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Effect of Macrophage Expressed α7 Nicotinic Acetylcholine Receptor (α7nAChR) on Migration of Macrophages During Inflammation

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ABSTRACT

Effect of Macrophage Expressed α7 Nicotinic Acetylcholine Receptor (α7nAChR) on Migration of Macrophages During Inflammation

by

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Sepsis is a life-threatening condition characterized by overwhelming inflammation, resulting in organ system damage, leading to a high mortality rate. Care in the clinical setting is supportive, and there are no approved sepsis-specific treatments. In septic mice, activation of the cholinergic anti-inflammatory pathway decreases cytokine secretion by leukocytes and improves survival. The cholinergic anti-inflammatory pathway is a reflex of the parasympathetic nervous system, converging on the α7 nicotinic acetylcholine (α7nAChR) at the surface of macrophages. Signaling through the receptor blocks NF-kB activation, thus cytokine secretion. Receptor activation has other effects on macrophages, including modulating their migration to target tissues during inflammation. The goal of this study was to describe the contribution of α7nAChR to macrophage migration during sepsis, using both activation with agonist PNU-282987 and α7nAChR-/- mice. We showed that α7nAChR-deficiency impedes migration to inflamed tissues, and that α7nAChR activation promotes macrophage accumulation in tissues, an effect mediated through altered expression of integrin αMβ2.

DEDICATION

This dissertation is dedicated to my husband Yihao, my parents, and my extended family for their unwavering support and encouragement during my PhD studies. Lastly, my cat Jolie for her companionship during my many hours in zoom lectures and reading papers.

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CHAPTER 1. INTRODUCTION

Structure and Function of the α7nAChR

The α7 nicotinic acetylcholine receptor (α7nAChR) is a receptor and ion channel belonging to the cysteine-loop ligand gated ion channel superfamily (Changeux 2012; Tsetlin et al. 2011). It is homopentameric in structure, meaning it is composed of five identical α7 subunits. Each subunit contains four transmembrane α-helices (TM), with a cytoplasmic loop located in between TM3 and TM4 (Albuquerque et al. 2009; Noviello et al. 2021). The ligand binding site is within the extracellular N-terminus of each α7nAChR subunit, with a complete receptor having five ligand binding sites in total. The central ion pore of the receptor is formed by TM2 in each subunit and has exceptional permeability to Ca^{2+} when compared to Na^{+} (Noviello et al. 2021). Recently, the gating cycle of the α7nAChR was mapped and the structure of the receptor was found to have three distinct conformations: a closed-channel ("resting") state, an open-channel ("activated") state, and a desensitized state (Noviello et al. 2021). The receptor rapidly desensitizes after ligand binding. However, ion translocation is not the only potential function of the α7nAChR, as due to the cytoplasmic loop between TM3-TM4 it is capable of interacting with signaling cascade proteins (Stokes et al. 2015). Activation of α7nAChR can trigger multiple downstream signaling pathways that have wide-spread effects on cellular functions, including JAK2/STAT3 (Marrero & Bencherif, 2009), MAPK, and ERK1/2/AP-1 (Jull et al. 2001; Liu et al. 2017). The activation of these intracellular signaling pathways induces changes in several cellular processes, including apoptosis, cell proliferation, phagocytosis, and cell migration (Guan et al. 2015; Lee and Vazquez 2013; Ulleryd et al. 2019b; Yang et al. 2015).

There are multiple ligands able to bind to the α7nAChR that can be classified into three broad categories: antagonists, agonists, and allosteric modulators. A complete list of these

molecules can be found in Table 1. Antagonists of α7nAChR are molecules that bind to the receptor but do not change the conformation, open the ion channel, or result in signal transduction. Examples of antagonists that have been used in the laboratory are α-bungarotoxin, also called α-cobratoxin, and methyllycaconitine (MLA) (Arredondo et al. 2009; Li et al. 2011; Wang et al. 2003). Both have a high specificity for α7nAChR and are well studied. Agonists of α7nAChR are molecules that bind to the receptor and are able to open the ion channel and activate intracellular signaling pathways. PNU-282987 and GTS-21 are two well studied synthetic agonists of α7nAChR (Nullens et al. 2016; Pavlov et al. 2007; Pinheiro et al. 2017; Shi et al. 2022). There are other synthetic agonists used in studying α7nAChR, such as AZ6983, AR-R17779, and TC-7020 (Marrero et al. 2010; Mullen et al. 2000; Ulleryd et al. 2019a). The endogenous agonist for α7nAChR is the ubiquitous neurotransmitter acetylcholine. Another well studied, but less specific, agonist of α7nAChRs is nicotine, most commonly known as the addictive agent in tobacco products (Saeed et al. 2005; Wang et al. 2004). PNU-2828987 is the agonist molecule chosen for our experimental designs due to its high specificity. Positive allosteric modulators of α7nAChR are molecules that bind to the receptor's allosteric site and sustain the activation of the receptor. One such modulator is PNU-120596 (Li et al. 2012). In experimental setups, they're frequently used in combination with an agonist like acetylcholine or choline.

Expression of α7nAChR on Macrophages

Because of its initial cloning from neurons, the receptor was at first termed the "neuronal" α7nAChR. However, since its initial discovery it has been reported to be nearly ubiquitously expressed in other cell types, such as adipocytes, endothelial cells, and leukocytes. Macrophages are a key leukocyte population in the innate immune system. They carry out

multiple effector functions ranging from efferocytosis to promoting wound healing and engulfing and killing bacteria. Expression of the α7nAChR was verified in macrophages both at the protein (cell surface) and at the mRNA level (Sato et al. 1999; Wang et al. 2003). On macrophages, the α7nAChR does still retain its ion translocating abilities in response to activation, but it's primary function may instead be the activation of downstream signaling cascades (daCosta et al. 2011; Kabbani and Nichols 2018). It was demonstrated by Kevin Tracey's research team that the α7nAChR expressed on macrophages is essential for the function of the cholinergic anti-inflammatory pathway, introduced in the section below (Wang et al. 2003).

The Cholinergic Anti-inflammatory Pathway. The cholinergic anti-inflammatory pathway (CAP) describes a connection between the nervous and immune systems that acts to control inflammation. This connection was first described by Kevin Tracey's group, and subsequent work elucidated the hypothesized steps of the pathway. The CAP begins with the perception of inflammatory cytokines by the central nervous system, signaling the presence of inflammation (Pavlov et al. 2018; Tracey 2002). After the integration of this signal, the parasympathetic nervous system sends impulses through the efferent vagus nerve, which controls a population of sympathetic neurons in the celiac ganglion. The splenic nerve, a noradrenergic nerve extending from the celiac ganglion, then releases norepinephrine in the spleen (Andersson and Tracey 2012; Pavlov and Tracey 2015; Rosas-Ballina et al. 2008). Norepinephrine next binds to the β-adrenergic receptors on the surface of T-cells, which respond by synthesizing and secreting acetylcholine (Rosas-Ballina et al. 2011; Vida et al. 2011a; Vida et al. 2011b). On the surface of neighboring macrophages, acetylcholine binds to α7nAChRs, activating downstream signaling pathways that prevent translocation of NF-kB into the nucleus. Because NF-kB is a master transcription factor for inflammatory genes, blocking its activation prevents the synthesis

and secretion of inflammatory mediators like IL-8, HMGB1, and TNF-α (Borovikova et al. 2000; Huston et al. 2007; Li et al. 2011; Rosas-Ballina et al. 2009). Vagotomy completely ablates any protective effects of the cholinergic anti-inflammatory pathway (Munyaka et al. 2014; Zhou et al. 2022). Experiments performing splenectomies have demonstrated that the spleen is necessary for the function of this pathway (Huston et al. 2006; Zhou et al. 2022). While there is strong evidence supporting the spleen as a necessary organ in the CAP, the vagus nerve provides input to ganglia in other immune-cell rich tissues such as the gastrointestinal tract, which also has immunomodulatory effects (Matteoli and Boeckxstaens 2013). The cholinergic anti-inflammatory pathway is a promising target for controlling inflammation in human diseases such as sepsis and atherosclerosis, but it's important for future translational research to consider a recently discovered duplicate of the α7nAChR subunit gene: CHRFAM7A.

CHRFAM7A, a Human Specific Duplicate. CHRFAM7A is a partially duplicated form of the α7 gene, CHRNA7. The gene is chimeric, resulting from the combination of exons 5-10 of the CHRNA7 gene, exons A-C and E of unc-51 like kinase 4 gene (ULK4), and exons D and F from the GOLGA8B gene (Di Lascio et al. 2022). CHRFAM7A is inserted on chromosome 15 near the CHRNA7 gene in either an inverse or direct orientation. In addition to orientation, more variation is introduced by a 2-bp deletion and varying copy numbers found in some individuals (Costantini et al. 2015; Di Lascio et al. 2022; Peng et al. 2022).

Because of the replacement of exons 1-4 in the CHRNA7 gene, the encoded protein, dupα7, partially lost the N-terminal ligand binding site. Dupα7 forms heteropentamers with α7 in varying proportions, with at least two α7 subunits required for the ion channel to retain functionality (Di Lascio et al. 2022). Combined, the lack of a ligand binding site and formation of heteropentamers is thought to be how dupα7 influences the functions of α7nAChRs. For

example, microinjection experiments show that few functional heteropentameric receptors reached the membranes of *Xenopus* oocytes, which therefore decreased activity in response to nicotine stimulation (De Lucas-Cerrillo et al. 2011). In addition to this observation, Benfante et al. (2011) reported that mRNA transcripts of dupα7 decreased in THP-1 cells in response to LPS stimulation. The effect was determined to be regulated by NF-κB, as treating cells with an inhibitor prevented the down-regulation of the dupα7 transcript (Benfante et al. 2011). Decreased macrophage migration following chemotactic signals was also reported in human macrophage-like cell lines in response to transfection of the CHRNAFAM7A gene (Chan et al. 2020). These studies highlight the growing consensus that dupα7 plays a critical role in the regulation of the immune response through the human α7nAChR.

Correlations between dupα7 and inflammation during sepsis have been proposed in the literature. A higher level of dupα7 expression in sepsis patients was associated with a higher level of inflammatory markers, and thus sepsis severity. The converse was also true: patients with lower levels of dupα7 had better prognoses (Cedillo et al. 2015; Di Lascio et al. 2022). Combined with existing scientific research, the discovery of CHRFAM7A introduces new perspectives on the regulation of inflammation during diseases like sepsis. Mouse models with a "knock-in" of this human specific gene are currently being created and validated. They show promise for bridging animal research to clinical applications (Costantini et al. 2019; Costantini et al. 2022).

