subunit (2 μg/ml, ab23832; Abcam, Cambridge, UK) in PBS containing 1% bovine serum albumin (BSA). Equivalent concentrations of irrelevant control rabbit polyclonal antibodies were used as isotype control. Subsequently, the sections were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (2.5 μg/ml; Dako, Glostrup, Denmark) in PBS containing 1% BSA and 10% normal human serum for 30 minutes at room temperature. Signal amplification was performed by incubating the slides for 15 minutes with biotinylated tyramine (Perkin-Elmer, Boston, MA) followed by HRP-labeled streptavidin (Dako) as previously described (34). Peroxidase activity was revealed using the aminoethylcarbazole substrate kit (SK-4200; Vector, Burlingame, CA). Sections were briefly counterstained with Mayer’s hemalum solution (Fluka Biochemika, St. Gallen, Switzerland) and mounted with Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany).
sections were washed extensively with PBS between all steps, unless otherwise specified. To quantify the data, a semiquantitative scoring system was used, ranging from 0 (no staining) to 3 (abundant staining) (34).

**Immunofluorescence microscopy**

For immunofluorescence stainings on cryosections of synovial biopsy samples and FLS cultured on chamber slides, sections were incubated with fluorescein isothiocyanate (FITC)-labeled α-bungarotoxin (1 μg/ml; Sigma, St. Louis, MO) overnight at 4°C in the dark. Where indicated, AR-R17779 was added to a final concentration of 10 μM, 1 hour before the addition of FITC-labeled α-bungarotoxin. The staining with FITC-labeled α-bungarotoxin with and without pretreatment with AR-R17779 in the synovial tissue of RA patients was analyzed with a semiquantitative scoring system, as described earlier (34). For immunofluorescence double-staining experiments, sections were washed after overnight incubation with FITC-labeled α-bungarotoxin (1 μg/ml), followed by incubation with a mouse monoclonal antibody against human CD55 (clone 67; Serotec, Oxford, UK) for 1 hour at room temperature. Sections were then stained with Alexa 594-labeled goat anti-mouse antibody (Molecular Probes, Leiden, The Netherlands). After labeling, slides were mounted in Vectashield (H-1000; Vector). To rule out the possibility of nonspecific binding, control sections were stained with mouse isotype control or PBS. The slides were analyzed using a Leica DMRA fluorescence microscope (Leica, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Dutch Vision Components, Breda, The Netherlands).

**Polymerase chain reactions (PCRs)**

Total RNA was isolated from FLS from 8 RA patients using TRIzol reagent (Invitrogen, Carlsbad, CA). complementary DNA (cDNA) was prepared according to the manufacturer’s instructions (iScript; Bio-Rad, Hercules, CA). Quantitative reverse transcriptase (RT)-PCR was performed using TaqMan RT-PCR reagents (Applied Biosystems, Foster City, CA). The primer set specific for the classical α7nAChR, amplifying 101 bp between exons 3 and 4, is assay ID Hs01063372 (Applied Biosystems). The primer set specific for dupα7, amplifying a region of 88 bp spanning from exon D to exon C is assay ID Hs00415199 (Applied Biosystems). The resulting cDNA templates were subjected to 40 cycles on a StepOnePlus Thermal Cycler with a 96-well reaction plate (Applied Biosystems). Cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. As a positive control, cDNA of the human neuroblastoma cell line SKNSH was used (HTB-11; American Type Culture Collection, Teddington, UK) (35). Duplicates were performed for each sample. Expression levels were normalized to GAPDH using the recommended change in threshold cycle (Δ Ct) method. Ct is defined automatically by the instrument using the auto mode of Ct determination.
Statistical analysis

Differences between 2 groups were analyzed for statistical significance using the Mann-Whitney U-test (GraphPad Prism, version 4.03; GraphPad Software, San Diego, CA). P values < 0.05 were considered significant. Since the distribution of the data obtained in the shRNA library screen revealed a long tail upward (up to 15-fold the average of the plate), a non-parametric analysis employing quartiles was used (36).

RESULTS

Knockdown of the α7nAChR by a specific shRNA results in increased IL-8 production by RA FLS

To identify genes which, upon Ad.shRNA-mediated knockdown, synergize with a low concentration of TNFα to induce IL-8 release by FLS, a high-throughput assay in 384-well format was screened with a set of 2,343 adenoviral vectors expressing shRNAs coding for 807 transcripts (32). IL-8 was chosen, since this is a key proinflammatory chemokine produced by RA FLS derived from actively inflamed joints (37). The screening data were analyzed on a plate-by-plate basis to identify statistically significant hits (Figure 1). The activity of the shRNA was expressed as the fold distance interquartile range (IQR) between the median and 75% quartile away from the median of the plate. Hits were called in biologic duplicates at 2 IQR above the median of the plate, yielding 152 duplicate hits.

