Cholinergic nervous system as therapeutic approach for the treatment of arthritis
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Summary and general discussion
This thesis focuses on the role of the cholinergic anti-inflammatory pathway in arthritis. Specifically, we investigated the role of the α7 subunit of the nicotinic acetylcholine receptor (α7nAChR) as a therapeutic target for the treatment of arthritis.

**SUMMARY AND MAIN FINDINGS**

In Chapter 1, a general introduction to the present knowledge of rheumatoid arthritis (RA), the pathogenesis of RA and current treatment is given. Furthermore, the cholinergic antiinflammatory pathway is introduced, focusing on the role of α7nAChR as a target for treatment of RA.

Chapter 2 provides a perspective on the role of the cholinergic antiinflammatory pathway in the treatment of RA. This chapter describes the current knowledge about the cholinergic pathway as a novel therapeutic approach for inflammatory diseases, and specifically the role of α7nAChR as target for novel treatment. Furthermore, this chapter contains detailed information about the structural and functional aspects of α7nAChR and its duplicate variant. The therapeutic implications of the cholinergic antiinflammatory pathway in RA and the possible underlying mechanisms are discussed; vagus nerve stimulation (VNS) as well as a pharmacologic approach are described. VNS has already shown antiinflammatory effects in several animal models of systemic inflammatory diseases (1-3). A possible role for VNS in the treatment of arthritis was suggested by a study showing an antiinflammatory effect of VNS on carrageenan-induced paw inflammation (4) and it has recently been shown that VNS is able to suppress the development of collagen-induced arthritis (CIA) in rats (5). The recent discovery of α7nAChR expression on immune cells and fibroblast-like synoviocytes (FLS) (6) raises the possibility of suppressing inflammation in RA by specifically targeting this receptor. The studies in Chapter 3-5 show the first results of targeting the cholinergic antiinflammatory pathway, and specifically α7nAChR, in the treatment of experimental arthritis.

Chapter 3 describes for the first time a role of the cholinergic nervous system in CIA, a well known animal model for RA. Inhibition of the cholinergic antiinflammatory pathway using unilateral cervical vagotomy resulted in a trend towards increased severity of arthritis whereas oral nicotine treatment starting before the onset of disease resulted in a significant decrease in clinical signs of arthritis, joint destruction and TNFα expression in the synovium. Pharmacologic activation of the nAChR with intraperitoneally (IP) injected nicotine resulted in a reduction of arthritis activity and a trend toward reduced bone destruction. IP injections with the specific and highly selective α7nAChR agonist AR-R17779 resulted in clinical improvement associated with significantly reduced bone destruction. The level of TNFα in the synovium as well as in the serum was clearly decreased after treatment with both IP injected nicotine and AR-R17779.
In Chapter 4, the role of α7nAChR in arthritis was further investigated. The objective of the study was to determine the role of the endogenous cholinergic antiinflammatory pathway, rather than the effect of exogenous stimulation of this mechanism, in CIA in α7nAChR deficient (α7nAChR−/−) and wild-type (WT) littermate mice. Mice deficient for the α7 subunit showed a clear increase in clinical arthritis scores in the acute phase (56%) and chronic phase (40%) of disease. This was accompanied by an increase in the incidence of arthritis, synovial inflammation, and joint destruction. It has been reported previously that splenectomy and selective abdominal vagotomy inactivate the antiinflammatory effects of either vagus nerve stimulation or administration of α7nAChR agonists, suggesting that the spleen is a specific and essential target of the cholinergic antiinflammatory pathway (7-9). Therefore, we investigated the antigen-specific production of TNFα by splenocytes from α7nAChR−/− mice and WT littermates in vitro. In splenocytes of α7nAChR−/− mice the production of TNFα was more pronounced. This can in part explain the observed increase in severity of arthritis since, as in human RA, TNFα plays a pivotal role in the pathogenesis of CIA (10;11). These data indicate that immune cell function in murine CIA is regulated by the cholinergic system which is, at least in part, mediated by α7nAChR.