The Role of α7nAChR in Inflammatory Disease

Sepsis and Endotoxemia

Sepsis is a life-threatening acute condition, typically secondary to bacterial infection, resulting from overwhelming systemic inflammation that often leads to organ damage. A high

level of inflammatory cytokines secreted by immune cells, also termed “cytokine storm”, is largely responsible for this intense inflammatory state. Due to immune cell exhaustion, immunosuppression will follow this dire inflammatory state (Angus and van der Poll 2013; Prescott and Angus 2018). The prognosis for patients is poor even if they recover from sepsis, with up to one third dying in the first year after convalescence, and one sixth having permanently decreased quality of life due to cognitive impairments and difficulties performing activities of daily living (Prescott and Angus 2018). Due to the ability of α7nAChR activation to reduce the secretion of pro-inflammatory cytokines, it has been studied extensively in mouse models of sepsis, like cecal ligation and puncture (CLP) and LPS-induced endotoxemia. In these models, mice have been treated with specific agonists or with vagal nerve stimulation (VNS).

Using human primary macrophages and macrophage-like cell line RAW264.7 treated with LPS, Wang et al (2004) reported that treatment with acetylcholine reduced their secretion of HMGB1 into the culture media. In the same study, nicotine was shown to improve survival in endotoxemic mice and decrease their serum HMGB1 concentrations. Nicotine also reduced HMGB1 in CLP mice and increased their survival, even when the dose was delayed 24h after surgery (Wang et al. 2004). Similar protective effects have been observed for the agonist GTS-21 in septic mice. Pavlov et al. (2007) reported that GTS-21 treatment improved survival in both CLP mice and those with LPS-induced endotoxemia (Pavlov et al. 2007). Additionally, Nullens et al. (2006) reported that intestinal permeability and serum levels of IL-6 and IL-2 were reduced in CLP mice receiving GTS-21. In the same study, α7nAChR-deficient mice also had significantly increased mortality within 48 hours of CLP when compared to WT controls (Nullens et al. 2016). In mouse models of acute lung injury, which mimic the pulmonary damage during sepsis/endotoxemia, intraperitoneal treatment with specific agonist PNU-282897 before

LPS instillation reduced inflammatory leukocytes and cytokines within bronchoalveolar lavage fluid. The same effect was seen when PNU-282897 treatment was given after the instillation of LPS. NF-kB phosphorylation was also found to be decreased by PNU-282897 treatment, reinforcing its role in the CAP (Pinheiro et al. 2017).

Vagal nerve stimulation (VNS) was also shown to reduce the secretion of inflammatory mediators in septic mice. When using transcutaneous VNS, secreted HMGB1 was reduced (Huston et al. 2007). TNF-α also showed reductions in endotoxemic rats after direct electrical stimulation of the vagus nerve (Borovikova et al. 2000). Together, these results support a role for α7nAChRs in sepsis and endotoxemia, providing a rationale for developing new treatments targeting the CAP. Such treatments would greatly benefit patients, as current care for septic patients is primarily supportive. The potential to harness the cholinergic anti-inflammatory pathway to reduce cytokine storm and organ damage is a promising avenue to improve patient prognoses.

Atherosclerosis

In contrast to sepsis, atherosclerosis is a disease resulting from chronic inflammation. Atherosclerosis is a cardiovascular disease that causes plaques to form in the walls of large arteries. Plaque formation is driven by infiltration of monocytes/macrophages that become foam cells, which are macrophages laden with oxidized lipids (Gerrity 1981). The pathogenesis of atherosclerosis results from a combination of clinical factors, including hypertension, hypercholesterolemia, genetics, smoker status, and family history (Ros et al. 2022). Studies in ApoE-/- and Ldlr-/- models of atherosclerosis have shown that activating α7nAChR can attenuate disease progression. In a study giving ApoE-/- mice high specificity agonist AR-R17779 in drinking water, Hashimoto et al. (2014) reported reduced plaque area in the thoracic

aorta, reduced serum cholesterol, and decreased expression of inflammatory cytokines (Hashimoto et al. 2014). Treatment with agonist AZ6983 in the same mouse model resulted in reduced CD68+ macrophages in atherosclerotic plaques and a general reduction in disease severity (Ulleryd et al. 2019a). Other agonists such as GTS-21 and PNU-282897 have been studied in atherosclerosis and have yielded similar results (Qian et al. 2021; Ulleryd et al. 2019c).

Johansson et al. (2014) reported that human macrophages in atherosclerotic plaques express α7nAChR. Staining for α7nAChR coincided with CD68+ and CD13+ macrophages within human atherosclerotic lesion surgical specimens. The effects of α7nAChR-deficiency were also examined, by reconstituting Ldlr-/- mice with α7nAChR-/- bone marrow. These bone marrow chimeras showed increased atherosclerosis when evaluated for lipid content and lesion stage (Johansson et al. 2014). Coupled with the wealth of experimental data demonstrating the anti-inflammatory effects of α7nAChR in atherosclerosis, the confirmation that macrophages within human specimens express α7nAChR is crucial evidence that treatments targeting this receptor may be beneficial in the overall care plans of patients.

Treatments Targeting α7nAChR

Because animal models have revealed protective effects of α7nAChR, there has been growing interest in developing therapies to target it, with the goal of mitigating inflammatory disease symptoms and progression. The α7nAChR can be targeted by both bioelectronic and pharmaceutical means, such as vagal nerve stimulation (VNS) and acetylcholinesterase inhibitors.

Vagal Nerve Stimulation (VNS). The vagus nerve is the endogenous means by which the CAP can be activated to reduce inflammation. It can be stimulated bioelectronically, using either

a transcutaneous electrode or an implanted device. The implanted bioelectronic device has been approved for decades to use in patients with depression and epilepsy unresponsive to medication but requires surgery to begin treatment (Nemeroff et al. 2006; Rincon et al. 2021; Yap et al. 2020). An electrode is wrapped around the cervical branch of the vagus nerve, and the device producing electrical impulses is placed below the skin of the upper chest or axillary border (Howland 2014). Transcutaneous VNS is being evaluated clinically to be approved for use in inflammatory diseases, including long COVID-19 and Crohn's disease, and has the advantage of being non-invasive (Badran et al. 2022; Sinniger et al. 2020). The transcutaneous device takes advantage of a vagus nerve branch in the left auricle of the ear, allowing for stimulation of the nerve with a removable, external electrode (Butt et al. 2020). Vagus nerve stimulation using either of these two bioelectronic devices is generally regarded as safe, with mild side effects such as coughing. However, invasive VNS comes with the risk of post-surgery infection (Toffa et al. 2020).

At the molecular level, VNS was demonstrated to have many of the same anti-inflammatory effects as activation of α7nAChR using specific agonists (Wang et al. 2016). Huston et al. (2007) revealed reduced secretion of HMGB1 in response to transcutaneous VNS (Huston et al. 2007). Direct stimulation of the vagus nerve was also able to reduce the level of TNF-α in the serum of endotoxemic rats (Borovikova et al. 2000). Due to existing approvals and evidence showing it is able to modulate inflammation, VNS is worthy of evaluation for efficacy in sepsis patients.

Pharmacological Interventions. Interest in developing drugs to directly activate α7nAChRs has been growing in recent years due to their potential to treat inflammatory diseases. Currently, compounds to directly stimulate α7nAChRs have not been approved for

human use. There are, however, drugs already introduced to the market that can modulate cholinergic activity, such as galantamine (Razadyne). Galantamine is a drug labeled for use in dementia, a neurodegenerative condition that adversely affects learning and memory. It is designed to block the activity of acetylcholinesterase and thus enhance cholinergic signaling in the central nervous system (Metz and Pavlov 2021). A study conducted in mice showed that inhibiting acetylcholinesterase in the brain can alleviate systemic inflammation (Pavlov et al. 2009).

α7nAChR Modulation of Macrophage Migration

Immune cells, including macrophages, need to travel to the site of inflammation in order to carry out their effector functions such as engulfing bacteria, efferocytosis, and wound healing. The leukocyte migration process is initiated by molecules such as pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) that indicate infection or injury (Medzhitov 2008). The endothelium, as well as immune cells, secrete chemoattractant molecules that attract immune cells to a targeted site (Nourshargh and Alon 2014). There are several steps in the recruitment process that could be altered by α7nAChR signaling or deficiency: rolling and arrest on the venule wall, transmigration through endothelial cells and pericytes, and movement through the extracellular matrix (Nourshargh and Alon 2014).

While modulation of macrophage migration is a potential function of the α7nAChR and therefore could be a unique therapeutic opportunity for inflammatory disease, there have been no studies examining this function in detail. The majority of studies reporting differences in accumulating macrophages with α7nAChR agonists rely on histological techniques or simplified migration setups. For example, in mice with collagen-induced arthritis, those that received nicotine showed fewer macrophages infiltrating into synovial joint sections. Vagotomy verified

that this effect was mediated by the CAP and therefore α7nAChR (Li et al. 2016). Reduced macrophage accumulation in the joint space is correlated with reduced arthritic scores and disease severity. The same pattern is observed with atherosclerosis: fewer macrophages migrate to the sites of forming plaques in response to α7nAChR stimulation (Ulleryd et al. 2019a). Additionally, reductions in disease severity and incidence of abdominal aortic aneurysm have been observed in mice (Hashimoto et al. 2014; Ulleryd et al. 2019c).

Yang et al. conducted a study on macrophage migration in response to agonist treatment. They used a transwell migration setup in which RAW264.7 cells were placed on top of the uncoated membrane and allowed to migrate through to the opposite surface. Before the start of migration, cells were pre-treated with PNU-282987 or acetylcholine before activating them with LPS. Both agonists decreased migration to the opposing side of the membrane when compared to LPS only. The expression and activity of MMP-9 were also studied via qPCR and gelatin zymography, respectively. MMP-9 is a secreted matrix metalloproteinase key in the breakdown of extracellular matrix proteins so that immune cells can move through a tissue. They reported that MMP-9 expression and activity were both decreased by agonist treatment (Yang et al. 2015). While this data is some of the first to report that α7nAChR influences macrophage migration, it has some limitations. The first is the simplified migration setup, as the protocol does not prevent confounding of results by passive movement with gravity. Chemokine gradients were absent as well, so directional and intentional migration of macrophages could not be verified. The report of decreased MMP-9 activity and expression is crucial, yet it could not be corroborated by the migration assay as there was no protein gel, such as fibrin or Matrigel, for the RAW264.7 cells to make their way through.

The primary goal of this project was to improve the understanding of how monocyte/macrophage migration is modulated by α7nAChR expression, as the currently available published data is limited. Experiments were designed to comprehensively test both *in vivo* and *in vitro* migration while accounting for the limitations of previous studies. Because migration depends on coordinating chemotactic cues and adhesion molecule activation, a thorough investigation of how each was affected by α7nAChR was also conducted. The primary inflammatory disease model used was LPS-induced endotoxemia in C57 and α7nAChR-/- mice, with an experiment in the ApoE-/- model of atherosclerosis collected as preliminary data for future exploration.