Figure 1. Screening data of 2,343 adenoviral short hairpin RNA (shRNA) in biologic duplicate (averages shown) on the interleukin-8 (IL-8) release assay in fibroblast-like synoviocytes from patients with rheumatoid arthritis. Induction of IL-8 is represented in fold interquartile range (IQR) from the median of the plate (see Results). The hit for the α7 subunit of the nicotinic acetylcholine receptor (α7nAChR) is indicated by an arrow.
One of the strongest hits (at 22.05 and 12.60 IQR above the median) was a shRNA against α7nAChR (5'-CAAATGTCTTGGACAGATC-3', complementary to the junction of exon 3 and 4 of the α7nAChR gene). The 152 hit shRNA viruses were repropagated in a different plate layout on a 96-well format including 40 individual negative control viruses per 96-well plate (empty shRNA, luciferase shRNA, and enhanced green fluorescent protein shRNA). The repropagated hits were rescreened in the same assay, using the median and SD of the negative controls as a reference point for the activity of the library viruses. In total, 85 hits were confirmed. The α7nAChR shRNA again scored very strongly in biological duplicate, now 15.8 and 10.4 SD from the median of the negative controls ($P < 1 \times 10^{-43}$). Moreover, the 85 confirmed hits were rescreened in 3 primary RA FLS cell lines from 3 different donors using the IL-8 readout, revealing similar results. In addition, in the supernatants of these cells we measured levels of matrix metalloproteinases (MMPs) 1 and 3, both of which were strongly affected in the cells treated with the α7nAChR shRNA (data not shown).

Expression of the α7 subunit of nAChR in synovial tissue from RA patients

Having identified α7nAChR as a potential therapeutic target, we performed immunohistochemical analysis to examine the in situ expression of α7nAChR in synovial

Figure 2. Expression of the α7 subunit of the nicotinic acetylcholine receptor in synovial tissue from patients with rheumatoid arthritis (RA). A representative synovial tissue section of 1 RA patient is shown. A, Immunohistochemical staining with rabbit polyclonal antibody against the α7 subunit showed strong binding in the intimal lining layer and to a lesser extent in the synovial sublining (n = 10). B, No detectable staining was observed in synovial tissues stained with irrelevant rabbit polyclonal antibodies. C, Strong binding of fluorescein isothiocyanate–labeled α-bungarotoxin was observed throughout the intimal lining layer. D, AR-R17779 pretreatment markedly reduced the intensity of staining. (Original magnification 100 X.) (See also section Color figures).
tissue from RA patients (n=10). The analysis was performed using an α7nAChR-specific polyclonal antibody, which binds to the extracellular domain of the receptor. In all RA synovial tissues the α7nAChR was predominantly expressed throughout the intimal lining layer and to a lesser extent in the synovial sublining (a representative picture is shown in Figure 2A). No detectable staining was observed in synovial tissues stained with irrelevant rabbit polyclonal antibodies (Figure 2B). Next, we examined the expression of α7nAChR using FITC-labeled α-bungarotoxin, a selective antagonist with high affinity for the α7 subunit (38;39). The binding domain of this antagonist is contained in the extracellular N-terminus. Strong binding of FITC-labeled α-bungarotoxin was observed throughout the intimal lining layer and to a lesser extent in the synovial sublining (Figure 2C). Pretreatment with AR-R17779, interfering with the binding domain of α-bungarotoxin (39), markedly reduced the intensity of the staining (Figure 2D), indicating that the α-bungarotoxin was most likely specific for the α7 subunit of the nAChR. Semiquantitative analysis of the sections after preincubation with AR-R17779 revealed a significant reduction in cells staining positive for α-bungarotoxin (from 2.75 ± 0.5 to 0.75 ± 0.96; P < 0.05).

To determine whether the strong staining shown in the intimal lining layer was, at least in part, due to expression of the α7nAChR on FLS, we performed a double-labeling immunofluorescence analysis of RA synovial tissues using an anti-CD55 antibody (a marker for FLS (40)) and FITC-labeled α-bungarotoxin. These stainings revealed that expression of α7nAChR was observed in CD55+ FLS, among other cells like macrophages, T-cells, B-cells and dendritic cells (Figure 3).