In Chapter 5, the functional activity of two novel α7nAChR agonists, CTI-15311 and CTI-15072, at α7nAChR expressed in Xenopus oocytes, was tested. Thereafter, the therapeutic effect of both agonists was tested in CIA in mice. Patch clamp electrophysiological experiments showed that CTI-15311 acts like a classical agonist of α7nAChR, but that CTI-15072 does not produce a current. Because CTI-15072 can bind to α7nAChR, it can actually act as an ion channel antagonist. Moreover, CTI-15072 was clearly distinct from typical competitive antagonists, since it was able to synergize with the allosteric modulator PNU-120596, suggesting that it is a selective desensitizer of α7nAChR. Furthermore, both agonists poorly pass the blood-brain barrier and could be delivered orally. It was hypothesized that the difference in characteristics could result in a different clinical and histological effect on arthritis. Both compounds can improve arthritis but the doses-response study showed that treatment with CTI-15311 2 mg/kg started before onset of arthritis, resulted in the most pronounced reduction of arthritis activity and a significantly reduced disease incidence and delayed onset of disease. Treatment with CTI-15311 2 mg/kg also resulted in a significant decrease in synovial inflammation and joint destruction. In conclusion, these data show the importance of α7nAChR in CIA, supporting the view that the α7nAChR may be a future target for the treatment of RA.

The ultimate goal is to develop new therapeutic agents for the treatment of patients with RA. Therefore, in Chapter 6 we focused on the role of the cholinergic antiinflammatory pathway in human RA. The ability of the cholinergic antiinflammatory pathway to suppress peripheral
inflammation in CIA prompted us to determine if α7nAChR is expressed in human synovial tissue samples and on primary cultured FLS.

A target screen using a library of adenoviral short hairpin RNA (Ad.shRNA) against 807 transcripts revealed that knockdown of α7nAChR by a specific shRNA results in increased interleukin (IL)-8 and matrix metalloproteinase expression (MMP)-1 and MMP-3 by RA FLS. Thus, having identified α7nAChR as a key regulator of RA FLS chemokine and MMP production using a hypothesis-free target screen, we performed immunohistochemical and immunofluorescence analysis to examine the in situ expression of α7nAChR in synovial tissue from RA patients. Synovial tissue sections of all RA patients tested showed expression of α7nAChR in the inflamed synovium, predominantly in the intimal lining layer and to a lesser extent in the synovial sublining. Double-labeling immunofluorescence analysis of the tissue sections with anti-CD55 antibody and FITC-labeled α-bungarotoxin revealed expression of α7nAChR in CD55-positive FLS in situ. Moreover, we also found α7nAChR expression in cultured RA FLS, both at the mRNA as well as the protein level. We demonstrated for the first time the expression of the duplicate α7 transcript (dupα7nAChR) in FLS; however, its role is still unclear. In order to get more insight into the functional role of the α7nAChR in FLS under inflammatory conditions, we examined TNFα-induced cytokine and chemokine secretion by FLS pretreated with nicotine or the α7nAChR-specific agonist AR-R17779. AR-R17779 inhibited IL-6 and IL-8 release, two important proinflammatory cytokines involved in the pathogenesis of RA (12-14), more effectively than nicotine. 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. Take together, from chapter 6 we can conclude that the α7nAChR is expressed in RA synovium and RA FLS, where they may play a critical role in regulating inflammation. This suggests that α7nAChR could represent a new target for the treatment of RA. After submission of our manuscript, another study confirmed our data, showing expression of α7nAChR in the synovium and on FLS (6). Preincubation of IL-1 stimulated FLS with the α7-specific agonist PNU-282987 resulted in a decreased release of proinflammatory cytokines. This was, at least in part, regulated by a decrease in mRNA stability.

**THERAPEUTIC IMPLICATIONS**

The work described in this thesis provides for the first time a clear link between the cholinergic nervous system and the inflammatory process in inflamed joints, leading to the identification of the cholinergic antiinflammatory pathway and especially the α7nAChR, as a novel therapeutic target for the treatment of arthritis.
In RA patients, the cholinergic antiinflammatory pathway appears to be suppressed (15). Consistent with this notion, a recent study has shown a correlation between depressed levels of vagus nerve activity and elevated levels of the proinflammatory cytokine HMGB1 in patients with RA (16). Accordingly, an other study showed that the adverse autonomic profile in RA patients, characterized by increased sympathetic and decreased parasympathetic tone, is also associated with reduced efficacy of anti-TNFα treatment (17). This opens the possibility that manipulating the cholinergic antiinflammatory pathway might be used to enhance the treatment response to anti-TNFα therapy and supports the hypothesis that the cholinergic pathway may be a target to control unrestrained inflammation in RA.