CHAPTER 2. NEUROIMMUNE NEXUS IN THE PATHOPHYSIOLOGY AND THERAPY OF INFLAMMATORY DISORDERS: ROLE OF α7 NICOTINIC ACETYLCHOLINE RECEPTORS

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Abstract

The α7-nicotinic acetylcholine receptor (α7nAChR) is a key protein in the cholinergic anti-inflammatory pathway (CAP) that links the nervous and immune systems. Initially, the pathway was discovered based on the observation that vagal nerve stimulation (VNS) reduced the systemic inflammatory response in septic animals. Subsequent studies form a foundation for the leading hypothesis about the central role of the spleen in CAP activation. VNS evokes noradrenergic stimulation of ACh release from T cells in the spleen, which in turn activates α7nAChRs on the surface of macrophages. α7nAChR-mediated signaling in macrophages reduces inflammatory cytokine secretion and modifies apoptosis, proliferation, and macrophage polarization, eventually reducing the systemic inflammatory response. A protective role of the CAP has been demonstrated in preclinical studies for multiple diseases including sepsis, metabolic disease, cardiovascular diseases, arthritis, Crohn's disease, ulcerative colitis, endometriosis, and potentially COVID-19, sparking interest in using bioelectronic and pharmacological approaches to target α7nAChRs for treating inflammatory conditions in patients. Despite a keen interest, many aspects of the cholinergic pathway are still unknown. α7nAChRs are expressed on many other subsets of immune cells that can affect the development of inflammation differently. There are also other sources of ACh that modify immune cell functions. How the interplay of ACh and α7nAChR on different cells and in various tissues contributes to the anti-inflammatory responses requires additional study. This review provides an update on basic and translational studies of the CAP in inflammatory diseases, the relevant pharmacology of α7nAChR-activated drugs and raises some questions that require further investigation.

1. Introduction

Interest in the α 7 nicotinic acetylcholine receptor (α7nAChR) as a target for suppressing adverse inflammatory responses has its origin in the early 2000's when research established that stimulation of the vagus nerve suppressed the inflammatory response to injection of lipopolysaccharide (LPS) in rats [1]. Early work showed that the spleen was required for this vagal anti-inflammatory effect and determined essential roles for nicotinic receptors and sympathetic nerves [1–3]. Ultimately, a series of experiments using α7nAChR deficient mice and cholinergic phenotype reporter mice, along with neuroanatomical techniques and flow cytometry, delineated the current model for the cholinergic anti-inflammatory pathway (CAP), which is illustrated in Figure 1. This pathway originates in the brainstem with preganglionic cholinergic neurons of the dorsal motor nucleus, which sends long axonal projections to the periphery by mean of the vagus nerve. While many of these axons innervate parasympathetic ganglia in various end organs, some also control a subpopulation of sympathetic neurons in the celiac and superior mesenteric ganglia, which send noradrenergic nerves to the splenic via the splenic nerve. Activation of this circuitry causes the release of norepinephrine (NE) from varicosities of noradrenergic nerves distributed within the splenic white pulp. Next, NE stimulates β_2 adrenergic receptors located on a special population of CD4+ T cells that can synthesize and release ACh. These cells contain the ACh synthetic enzyme, choline acetyltransferase (ChAT), but release of ACh occurs by a transporter mechanism instead of exocytosis as occurs with cholinergic nerves. While close juxtaposition of noradrenergic nerves with cholinergic T cells was considered important initially, it now appears that diffusion of transmitter to the target T cell population is sufficient. For the final step in the pathway, ACh diffuses to monocyte- and macrophage-rich areas of the spleen and stimulates α7nAChR

receptors located in their cell membrane. Binding of ACh activates a signaling cascade that inhibits the synthesis and release of pro-inflammatory cytokines such as tissue necrosis factor- α (TNF-α). Given the central role that inflammation plays in many diseases, there has been explosive growth in studies investigating potential therapeutic applications for activation of the CAP in the spleen and similar mechanisms in other tissues by using bioelectronic and pharmacologic approaches. Furthermore, recent work has identified novel α7 dependent mechanisms in the bone marrow that regulate hematopoiesis and leukocyte trafficking [4], suggesting an additional target for α7 based immunomodulatory therapy.

2. Therapeutic Approaches

2.1 Bioelectronic methods for activating the CAP

2.1.1 Invasive VNS.

Stimulation of the vagus nerve by means of electrodes applied directly to the cervical vagus has been instrumental in the discovery and characterization of the CAP in preclinical studies [1–3,5,6]. Furthermore, potential translation of this approach to patients to achieve anti-inflammatory responses should be facilitated by decades of experience gained from the use of VNS to treat patients with drug-resistant epilepsy or depression [7–9].

The neuroanatomical circuitry that mediates activation of the CAP in the spleen after direct stimulation of the cervical vagus has been subject to debate, the main point of contention being the role of vagal efferent input to the celiac/superior mesenteric ganglia. There is much functional evidence supporting the idea that excitatory cholinergic input to these ganglia is activated directly by VNS. However, preganglionic cholinergic input that drives sympathetic ganglia normally comes from the spinal cord, and there is solid anatomical evidence for spinal projections to the celiac/superior mesenteric ganglia via the splanchnic nerve [10]. The latter

pathway requires vagal afferent input to the brain and activation of central circuity to increase splanchnic nerve activity. Most evidence links the vagal efferent circuit to activation of the spleen CAP, while the splanchnic circuit evokes an α7 independent anti-inflammatory response [10,11]. The latter is mediated by noradrenergic stimulation of β_2 receptors on macrophages. Given the broad distribution of vagal efferent nerves (Fig. 1), it is not surprising that cholinergic anti-inflammatory effects can also occur in other tissues, such as the gut, via direct effects of neuronal ACh on α7nAChR expressing macrophages [12].

Two factors that limit the utility of invasive VNS clinically are the need for surgery to implant the electrode and stimulator and the occurrence of off-target effects such as coughing [13]. There is also the potential for surgical complications and infection. This has led to the development of non-invasive approaches such as transcutaneous VNS (tVNS) and peripheral focused ultrasound stimulation (pFUS).

2.1.2. Transcutaneous VNS (tVNS)

As the name implies, this approach involves passing current through the skin to activate vagal nerve fibers [14]. Some studies have used this approach in acute experiments to stimulate in the cervical vagus region, and others have targeted the auricular branch of the vagus nerve by applying transcutaneous stimulation of the concha and inner tragus of the outer ear [13]. Precise neuroanatomical mechanisms by which these approaches modulate autonomic efferent tone and the full extent of their therapeutic spectrum for treating peripheral disease and suppression of inflammation are topics of intense investigation [13,15,16]. While there is some evidence that tVNS can suppress inflammatory responses [16], the ability to recruit the CAP and α7nAChRs in the spleen has not been well tested.

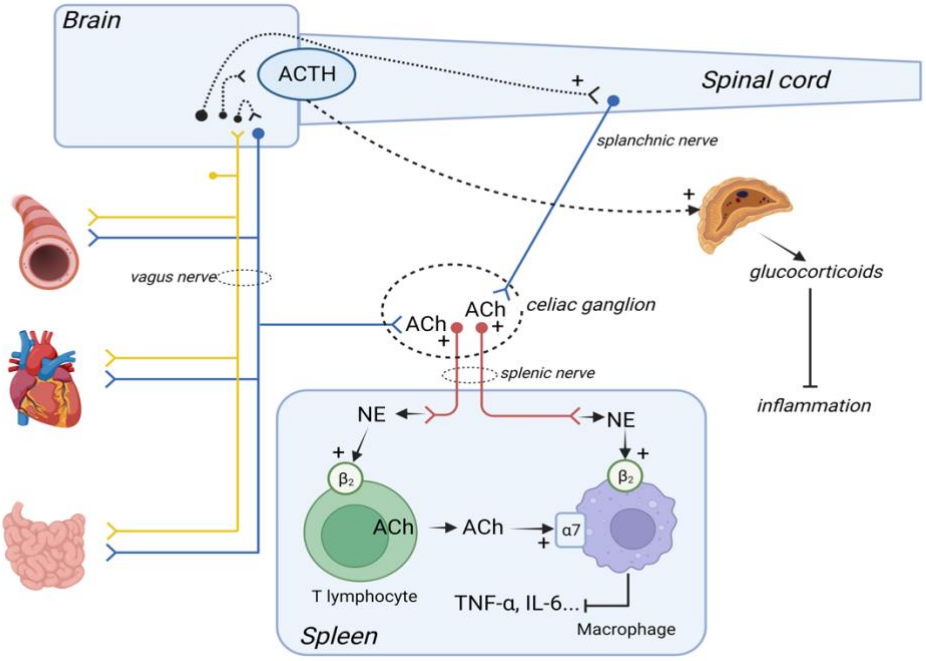

Figure 2.1: Anti-inflammatory effects of the vagus nerve.

Diagram showing pathways for anti-inflammatory effects that can be evoked by stimulation of the vagus nerve. The CAP is activated by stimulation of efferent cholinergic nerves (blue) that project to the celiac ganglia. ACh release from these nerves stimulates postganglionic sympathetic nerves (red) that project to the spleen. NE released from these noradrenergic nerves stimulates β2-adrenergic receptors on CD4+ cholinergic T cells causing release of ACh. Subsequent stimulation of α7 receptors on monocytes/macrophages by ACh inhibits the release of proinflammatory cytokines. Vagal stimulation can also activate afferent nerve fibers (yellow) that project to the medulla. This afferent input can activate different central pathways to stimulate 1) the CAP, 2) spinal sympathetic input to the celiac ganglia and spleen that inhibits inflammatory cytokines release by stimulation of β2-adrenergic receptors on macrophages, and 3) release of ACTH from the pituitary gland and subsequent release of anti-inflammatory glucocorticoids from the adrenal cortex. Cholinergic anti-inflammatory effects can also be evoked by preganglionic vagal input (blue) to cholinergic ganglia in the airways, heart, and intestines. Release of ACh from these postganglionic nerves can activate α7 receptors on macrophages in these tissues.

2.1.3 Peripheral Focused Ultrasound Stimulation (pFUS)

Focused low intensity ultrasound stimulation is an emerging technique that can safely modulate neuronal activity deep within the brain [17], and recent work aims to apply this promising technology to alleviate disease in peripheral tissues by neuromodulation [18]. One obvious target for this approach is the CAP in the spleen. Here the goal is to stimulate release of NE from sympathetic nerves by pFUS. In theory, this should allow activation of the CAP with no off-target effects. Recent preclinical studies using rodent models have provided extensive evidence that FUS applied to the spleen can activate the CAP by stimulating sympathetic nerves in the tissue, bypassing the need for upstream VNS [19–21]. Application of FUS to the spleen produced equivalent inhibition of LPS-evoke inflammation to that achieved by invasive VNS and did so without producing bradycardia or altering companion LPS-evoked hyperglycemia [20]. The anti-inflammatory response to FUS was absent in mice lacking α7nAChRs (knockouts) or cholinergic T cells (genetic ablation) and after treatment with reserpine to deplete catecholamines. In contrast, FUS applied to a specific hepatic site, blocked LPS-induced hyperglycemia by a non-CAP mechanism involving hepatic sensory fibers but did not alter the inflammatory response to LPS [20]. Anti-inflammatory effects of FUS have also been demonstrated in mouse models of arthritis [21] and pneumonia [19].