**Figure 3.** Double-labeling immunofluorescence analysis of rheumatoid arthritis (RA) synovial tissue using an anti-CD55 antibody (red) and fluorescein isothiocyanate–labeled α-bungarotoxin (green). A, Expression of the α7nAChR was observed in CD55+ fibroblast-like synoviocytes (FLS). Synovial tissue sections from 2 representative RA patients are shown. B, High-magnification views of the CD55+ FLS. (Original magnification 100 X in A; 400 X in B.) (See also section Color figures).
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FLS of RA patients express the α7 subunit of nAChR

To confirm the expression of α7nAChR on FLS, immunocytochemical and immunofluorescence studies were performed using primary FLS from RA patients (n=5). The cells were stained with

Figure 4. Expression of the α7 subunit of the nicotinic acetylcholine receptor (α7nAChR) on primary fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA). A, The majority of FLS stained positive for the α7 subunit using the α7nAChR-specific antibody. FLS from 1 representative RA patient are shown. B, No staining was seen with the rabbit isotype control. C, A similar staining pattern was seen using fluorescein isothiocyanate (FITC)-labeled α-bungarotoxin. D, Pretreatment with excess of the α7nAChR agonist AR-R17779 resulted in a marked reduction of fluorescence intensity of FITC-labeled α-bungarotoxin. (Original magnification 100 X in A and B; 400 x in C and D.) (See also section Color figures).

Figure 5. Gene expression of mRNA coding for the classic α7 subunit of the nicotinic acetylcholine receptor and the dupα7 variant, using specific primers for the respective mRNA. Shown are relative expression levels of quantitative polymerase chain reaction products in fibroblast-like synoviocytes from 8 patients with rheumatoid arthritis. Expression levels were normalized to GAPDH using the recommended change in threshold cycle (ΔCt) method. Ct is defined automatically by the instrument using the auto mode of Ct determination.

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A polyclonal antibody against α7nAChR or with FITC-labeled α-bungarotoxin. The majority of FLS from all RA patients (mean ± SD semiquantitative score 2.6 ± 0.5) stained positive for the α7 subunit using the antibody (representative picture is shown in Figure 4A), whereas no staining was seen with the rabbit isotype control (Figure 4B). Similar staining was observed using FITC-labeled α-bungarotoxin (mean ± SD semiquantitative score 2.8 ± 0.4) (Figure 4C). Pretreatment with excess of the α7nAChR-specific agonist AR-R17779 resulted in a significant reduction of cells staining positive for FITC-labeled α-bungarotoxin (mean ± SD semiquantitative score 0.75 ± 0.5) ($P < 0.05$) (Figure 4D).

Using quantitative PCR, we next examined the expression of messenger RNA (mRNA) coding for the α7 subunit. We analyzed the expression of both α7 and its dupα7 variant, using specific primers for the respective mRNA of FLS from 8 RA patients. FLS from 5 out of 8 RA patients expressed detectable levels of the classical α7 subunit, whereas all FLS expressed dupα7 mRNA (Figure 5). As a positive control, mRNA of human neuroblastoma cell line SKNSH was used. No statistically significant differences between expression levels of the classic α7nAChR and those of the dup variant were observed in RA FLS that expressed both variants.

**Figure 6.** Cholinergic agonists inhibit tumor necrosis factor α (TNFα)–induced interleukin-6 (IL-6) and IL-8 secretion in primary fibroblast-like synoviocyte cultures of 8 rheumatoid arthritis patients. Values are the mean and SD. Shown are inhibition of IL-6 and IL-8 release by nicotine and AR-R17779 after stimulation with TNFα for 4 hours. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control.
Inhibition of IL-6 and IL-8 release by nicotine and AR-R17779 in TNFα stimulated FLS

To provide more insight into the functional role of the α7nAChR in FLS under inflammatory conditions, we examined TNFα-induced cytokine and chemokine secretion by FLS (from 8 RA patients) pretreated with nicotine or the α7nAChR-specific agonist AR-R17779. The levels of cytokines and chemokines secreted in the culture supernatants were measured stimulation with TNFα for 4 hours. As shown in Figure 6, AR-R17779 inhibited IL-6 and IL-8 release ($P < 0.001$ for nicotine [1-10 μM] and AR-R17779 [10 μM]).

DISCUSSION

We identified a role for the α7nAChR in regulating the inflammatory response in primary RA FLS in a target screen using a library of Ad.shRNA against 807 transcripts. The screen was performed in independent RA FLS cell lines and showed upregulation of IL-8, MMP-1, and MMP-3 production upon knockdown of the target genes. These results suggest a strong involvement of the α7 subunit of the nAChR in modulating the inflammatory response. Consistent with the notion that the α7nAChR is involved in regulation of the inflammatory response in RA, we subsequently found that α7-specific agonists reduce TNFα-induced IL-6 and IL-8 production by FLS. Moreover, expression studies presented here show that the α7 subunit of the nAChR and its dupα7 variant are expressed by RA FLS and the inflamed synovium.