With the discovery of the cholinergic antiinflammatory pathway comes the possibility of developing therapies aimed at controlling the inflammatory response. Suppression of proinflammatory cytokine production may be possible by either altering vagus nerve activity or targeting specific components of the cholinergic pathway by pharmacologic compounds, described in chapter 2. Direct stimulation of the vagus nerve requires implantation of a small device. VNS has been an approved treatment for refractory epilepsy and is being tested in clinical trials for resistant depression (18). Unexpectedly, in a study of VNS in patients with resistant depression, circulating levels of both pro- and antiinflammatory cytokines were found to be markedly raised and this increase was unlikely to be a nonspecific inflammatory reaction (19). In addition, it is not known whether the ACh release mechanisms are compromised in patients with RA, which could make stimulation of the vagus nerve inefficient as a treatment option (20). Non-invasive transcutaneous VNS has been shown to improve survival in murine sepsis (21), and future studies should address the question whether this approach can be translated into treatment of RA.

It is interesting to note that the joint is not innervated by the vagus nerve. Still, we and others have shown that electrical and pharmacological stimulation of the vagus nerve results in decreased carrageenan- and collagen-induced joint inflammation in mice and rats. This could be explained by 2 mechanisms. First, recent data indicate that the spleen may have role in exerting the antiinflammatory effects of vagus nerve activity, as VNS fails to attenuate serum TNF levels in splenectomized mice treated with endotoxin (8). This neural connection between the vagus nerve and the spleen can allow for rapid and precise control of systemic cytokine production (8). It may also control the trafficking of inflammatory cells to the site of inflammation, the synovium (22). Second, the existence of a marked non-neuronal cholinergic system in human synovial tissue from RA patients has recently been shown (23). Immunohistochemistry and in situ hybridization revealed both choline acetyltransferase (ChAT) protein and mRNA expression in FLS and mononuclear-like cells in the synovium. These results support earlier reports that immune cells are able to produce ACh (24-26). This underscores the possibility that local release of ACh modulates...
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the inflammatory process in the joint. In addition, the results described in chapter 6 together with the recent paper by Waldburger et al. (6) show expression of α7nAChR in the synovium and on FLS of RA patients.

Collectively, these data support the development of therapeutic strategies aimed at specific targets of the cholinergic pathway, especially the α7nAChR. There are several well characterized specific α7nAChR agonists among which GTS-21, AR-R17779 and PNU-282987. AR-R17779 was the first full agonist of α7nAChR (efficacy 96%) and until recently the most selective α7nAChR-specific agonist known (27). This unique potency and selectivity of AR-R17779 in combination with the minimal ability of crossing the blood-brain barrier make AR-R17779 an important tool for studying the function of α7nAChR in arthritis. Consistent with the notion that α7nAChR is expressed in the synovium, we have shown the antiinflammatory effects of AR-R17779 in experimental arthritis. Recently, two novel α7nAChR-specific agonists were developed with higher specificity and better affinity for the α7 subunit than AR-R17779 with negligible brain penetration; CTI-15311 and CTI-15072. We tested both agonists in CIA and CTI-15311 has been shown to be more effective than AR-R17779. So far GTS-21 is the only specific α7nAChR agonist that has been tested in humans. Recently, GTS-21 was tested in a phase 2 trial in patients with schizophrenia. The trial showed a clear effect on core negative symptoms whereas no effect on cognition was shown (28). Future studies are required to evaluate the potential use of specific α7nAChR agonists for the treatment of RA. It seems likely that in RA, different mechanisms have a role in the cholinergic antiinflammatory pathway, including involvement of a variety of different immune cells, neurotransmitters and ACh receptors, ultimately leading to downregulation of inflammatory responses.

In conclusion, although significant progress has been made in understanding the changes in the systemic immune response in RA, in particular in anti-citrullinated peptide antibody (ACPA) positive RA, it is still unclear why the joints are targeted and how the symmetrical distribution of the clinical manifestations can be explained. Our research suggests that neuroinflammatory pathways may play a crucial role. This work could also lead to novel therapeutic strategies, by targeting the cholinergic antiinflammatory pathway and especially the α7nAChR. Future studies, both clinical and preclinical, are needed to explore the protective effects of these methods in the treatment of RA.
REFERENCES