2.2. Pharmacological Methods for Activating/Blocking the CAP

2.2.1. Direct Stimulation/Blockade of the α7nAChR

There is substantial interest in developing drugs that can stimulate α7nAChR selectively for potential use in treating inflammatory conditions as well as diseases of the central nervous system (CNS) such as depression, schizophrenia, and Alzheimer's disease. Interestingly, the later CNS diseases are now recognized to also have inflammatory components. Work to develop

α7nAChR active drugs and characterize their pharmacology at the cellular level has contributed to improved understanding of the structure and function of this unique receptor [22].

Nicotinic receptors are pentamers comprising specific subunits, which give each variant its distinctive properties. While most nicotinic receptors are heteromeric, having different combinations of specific α and β subunits, α7nAChRs are unique in being homomers containing five α7 subunits. All nicotinic receptors function as ligand-gated cation channels when activated by agonists such as ACh, but α7nAChRs differ in having a preferential permeability to calcium compared to sodium. They also undergo rapid and reversible desensitization to a non-conducting state when stimulated by ACh. Non-neuronal localization of α7nAChRs is common in the periphery, where their presence on macrophages is central to this review because they mediate anti-inflammatory effects. Accumulating evidence gained from biochemical studies of macrophage responses to α7 receptor activation suggests that signaling in these cells is not mediated exclusively by channel activity. Rather, such responses have characteristics typical of metabotropic signaling [22]. Specifically, stimulation of α7 receptors in these cells stimulates the Jak2/STAT3 pathway, which inhibits NF-κB activation and thereby decreases production of inflammatory cytokines while increasing anti-inflammatory cytokines. How α7 receptors connect to this pathway remains unknown, but it is thought that such signaling is associated with the desensitized, non-conductive state [22].

Signaling through α7nAChRs in humans also involves complications introduced by CHRFAM7A, a human-specific duplication of the α7nAChR gene, consisting of exons A, B, C, and E of the ULK4 gene and exons 5-10 of the CHRNA7 gene. It is located on chromosome 15 in either a direct or inverted orientation to CHRNA7 [23]. In addition to these two orientations, a 2-bp deletion and variable copy numbers have been identified [24]. The CHRFAM7A gene

encodes a functionally altered α7 subunit, dup-α7, which is thought to be regulated at the transcriptional level [25]. Dup-α7 can form heteropentamers with functional α7 subunits in varying proportions, requiring at least two α7 subunits for the ion channel to retain function [23,26]. Data show that dup-α7 may negatively modulate α7nAChR activity as part of a heteropentamer, hypothetically through the reduction of ligand binding sites, which are located in the N-terminal region (exons 1-7) of the subunits, and partially lost in the CHRFAM7A chimera [23].

Its expression during inflammatory disease is related to the presence of inflammatory stimuli, which down regulate CHRFAM7A expression and increase CHRNA7 expression in an NF-κB dependent manner. It was suggested that this method of regulation blocks unneeded CAP activation in homeostatic state and promotes CAP activation in the presence of inflammatory mediators [23]. The proportions of CHRFAM7A:CHRNA7 vary between inflammatory diseases, and further study regarding the role of CHRFAM7A is warranted, as this duplicate gene may be a translational gap between laboratory animal and human studies.

Many drugs have been synthesized and tested with the goal of identifying agents that can elicit α7 mediated responses with high selectivity and potency [22]. One limitation of this work has been the need to measure α7 channel activity as a functional assay. Nevertheless, this work has resulted in several drugs that evoke anti-inflammatory activity in vitro and in animal models of inflammation. These include agonists, positive allosteric modulators, and silent agonists. There has also been progress in discovery of selective antagonists, which have an important role in mechanistic studies required to establish that anti-inflammatory effects of drugs and vagal stimulation are mediated by the α7nAChR. Table 1 lists major drugs that have been used in

preclinical studies of cholinergic anti-inflammatory effects.

Table 2.1 Cholinergic drugs used to study α7 mediated anti-inflammatory responses.

Drug class/name	Mechanism	References
α7 agonists		
GTS-21	Partial agonist and causes long-lasting desensitization of α7. Blocks $5HT_{3a}$ and other nAChR subtypes	22
PNU-282987	α7 selective	22, 28-30
PHA-543,613	α7 selective	22, 28-30
PHA 568487		182
AR-R17779	Selective for α7nAChRs in binding and functional assays. Not active at 5HT3aR	180
AZ6983	Activity at α7nAChR but not at α4β2nAChR. High affinity at α7nAChR (19 nM) but low at α4β2nAChR (4.9 μM) and $5HT_{3a}$R (6.6 μM).	91
CAP55	Binding assays and nAChR-mediated anti-inflammatory actions	98

TC-7020	α7 selective but effects on $5HT_{3a}$ not studied. Ki ~2 nM against [^{3}H]MLA. Minimal activity at ganglion and muscle nAChRs. No competition at ~60 other receptors. Effects blocked by MLA	22, 148
ICH3	α7 selective	149, 181
Varenicline	Binding affinity: α4β2>>α3β2>α7>	22, 31-35
Tropisetron	Potent α7 partial agonist and $5HT_{3a}$ antagonist	22, 31-35
Novel drug classes acting at α7nAChR		
PNU-120596	Positive allosteric modulator (PAM)	22, 31-35
GAT107	Direct allosteric activation and PAM	22, 39-41
NSA6740	Silent agonist (long lasting desensitization)	22, 42-44
α7 Antagonists		
α-Bungarotoxin (α-BTX)	High affinity blockade of α7 and skeletal muscle nAChR	22
Methyllycaconitine (MLA)	α7 selective	22
Drugs acting centrally to stimulate CAP		

McN-A-343	M1 muscarinic receptor agonist	52
CNI-1493	M1 agonist	52
Xanomeline	M1 agonist	54
BQCA	M1 positive allosteric modulator	51, 56, 57
Galantamine	Centrally active ChEI	51, 53, 58
Donepezil	Centrally active ChEI	58, 130-132
Drugs acting by inhibition of peripheral ChE		
Pyridostigmine		59-63

Selective agonists: These drugs evoke channel opening by binding to active orthosteric sites on the receptor, just as ACh. GTS-21 is the most widely used drug in this class and has well-established α7-mediated anti-inflammatory effects. It is a partial agonist that produces long-lasting desensitization, which could be important for metabotropic signaling [22,27]. It also blocks 5-HT_{3a} serotonin receptors and, at higher concentrations, blocks some other nicotinic receptors [22]. PNU-282987 and PHA-543,613 are full, rapid desensitizing agonists at the α7nAChRs [22,28–30], and both have documented anti-inflammatory activity. Tropisetron is another agent in this class with some properties like GTS-21 [31]. It is approved for clinical use as an antiemetic, due to its antagonist activity at 5-HT_{3a} receptors, but it is not available in the United States. Limited evidence from preclinical and clinical studies suggests tropisetron has α7-mediated anti-inflammatory activity [32–35].

α7-Positive allosteric modulators (PAMs): Nicotinic receptors have both orthosteric and allosteric binding sites, and binding of drugs to allosteric sites can alter the response to orthosteric agonists like ACh. Two types of PAM have been identified, and these potentiate current responses evoked by ACh while not opening channels when applied alone [22]. Both

types increase peak current, but only Type II drugs prolong responses. There is limited evidence that one of these compounds, PNU-120596 [36], can elicit α7-mediated anti-inflammatory responses [37,38].

Other drugs with α7 activating activity: Two additional profiles have been identified for novel drugs acting at the α7 receptor. GAT107 appears to activate the receptor directly through a distinct allosteric activation site and have PAM activity as well. Such drugs have been termed "ago-positive allosteric modulators" [22]. Effects of GAT107 on the CAP have not been evaluated, but there are reports that this agent reverses inflammatory pain by stimulation of α7 receptors [39] and macrophage dysfunction caused by hyperoxia [40]. Other studies have shown that GAT107 can cause α7-mediated suppression of neuroinflammation and pathology in a murine model of experimental autoimmune encephalomyelitis [41]. Interestingly, the latter effects occur at least in part by stimulation of α7 receptors on T cells, although the same study also established that GAT107 reduced the inflammatory response of RAW267.4 cells (macrophage line) to LPS. Lastly, NS6740 is an early representative of the silent agonist class [22,42]. Such drugs have minimal channel activating activity but do induce a long-lasting non-conducting state (desensitization). If this condition is associated with metabotropic signaling, as proposed [22,42], then silent agonists could be especially effective agents for treating chronic inflammatory diseases. A few studies found that NS6740 can attenuate pro-inflammatory effects of LPS on primary microglia from rats [43] and on a microglia cell line [44].

α7nAChR antagonists: While selective antagonists have no clinical applications, such compounds are valuable for mechanistic studies aimed at confirming the role of α7nAChRs in anti-inflammatory effects evoked by nerve stimulation or drugs (e.g., GTS-21). α-Bungarotoxin (α-BTX) is a snake toxin that has a high affinity for blocking α7nAChRs, but it also blocks

nicotinic receptors at the neuromuscular junction. This limits its utility for in vivo studies, although it can be given directly into the central nervous system. Nevertheless, it can be useful for in vitro studies to evaluate the role α7 receptors on leukocytes and labeled forms of α-BTX can be used to study the cellular localization of these receptors.

Methyllycaconitine (MLA) is the most widely used α7 antagonist, but its selectivity is concentration dependent, and blockade of ganglia can occur at higher doses [22]. While it is generally regarded as a competitive antagonist, a few studies indicate it can have more complicated actions, at least in some test systems. One cell culture study of microglial cells found that MLA can attenuate LPS-induced release of TNF-α [43], which is contrary to results from many other studies of macrophages [45–48]. Recent voltage-clamp studies of α7nAChRs expressed in Xenopus oocytes also suggest that MLA might function as an inverse agonist rather than a simple competitive blocker of ACh [49]. These findings highlight the importance of using complementary approaches (e.g., α7 knockout) to confirm the role of α7nAChRs in neuroimmune mechanisms.

2.2.2. Drugs that Stimulate the CAP Centrally

Specific cholinergic neurons in the brain have output that stimulates the CAP via the vagal efferent pathway (Fig. 2) [50–54]. Although precise circuitry needs to be defined, recent evidence has implicated cholinergic neurons in the medial septum/diagonal band regions, which have projections to downstream areas where cholinergic transmission occurs by activation of postsynaptic M1 muscarinic receptors [51]. This pathway can be activated by centrally acting cholinesterase inhibitors (ChEIs) like galantamine, which enhance cholinergic neurotransmission [51,53]. It can also be activated by intracerebral injection of M1 muscarinic agonists, such as McN-A-343 and CNI-1493, as well as the nonselective muscarinic agonist muscarine [52].