Immunohistochemical and double-labeling immunofluorescence analysis demonstrated expression of the α7nAChR on FLS in the intimal lining layer. We confirmed these results by labeling experiments with FITC-labeled α-bungarotoxin, which binds specifically to the α7nAChR. It is known that various other immune cells also express α7nAChR, explaining the staining in the synovial sublining from RA patients. Consistent with the results of double immunofluorescence, cultured primary FLS derived from RA patients expressed the α7nAChR, as shown by labeling with either α7-specific antibody or FITC-labeled α-bungarotoxin.

In view of the recently described dupα7 receptor on leucocytes, we also investigated the expression of the dupα7 gene in FLS. We performed quantitative PCR with specific primers for the classical α7 variant and dupα7 variant. As in leukocytes, FLS from all patients expressed dupα7 mRNA. Expression of the gene for the classical α7 receptor was not shown in FLS from all patients, but we could detect the expression of α7nAChR on the protein level in all patients. We can only speculate about the reason for this observation, but a possible explanation could theoretically be the rapid turnover of the mRNA in some of the RA FLS. Since specific antibodies to the dupα7 variant are not yet available, we currently do not know how these results translate into the protein level. It is widely accepted that the binding domain for α-bungarotoxin is contained in the extracellular N-terminus, encoded by exons 1-5 that are not conserved in the dupα7 gene (38;39). At this time the function of the dupα7 receptor on FLS remains unclear and is a subject of ongoing research in our laboratory. One possibility might be that the dupα7
variant can oligomerize with the classic α7 receptor in order to increase its binding affinity for α7-specific agonists.

To study the cellular response of FLS to α7nAChR activation, we investigated the effect of nicotine and AR-R17779 on stimulated FLS. AR-R17779 is a more selective α7nAChR agonist with a 35,000-fold higher selectivity and 5-fold higher affinity for the α7nAChR compared with nicotine (41;42). Previous in vivo studies have indicated that nicotine and α7-specific agonists have antiinflammatory properties. Pharmacologic stimulation with the α7-specific agonist CNI-1493 as well as electrical stimulation of the vagus nerve were shown to decrease acute inflammation (23).

Recently we have shown that in murine CIA, nicotine treatment as well as treatment with AR-R17779 resulted in a significant decrease in clinical signs of arthritis, joint destruction, and TNFα expression in the synovium and plasma (24). In the present study we show the significant decrease of IL-6 and IL-8 production by FLS after treatment with nicotine or AR-R17779; these are important proinflammatory cytokines involved in the pathogenesis of RA (37;43;44). These data suggest that stimulation of α7nAChR by specific agonists could rapidly deactivate FLS in the synovium, and the effects could potentially synergize with the previously reported antiinflammatory effects of α7-specific agonists on macrophages. Cells of the immune system tightly regulate the production of cytokines by repressing their expression at the posttranscriptional level, so the rapid decrease in IL-6 and IL-8 levels could be caused by a mechanism consistent with targeting mRNA stability (45). After 24-hour stimulation the antiinflammatory effects of both agonists were diminished in vitro (data not shown). It is known that the α7nAChR displays fast (millisecond) onset of desensitization upon agonist application (46), so this phenomenon could be caused by desensitization and loss of biologic response of the receptor due to sustained agonist stimulation.

Taken together, the results presented here reveal the expression of α7nAChR on synovial tissue and FLS from RA patients. Moreover, the data show that α7-specific agonists can modulate the inflammatory response of FLS, which suggests the importance of α7nAChR, and perhaps dupa7, in the regulation of proinflammatory cytokines and chemokines. Since the therapeutic use of nicotine is obviously not an option because of its pharmacologic nonspecificity and toxic side effects, specific α7nAChR agonists or perhaps neurostimulation of the cholinergic antiinflammatory pathway may be better candidates for the development as a novel therapeutic approach for the treatment of RA. At this time the most well-characterized specific α7nAChR are GTS-21, AR-R17779 and CAP55 (47). Treatment with GTS-21 produced beneficial effects in psychological and cognitive tests when given to healthy volunteers in clinical trials, and patients tolerated the treatment well (48-50). Development of agonists that do not cross the blood-brain barrier would improve the application of these kinds of agents for the treatment of chronic inflammatory diseases like RA. Future studies are needed to determine whether α7nAChR agonists can control the inflammatory process in patients with RA.
Chapter 6

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