Likewise, peripheral administration of the centrally acting M1 agonist xanomeline attenuated pro-inflammatory cytokine release evoked by LPS and increased survival after LPS administration and in a mouse model of sepsis [54]. Such effects of M1 agonists are centrally mediated since they were blocked by atropine, which penetrates to the brain, and unaffected by atropine methyl nitrate, which does not cross the blood brain barrier [51,52,54,55]. Blockade of inhibitory presynaptic M2 muscarinic autoreceptors on cholinergic nerve ending has the same effect by enhancing release of ACh, and thereby potentiating cholinergic neurotransmission [52]. Central activation of the CAP can also be triggered by central or peripheral administration of BQCA, an allosteric modulator of M1 muscarinic receptors [51]. This novel drug acts by augmenting the response evoked by endogenous ACh binding to M1 receptors [56,57]. Each of these approaches ultimately elicits an anti-inflammatory effect mediated by the CAP and peripheral α7nAChRs.

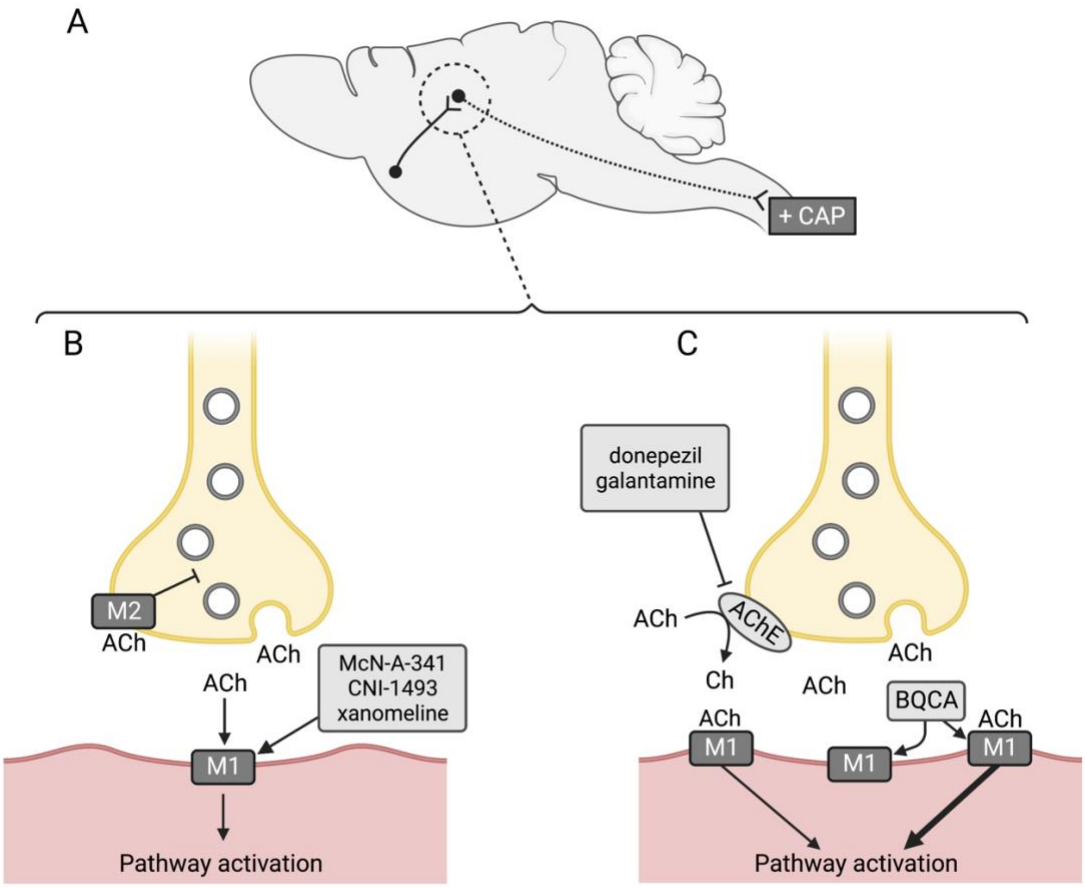

Figure 2.1: Central CAP activation.

Diagram showing drugs and mechanisms for central activation of the CAP. A. Parasagittal section of brain shown cholinergic neuron projecting from the basal forebrain to synapse with first neuron in descending pathway that activated the CAP. Synapse region indicated by dashed circle in shown at higher magnification in B and C. B. CAP pathway is activated by stimulation of postsynaptic M1 muscarinic receptors by endogenous ACh or M1 selective agonists. Presynaptic M2 autoreceptors mediate feedback inhibition of ACh release. C. CAP pathway can be activated by centrally acting cholinesterase inhibitors, which prolong the action of endogenous ACh, and by the M1 allosteric modulator (BQCA), which amplifies the response to M1 stimulation of endogenous ACh. BQCA alone produces no effect.

2.2.3. Drugs Potentiating ACh by Inhibition of ChEs in the Periphery

Drugs that inhibit ChEs in the periphery can amplify and prolong actions mediated by endogenous ACh, whether released from cholinergic nerves or non-neuronal sources such as T and B cells. Therefore, is it logical to expect that peripheral ChEIs might elicit anti-inflammatory effects by this mechanism. Several preclinical studies have evaluated the anti-inflammatory efficacy of pyridostigmine and related quaternary amines in a wide variety of models [58]. Many of these studies found that selective inhibition of peripheral ChE reduced inflammatory indices and most showed improved outcome [59–63], but others reported lack of anti-inflammatory efficacy [64,65]. Nevertheless, several studies that compared centrally active ChEIs to those that act only in the periphery, showed greater anti-inflammatory efficacy with the drugs that inhibit both central and peripheral ChE [58,66,67]. Such superiority of centrally acting ChEIs might be attributed to the fact that they activate the CAP and potentiate its activity by protecting ACh released at peripheral sites.

3. Expression of α7nAChR on Leukocytes

The α7nAChR was originally cloned from DNA libraries created from brain tissue, but subsequent work has shown that it is expressed by numerous additional cell types, including those of the immune system, where it has a major role in regulating inflammation. Expression of α7nAChR on leukocytes has been demonstrated in both RNA and cell-surface studies. The presence of α7nAChR is also indirectly identified when leukocytes respond to stimulation with nicotinic agonists, like ACh and nicotine, and such responses are blocked by MLA or α-BTX.

3.1 Monocytes and Macrophages

Expression of α7nAChR is well documented in monocytes and macrophages. Human donor monocytes were shown to express α7nAChR at the mRNA level [68]. Macrophages retain

α7nAChR expression after their differentiation from monocytes. Wang et al used FITC-labeled α-BTX to show α7nAChRs studding the surface of macrophages in fluorescent microscopy images [3]. For details on the cellular functions altered by α7nAChR on monocytes/macrophages, see Section 4.0 Cellular Functions Altered by α7nAChR activation.

3.2 Neutrophils

Monocytes and macrophages are not the only myeloid cells that express a functional α7nAChR, as the receptor is also expressed by neutrophils. Neutrophils not only express α7nAChR at the mRNA level, but they also respond to α7nAChR agonists. Stimulation of neutrophils with ACh and nicotine altered both adhesion and respiratory burst [69].

3.3 Lymphocytes

In addition to cells of myeloid lineage, both T-cells and B-cells express α7nAChR. Expression of nicotinic ACh receptors on T-cells was first investigated by examining the binding of labeled agonists to the receptor, such as radiolabeled ACh, which showed their presence on the surface of murine lymphocytes [70]. The expression of specific subunits was confirmed by measuring mRNAs. Sato et al [68] demonstrated the expression of α7nAChR in human donor T and B lymphocytes. T-cells themselves are critical for the function of the CAP, but their expression of α7nAChR is not [71]. However, activating the α7nAChR on T-cells by nicotine results in reduced antigen-mediated signaling [72].

B lymphocytic cell lines were used by Arredondo et al [73] to study both the expression of α7nAChR and effects of activation. B-lymphocytic cell lines showed the highest expression of α7nAChR in the lymphoblast stage (Daudi cells), with significantly lower expression at the mature stage (Ramos cells). Stimulation of the α7nAChR resulted in an increase in Bcl-6 and CD138 expression. Other effects of α7nAChR activation include many shared with

macrophages, like decrease in expression and secretion of cytokines and inflammatory markers [72,73].

3.4 Dendritic Cells

Dendritic cells also express α7nAChRs. Activation of α7nAChR on dendritic cells by agonists, like GTS-21 or ACh, can affect the way they interact with other immune cells. Co-stimulatory molecule CD80 as well as MHCII expression were down regulated after treatment of dendritic cells in a collagen induced arthritis model, possibly impacting the downstream activation of T and B lymphocytes [74]. Activation of α7nAChR can also result in decreased secretion of inflammatory cytokines, like IL-23 [75].

4. Cellular Functions Altered by α7nAChR Activation

4.1 Cytokine Secretion

The secretion of cytokines from macrophages is a highlight of the inflammatory response. The general concept of the α7nAChR-mediated anti-inflammatory pathway was discovered based on the analysis of cytokine expression during α7nAChR activation.

As mentioned above, the first discovery was done by Kevin Tracey's group, which showed the inhibition of TNF-α expression by macrophages after VNS. The critical role of α7nAChR in this mechanism was demonstrated by using α7nAChR-deficient mice where VNS did not affect TNF-α expression [3].

Since TNF-α expression is regulated via NF-κB transcription factor, it was tempting to hypothesize that NF-κB activation is also affected by α7nAChR-mediated signaling. Indeed, further studies demonstrated that α7nAChR-mediated signaling inhibits NF-kB -dimerization and translocation [76]. NF-κB is a master transcription factor that regulates more than 200 pro-inflammatory genes including different cytokines (IL-1β, Il-6, IL-8), chemokines (MCP-1,

RANTES), and adhesion molecules (VCAM-1, E-selectins). Accordingly, many studies have been focused on the effect of α7nAChR activation on the expression of NF-κB-dependent pro-inflammatory mediators, which are released in pathophysiological conditions or in vitro after NF-κB activation by endotoxin (i.e., LPS).

Endogenous α7nAChR ligands and pharmacological agonists have similar effects. Nicotine inhibits the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-12 in bone marrow monocytes, while stimulating the secretion of IL-10, an anti-inflammatory cytokine [77]. Acetylcholine and its derivatives inhibit IL-1β secretion from human and rat monocytes in vitro treated with 3'-O-(4-Benzoyl)benzoyl ATP , which a is P2X7 receptor agonist with potent pro-inflammatory functions [78].

GTS-21 attenuates TNF-α and IL-1β at the transcriptional level in human whole blood activated by exposure to endotoxin [79]. Notably, other pro-inflammatory cytokines such as IL-6, IL-8, IL-12 were not inhibited by GTS-21 in this study when evaluated 4 h after treatment, however the authors hypothesized that these cytokines may be inhibited with longer treatment.

Varenicline decreased cytokine and chemokine expression including TNF-α, IL-6, and IL-1β via α7nAChRs in RAW 264.7 murine macrophage cell line when evaluated at 24 h after LPS-stimulation [80]. Varenicline, a drug prescribed for smoking cessation, has full agonistic properties on α7nAChRs but is more potent as a partial agonist at α4β2-nAChRs [81].

Administration of α7nAChR agonists (nicotine, PNU 282987, or PHA 568487) reduced bronchoalveolar lavage MIP-2 (CXCL2) production [46] in endotoxin and live Escherichia coli-induced acute lung injury (ALI) in mice. MIP-2 assists in the recruitment of neutrophils to sites of injury or infection.

Regulation of pro-inflammatory stimulation via α7nAChR occurs naturally during inflammation, and α7nAChR-deficiency increases pro-inflammatory cytokine expression. For example, development of neuroinflammation was compared in wild type (WT) and α7nAChR-deficient mice with intraperitoneally inoculated E.coli. Infected α7nAChR-/- mice showed significantly higher mRNA expression of TNF-α, IL-6, MCP-1, and suppressor of cytokine signaling 1 (SOCS1) in the brain [82].

Macrophages are the major type of immune cells regulating acute immune response via cytokine secretion. Macrophage functions are regulated by T lymphocytes. The secretions of INFγ from Th1 lymphocytes or IL-4/IL-13 from Th2 lymphocytes promote classical or alternative activation of macrophages, which release different sets of cytokines directed toward pro-inflammatory or remodeling functions, correspondingly. Therefore, the modulation of T cell phenotype by α7nAChR activation is an important regulator of cytokine secretion. For example, during experimental autoimmune encephalomyelitis (EAE), GAT107, an ago-positive allosteric modulator of α7nAChRs, reduced the production of pro-inflammatory cytokines including IL-17, IFNγ, and IL-6, as well as increasing the production of the anti-inflammatory cytokine IL-10 in encephalitogenic T cells [41]. Accordingly, macrophages will be polarized toward the M2 phenotype (alternative activation), preventing NF-κB activation. Therefore, data regarding the role of α7nAChR in the regulation of IL-10 expression confirms the cholinergic anti-inflammatory mechanism. Taken together, these studies have revealed inhibition of NF-κB-dependent pro-inflammatory cytokines by α7nAChR activation.

4.2 Apoptosis and Proliferation

Apoptosis and proliferation are two opposite mechanisms that can control pathophysiological outcomes by regulating leukocyte accumulation in tissue. It has been

demonstrated that the proliferation of macrophages at the site of inflammation is critical for the development of inflammatory responses [83]. The role of macrophage apoptosis was also recently reconsidered as an important alternative for macrophage efflux during inflammation [84]. Proliferation and apoptosis may have a protective or pathological effect at different stages of diseases; therefore, it can represent an important part of anti-inflammatory mechanisms that depend on α7nAChR activation.

The effect of α7nAChRs on leukocyte proliferation starts with the regulation of hematopoiesis. Recently, it was shown that B-cell derived ACh in bone marrow reduces the proliferation of hematopoietic stem and progenitor cells (HSPCs) [4]. This leads to decreased proliferation of common myeloid progenitor cells, and thus monocytes, in circulation. Notably, deletion of α7nAChRs leads to higher levels of circulating Ly6Chi monocytes, Ly6G+ neutrophils and CD71+Ter119+ erythrocytes but unchanged platelet counts compared to C57BL/6 WT controls. The effects are mediated via increased proliferation of HSPCs and myeloid progenitors, demonstrating the critical role of α7nAChRs in progenitor proliferation [4].

α7nAChR activation can also directly regulate proliferation in circulation or tissue. For example, the effect GAT107 on development of neuroinflammation by regulating cell proliferation was demonstrated [41]. Administration of GAT107 during experimental autoimmune encephalomyelitis (EAE) reduced encephalitogenic T cell proliferation and the production of pro-inflammatory cytokines, as was shown above. Another example comes from study of nicotine effects in rats with cerebral ischemia. It has been shown that nicotine inhibits microglial proliferation. Notably, blockade of α7nAChRs with α-BTX could prevent the inhibitory effects of nicotine on cultured microglial proliferation, suggesting that nicotine inhibits microglial proliferation in an α7nAChR-dependent fashion [85]. Studying bone-marrow-

derived monocytes, St-Pierre and colleagues demonstrated that nicotine reduces M1 monocyte proportions in bone marrow cells ex vivo by increasing cell death and inhibiting their proliferation, but not by preventing their polarization [77].

Interestingly, in another study nicotine did not effectively reduce cell proliferation of Jurkat T cells or IL-2-dependent Kit-225 T cells over a period of 30 days in culture at concentrations ranging from 10nM to 100μM [86]. These results echo the recent finding that T cell number is not affected in blood after acetylcholinesterase inhibition or ACh inhibition [4]. For more detail, please see section 4.4 Hematopoiesis.

At the same time, in circulation and tissue, nicotine may act both as a survival factor or as an inducer of apoptosis in normal or transformed lymphocytes, and possibly other non-neuronal cells. Based on α7nAChR knock-down with siRNA, the effect of nicotine is, at least partially, regulated via α7nAChRs [86].

In several non-neuronal cells, activation of the α7nAChR promotes cell survival and protects cells from apoptosis. It was suggested that nicotine has an important role in promoting cellular survival in macrophages during *Mycobacterium avium paratuberculosis* infection [87]. The effect is related to increased expression of the anti-apoptotic protein Bcl2. Another example was obtained using ischemia-reperfusion injury model where vagal stimulation decreases infarct size and inflammatory markers due to antiapoptotic properties of the nicotinic pathway [88]. The mechanism of cell survival was linked to STAT3 pro-survival pathway, which was activated via α7nAChR and generated a protection of macrophages from endoplasmic reticulum (ER) stress-induced apoptosis [89]. Interestingly, it works specifically for M2 macrophages, while the apoptosis of M1 macrophages was not significantly affected. Remarkably, this mechanism is completely lost in α7nAChR-deficient M2 macrophages [89].

Notably, apoptosis has a protective effect during many pathological conditions, therefore a pro-survival role of α7nAChR cannot explain all potential anti-inflammatory outcomes.

4.3 Migration

NF-κB regulates different pro-inflammatory factors including the expression of adhesion molecules and chemokine receptors. Therefore, α7nAChR-mediated inhibition of NF-κB pathway may regulate monocyte/macrophage migration during inflammation. Notably, the presence of leukocytes at the site of inflammation may have protective or pathological outcomes depending on the type and stage of inflammation.

The potential role of α7nAChR in the regulation of macrophage migration was considered in a few publications without detailed analysis. First, the effect of α7nAChR stimulation or α7nAChR deficiency on the accumulation of macrophages in tissue was detected. Particularly, GTS-21 treatment in mice with LPS-induced acute lung injury reduced the number of macrophages in the bronchoalveolar lavage fluid [90]. Nicotine reduced the accumulation of CD11b-positive macrophages in the synovium and spleen during arthritis. However, tissue immunostaining for CD11b can also label neutrophils and other myeloid cells. Activation of α7nAChRs by agonists AZ6983 [91] or GTS-21 [92] reduces atherosclerosis development and the number of lesion macrophages in ApoE-/- mice. These results were obtained by immunostaining aortic roots with macrophage specific anti-CD68 antibodies. Surprisingly, another report detected the increased size of atherosclerotic lesions and enhanced intraplaque macrophage content in ApoE-/- mice [93]. However, all these studies indicate the role of α7nAChRs in regulation of macrophage accumulation in tissue that can be mediated either by alteration of monocyte recruitment or macrophage efflux.

As discussed above, in addition to migration, macrophage accumulation may also depend on proliferation and apoptosis. However, several other publications made the effort to measure direct migration. The ability of α7nAChR agonists to decrease migration of RAW264.7 cells was shown by simple testing of cell transmigration through uncoated trans-well membranes (Boyden chambers) without a chemokine gradient. Although this setup is simplified, the authors confirmed the well-defined fact that LPS treatment significantly promoted the migration of RAW 264.7 cells. Most importantly, they showed that the number of migrated cells was significantly less in the group treated with LPS and ACh compared to LPS alone [94].

A similar result was obtained by others who demonstrated that ACh can inhibit LPS-Induced RAW264.7 cell migration [95]. It was suggested that inhibition of migration was due to blocking MMP-9 or MMP-2 expression. MMPs contribute to 3D macrophage migration through the extracellular matrix (ECM) by degrading ECM proteins and generating space for cell movement. Although the concept is interesting, the presented experiments do not verify this hypothesis. Since this mechanism is not involved in any way in the proposed model of cell migration, where macrophages transmigrate via non-coated 8-μm pore-size membrane without a chemokine gradient. In such a setting, only gravity and diffusion regulate cell motility.

Treatment with an α7nAChR agonist, varenicline, generates a similar effect. The rate of LPS-induced cell migration was decreased with varenicline in the Boyden chamber toward serum gradient without any chemokine or post-coat [80].

As a separate line of evidence, it has been shown that the unique human variant of α7nAChR, CHRFAM7A alters monocyte transmigration in Boyden chamber toward MCP-1 gradient, demonstrating the regulatory role of this receptor variant [96]. Notably, CHRFAM7A

mice presented more anti-inflammatory phenotype and had improved survival compared to WT after a lethal dose of LPS [97].

Another potential mechanism for α7nAChR-dependent leukocyte migration was suggested by Saeed et al [98]. They showed that nicotine and cholinergic agent CAP55 inhibit expression of endothelial cell adhesion molecules (VCAM-1 and E-selectin) and significantly block leukocyte transmigration through endothelial monolayer [98]. Since expression of VCAM-1 and E-selectin is regulated by NF-κB activation, these data correspond to the accepted model that α7nAChR activation prevents NF-κB-mediated pro-inflammatory gene expression. These data agree with recent results of Nahrendorf's group (discussed below) that detected a reduced number of myeloid leukocytes in the circulation of mice after α7nAChR stimulation due to reduced proliferation of leukocyte progenitors [4]. Taken together, these observations suggest that α7nAChR activation reduced the number of monocytes and prevented their recruitment, while the α7nAChR-non-treated group may have a higher number of pro-inflammatory monocytes in circulation and increased monocyte transmigration/recruitment due to augmented levels of endothelial adhesive receptors.

Thus, current evidence points to the potential role of α7nAChRs in the regulation of macrophage migration. However, critical aspects such as the contribution to particular step(s) of leukocyte recruitment (adhesion to endothelium, transmigration, 3D migration in tissue), type of cell motility (amoeboid or mesenchymal) and important migratory receptors (chemokines or adhesion molecules) are not fully understood.

4.4 Hematopoiesis

Hematopoiesis occurs in bone marrow and spleen. In a comprehensive paper by Nahrendorf's group, the critical role of ACh in regulation of hematopoiesis was shown [4]. It is

known that sympathetic (noradrenergic) nerves promote hematopoiesis in the bone marrow. In this paper, the authors showed, that cholinergic stimulation reduces hematopoiesis [4]. The primary source of ACh in bone marrow is B lymphocytes, which secrete 3-5-fold more ACh compared to other leukocytes [4]

Using the ChEI pyridostigmine to amplify effects of endogenous ACh, the authors demonstrated that increased concentration of ACh reduced the number of CD11b+Ly6Chi monocytes and CD19+ B cells in blood, but did not change Ly6G+ neutrophils, CD3+T cells, CD41+CD61+ platelets and CD71+Ter119+ erythrocytes. These changes depend on hematopoiesis since pyridostigmine treatment reduces the number of common myeloid progenitors, but common lymphocyte progenitors were not affected. A similar result was obtained in human patients with Alzheimer's disease who were treated with the ChEI donepezil. This treatment led to a significant dose-dependent decrease in the number of leukocytes in blood compared to control samples obtained without treatment.

To verify these results, they generated Cd19CreChatfl/fl mice, in which ChAT, essential for producing ACh, was conditionally deleted in B cells. The level of ACh in bone marrow of these mice was reduced 3.6-fold. This reduction leads to an increased amount of SLAM LSK cells (most upstream hematopoietic progenitors), and common myeloid progenitors due to increased proliferation. According to this result, the number of Ly6Chi and Ly6Clo monocytes, Ly6G+ neutrophils and B220+ B cells was increased in the blood. The number of T cells, erythrocytes and platelets was similar in Cd19CreChatfl/fl and Chatfl/fl mice.

The critical role of α7nAChRs in this mechanism was shown by using knockout mice. Analysis of α7nAChR-/- mice demonstrated higher bone marrow numbers of common progenitors and myeloid progenitors as well as higher levels of circulating Ly6Chi monocytes,

Ly6G+ neutrophils and CD71+Ter119+ erythrocytes but unchanged platelet counts compared to C57BL/6 WT controls. Further, experiments with BrdU demonstrated increased proliferation in the bone marrow of α7nAChR-/- mice.

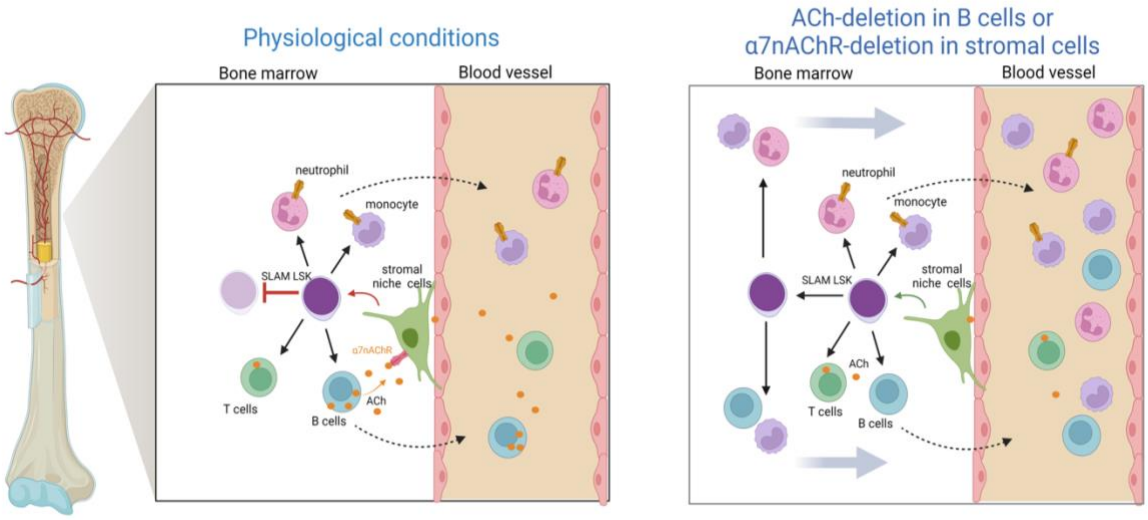

Figure 2.2: Regulation of leukocyte proliferation in bone and blood by ACh.

Diagram showing a potential mechanism of B lymphocyte-released ACh on the regulation of leukocyte proliferation in bone marrow and blood. Left panel. B cells are a major source of Ach in the bone marrow, which is critical for steady-state hematopoiesis. Released ACh is required for the prevention of proliferation of common leukocyte progenitors Lin–Sca-1+c-Kit+CD150+CD48− (SLAM LSK) cells that lead to the controlled proliferation of CD11b+Ly6Chi monocytes and CD19+ B cells. Right panel. The importance of ACh and α7nAChRs were confirmed in α7nAChR-/- mice and Cd19CreChatfl/fl mice, in which Chat, essential for producing ACh, was conditionally deleted in B cells. The absence of the cholinergic pathway leads to the elevated proliferation of SLAM LSK cells in the bone marrow and higher cell number of CD45+ leukocytes, monocytes, neutrophils, and B cells in circulation. Adoptive transfer of WT bone marrow cells to α7nAChR-/- mice demonstrated that ACh influenced hematopoiesis through signaling to stromal niche cells.

Most interestingly, these investigators detected that different stromal cells express α7nAChRs and stromal niche cells in bone marrow appear to sense ACh via α7nAChRs. Therefore, ACh released from B cells regulates steady-state hematopoiesis in bone marrow via α7nAChRs. Blocking of ACh or α7nAChR-deficiency leads to the upregulation of pro-inflammatory monocytes (Ly6Chi-positive) and neutrophils in the blood and common hematopoietic progenitors and myeloid progenitors in the bone marrow. Reduced hematopoiesis and leukocyte number in the circulation occur after pharmacological stimulation of the cholinergic system. Thus, these data provide the molecular mechanism for reduced leukocyte number after α7nAChR-mediated treatment (Fig. 3).

Notably, the ability of B cells to express ACh was shown previously when Reardon et al. [99]. used ChAT-GFP mice to detect ACh expression in different organs. They found ChAT-GFP positive cells in the spleen, lymph nodes and peripheral blood. In the bone marrow, GFP expression was not detected in immature B cells, but was observed in mature recirculating B cells (B220hi). Based on this information, they concluded that ChAT-GFP expression is induced in response to specific signals received after B-cell development in the bone marrow [99]. This result is mostly consistent with the Nahrendorf study that, in addition, detected ChAT-GFP activity in immature B cells, albeit 5-folds less compared to mature B cells [4].

The data of Nahrendorf's group are in agreement with previous findings by Constatini et al. [100]. They tested CHRFAM7A-transgenic mice. As we discussed above (2.2.1) CHRFAM7A is a unique human gene, which encodes a protein with structural similarity to α7nAChR. However, it has been shown that CHRFAM7A serves as an inhibitor of ligand binding to α7nAChR [101,102]. The authors found that CHRFAM7A increased the hematopoietic stem cell reservoir in the bone marrow and increased their differentiation to the monocyte lineage in vitro [100].

They also demonstrated that while the hematopoietic stem cell reservoir was depleted during Systemic inflammatory response syndrome, hematopoietic stem cells were spared in CHRFAM7A-transgenic mice and that these mice also had increased immune cell mobilization and myeloid cell differentiation. Interestingly, authors suggest that CHRFAM7A, as a unique human gene may contribute to discrepancies between the effectiveness of α7nAChR agonists in animal models and human clinical trials for inflammatory and neurodegenerative diseases [100].

Recently, Fielding et al provided another line of evidence that demonstrates the importance of α7nAChR-dependent cholinergic signaling for the regulation of hematopoiesis [103]. They demonstrated that the activation of α7nAChR on mesenchymal stromal cells in the bone marrow hematopoietic stem cell niche leads to increased CXCL12 expression that preserves hematopoietic stem cell quiescence, thereby helping protect stem cells from exhaustion during proliferative stress. Accordingly, the regulation of hematopoiesis depends on cholinergic signaling on immune progenitors and bone marrow niche cells.

The contribution of α7nAChR to the regulation of hematopoiesis in the spleen was described in several papers and demonstrated a similar outcome. Particularly, the administration of α7nAChR partial-agonist GTS-21 leads to significantly reduced spleen size and splenic leukocyte numbers. The effect was most apparent for the Ly6-Chi monocyte population. According to these data, reduced number of hematopoietic stem cells (HSPCs) and myeloid progenitor cells in the spleens of the treated mice was observed [92].

Using α7nAChR Cre:YFP mice, it has been shown that all hematopoietic organs including bone marrow, spleen, thymus and lymph nodes contain α7nAChR positive cells. However, only 15-30% of hematopoietic cells express α7nAChRs and these cells are CD11b+-myeloid cells and B lymphocytes [104].

5. α7nAChRs in Inflammatory Diseases and Pathologies

5.1 Sepsis

The potential link between the parasympathetic nervous system and immune response was discovered by Kevin Tracey's group when they showed the inhibition of TNF-α synthesis by VNS [3], as discussed earlier. In a further study, Ulloa and Tracey demonstrated that nicotine improves the survival of mice in LPS-induced endotoxemia and cecal-ligation and puncture (CLP) sepsis [76]. The effect was mediated via nicotinic inhibition of HMGB1 release and blocking of the NF-κB pathway via activation of α7nAChRs. This manuscript initiated the detailed study of the protective effect of α7nAChR stimulation in the treatment of sepsis. The effective contribution of α7nAChRs to the anti-inflammatory response was demonstrated using several α7nAChR agonists. Treatment with choline or GTS-21 improves survival of mice after CLP or LPS-induced endotoxemia [105,106]. Interestingly, choline administration 24 h after CLP-induced polymicrobial sepsis still significantly improved survival in mice, showing high impact of the CAP. In agreement with these results, activation of α7nAChRs by nicotine enhances survival in sepsis-induced ALI induced by instilling *E. coli* into the airways. The result was verified by using α7nAChR-deficient mice, which had significantly lower survival [107].

Further studies showed that the α7nAChR protective mechanism is tightly related to splenocyte functions, since VNS fails to inhibit TNF-α in splenectomized animals during lethal endotoxemia. Furthermore, the administration of nicotine actually increases proinflammatory cytokine production and lethality from polymicrobial sepsis (CLP) in splenectomized mice [2]. Based on these results, it was suggested that VNS activates T cells in the spleen that release ACh for macrophage anti-inflammatory shift, described in detail in introduction section. Notably,

recent data demonstrate that B lymphocytes in bone marrow and the circulation are alternative sources of ACh for activation of anti-inflammatory cholinergic response [4].

The NF-κB pathway can be activated via several pro-inflammatory signaling pathways. Signaling via different TLRs, formation of IL-1-initiated signalsome or TNF-α-mediated stimulation initiate NF-κB release from I-κBα complex and translocation to the nucleus. In several following publications, the effect of α7nAChRs on different NF-κB-mediated signaling pathways was evaluated. Accordingly, it has been shown that α7nAChR stimulation inhibits the expression of TLR4 and CD14 [108], and this mechanism can occur via α7nAChR/PI3K signaling [109]. Different endogenous agonists of the α7nAChR, including ACh, choline, and phosphocholine inhibit ATP-mediated IL-1β release in human and rat monocytes [78]. In agreement with these results, α7nAChR stimulation by GTS-21 inhibits the production of cytokines from monocytes activated by ligands for TLR2, TLR3, TLR4, TLR9 and RAGE [79]. These results demonstrate a comprehensive effect of α7nAChR activation on the prevention of NF-κB-mediated pro-inflammatory stimulation. Accordingly, the cholinergic anti-inflammatory mechanism may inhibit the development of different inflammatory diseases initiated by different pathogens.

Numerous data demonstrate that classical activation of macrophages in vivo or M1 macrophage polarization in vitro leads to NF-κB activation that indicates a direct link between NF-κB and pro-inflammatory macrophage phenotype. It would be logical to expect that α7nAChR stimulation modifies macrophage phenotype. Indeed, GTS-21 [90] or PNU-282987 [110] treatment significantly diminished the number of M1-polarized macrophages and increased the number of M2-polarized macrophages in lungs after LPS-mediated acute lung injury (ALI) in mice. Also, stimulation with nicotine decreases M1 and increases M2 macrophages in the

decidua of pregnant mice treated with LPS [111]. In agreement with this result, ACh inhibited LPS-induced IL-1β and IL-6 elevation (M1 phenotype) and promoted IL-4 and IL-10 production (M2 phenotype) in microglia, and knockdown of the α7nAChR abolished these effects of ACh [112]. Apparently, a shift in macrophage phenotype reduces cytokine storm and regulates the organism response to infection.

The generation of α7nAChR-mediated anti-inflammatory response by the inhibition of macrophage pro-inflammatory cytokine release is generally accepted. However, other potential mechanisms are still under consideration. For example, the contribution of α7nAChR to the reduction of inflammatory response in acute lung injury (ALI) by regulating maturation, phenotype, and quantity of DCs was described [113]. Another non-NF-κB-related mechanism was suggested by testing tissue edema formation. It is well known that sepsis increases microvascular permeability that dysregulates homeostasis. However, microvascular permeability was significantly reduced in animals treated with GTS-21 simultaneously and 1h after induction of endotoxemia [114]. Sepsis development strongly depends on the recruitment of myeloid immune cells to damaged organs [115]. Accordingly, the potential effect of α7nAChRs on monocyte and macrophage migration can make an important contribution. More details on macrophages migration are in section 4.3 Migration.

Notably, most in vivo data were obtained by using α7nAChR agonists but not by α7nAChR-deficient mice. To the best of our knowledge, only one study demonstrated the increased mortality in α7nAChR-deficient mice after CLP and the results are moderate - 80% survival of α7nAChR-/- mice versus 100% survival of WT [116]. Another paper demonstrates that a deficiency of α7nAChR worsens the reduced survival in *E. coli* pneumonia [46]. However, there is no data that compare the survival rate of α7nAChR-/- versus WT mice in the classical

LPS-induced endotoxemia model. Certainly, the exogenous stimulation of α7nAChR via vagus nerve or agonist mediates a protective anti-inflammatory response. However, the level of α7nAChR activation in natural conditions during sepsis development is not fully understood. These questions still require clarification.

5.2 SARS-CoV-2 Interactions with α7nAChRs

SARS-CoV-2, the causative agent of the global pandemic beginning in 2019, is a continuing threat with both health and economic implications for healthcare providers and patients. The SARS-CoV-2 virus primarily causes respiratory disease but is known to cause severe inflammation and damage in other organs, with multiple potential extrapulmonary sequelae. While comorbidities and immune status play a major role in determining the severity of disease in each patient, it has been reported that there are potential interactions between the α7nAChR and SARS-CoV-2 that could have immunomodulatory effects via the α7nAChR. Investigations into these interactions stemmed largely from the observation that fewer smokers developed severe SARS-CoV-2 disease compared to non-smokers [117]. Nicotine, the addictive agent in tobacco products, is a non-specific agonist of the α7nAChR. The observations published as a result of these studies may present opportunities for therapies aimed at preventing severe disease.

5.2.1 Spike Protein Region Y674-R685 Interacts with α7nAChRs

It has been suggested that SARS-CoV-2 spike protein has the potential to interact with the α7nAChR [117]. The spike protein contains a motif similar to known nicotinic ACh receptor antagonists, the Y674-R685 region, which is accessible in the fully glycosylated protein. This Y674-R685 region is homologous to the portion of α-BTX that interacts with α7nAChRs. This

led to the hypothesis that spike protein interacts with nAChRs, which was investigated with molecular dynamics simulations [118].

This in silico prediction was validated experimentally using the α7nAChR expressed on human cell lines and mitochondria extracted from mouse brains. In a competitive ELISA, the SARS-CoV-2 spike protein fragment Y674-R685 inhibited the binding of an antibody specific to the α7nAChR region 179-190. It was additionally reported that the SARS-CoV-2 fragment was able to disrupt cytochrome c release from mitochondria, through the interruption of α7-bax complexes in the mitochondrial membrane. Coronaviruses are known to express proteins that either initiate or delay apoptotic processes. This data suggests that spike protein fragment Y674-R685 attenuates apoptosis of the infected cell to support its viability while the virus replicates. Interestingly, this inhibition of apoptosis is only observed when the fragment interacts with mitochondrial α7nAChR, not with the α7nAChR on the plasma membrane [119].

5.2.2 α7nAChR in SARS-CoV-2 Disease

The α7nAChR has been studied in SARS-CoV-2 disease not only due to potential interaction with the virus's spike protein, but as a possible therapeutic target to combat the cytokine storm that often proves fatal for patients. Cytokine release syndrome is a characteristic of severe disease, where hyperinflammation of lung and other tissues result from the release of IL-6, IL-8, and TNF-α by both the adaptive and innate immune systems. Because of the well-characterized ability of α7nAChR activation to decrease pro-inflammatory cytokines, it was hypothesized that nicotine may be useful in controlling aberrant cytokine release. Additionally, it has been suggested that SARS-CoV-2 might impair macrophage function and upset M1/M2 balance [120]. Nicotine's widespread availability as well as its function as an α7nAChR agonist may prove useful in the treatment of SARS-CoV-2 [121].

Most recently, treatment with pyridostigmine has been studied in a phase 2/3 clinical trial in SARS-CoV-2 patients, resulting in reduced mortality [122]. Self-managed tVNS is also being investigated for the treatment of lasting symptoms after convalescence, termed “long COVID”, in a preliminary controlled trial. tVNS was reported to be safe and feasible for participants and resulted in reductions in mental fatigue scores. Mental fatigue, or “brain fog” is one of the most common sequelae of SARS-CoV-2 after convalescence [123]. VNS is a potential therapy in active disease, as a means to prevent severe illness by reducing inflammatory cytokine production [124].

5.3 Cardiac Disease and the α7nAChR

Coronary artery disease, cardiac ischemia, myocardial infarction (MI), and heart failure (HF) are related cardiac disorders that are major contributors to global morbidity and mortality. While multiple factors contribute to the underlying pathology, activation of the immune system and recruitment of leukocytes to the heart plays a pivotal role in adverse cardiac remodeling, at least in part due to excessive stimulation of pro-inflammatory pathways. In this regard, several preclinical studies have provided direct and/or indirect evidence for beneficial effects of α7nAChR stimulation on cardiac remodeling and function. Furthermore, preclinical and some clinical studies have established the therapeutic potential of VNS.

5.3.1 Impact of the α7nAChR and VNS in Ischemia Models of MI with and without Reperfusion

Occlusion of a coronary artery causes ischemia downstream, which leads to MI and impaired cardiac pump function. Two major responses to this damage are reflex activation of the sympathetic nervous system and stimulation of systemic and local immune responses. In addition to stimulating the heart, sympathetic activation can also stimulate myelopoiesis and trafficking of

monocytes from the spleen to the infarct region, where they become macrophages. Balanced activation of pro- and anti-inflammatory macrophage phenotypes is crucial to limiting infarct size and healing. However, excessive activation of the pro-inflammatory phenotype plays a major role in adverse cardiac remodeling.

Early work established beneficial effects of VNS in a rat ischemia/reperfusion model of MI [88]. For these experiments, ischemia was induced by ligating the left anterior descending (LAD) coronary artery for 30 min, and the ligature was then removed for reperfusion. Stimulation of the right cervical vagus nerve, starting 5 min before ischemia and ending 5 min after reperfusion, decreased infarct size and recruitment of macrophages to the area at risk when evaluated at 24 h. Beneficial effects of VNS were blocked by local cardiac administration of the non-selective nicotinic antagonist mecamylamine but remained when the left atrium was paced during VNS to prevent bradycardia. Thus, the beneficial effects of VNS were mediated by nicotinic receptors, most likely within the heart, but were not related to parasympathetic slowing of the heart rate and the resulting decreased oxygen demand. Double staining for CD68 and α7nAChR suggested the presence of α7nAChRs on macrophages, however, the specificity of nicotinic receptor antibodies has been questioned [22,125].

α7nAChRs have also been implicated in the beneficial effects of VNS in a mouse model of acute MI [126]. VNS delivered separately during ischemia or reperfusion reduced infarct size, determined 2 h after reperfusion, by two different cholinergic mechanisms. Atropine and wortmannin blocked the protective effect of VNS given during ischemia, implicating muscarinic receptors and the Akt/GSK-3β pathway, which was confirmed by Western blotting. In contrast, the protection achieved by VNS during reperfusion was blocked by MLA and not atropine, suggesting mediation by α7nAChRs. Further experiments found that effects of VNS during

reperfusion were also attenuated by blockade of the JAK2 pathway, which has been implicated in α7 mediated anti-inflammatory effects [12,22]. Interestingly, VNS during reperfusion still reduced infarct size after sectioning the vagus nerve below the diaphragm or splenectomy, suggesting mediation by α7nAChRs intrinsic to the heart rather than the classical CAP. While it is expected that α7nAChRs receptors are present on resident cardiac macrophages and monocytes-derived macrophages, this has not been demonstrated directly for cardiac tissue. In another study using rats, administration of the α7nAChR agonist, PNU-282987, just before reperfusion, reduced infarct size, serum markers of cardiac injury, and serum levels of the pro-inflammatory cytokines TNF-α, IL-6, and HMGB1 when measured after 2 h of reperfusion [127]. Levels of the anti-inflammatory cytokine IL-10 were unaffected in this study. Interestingly, postconditioning by brief hindlimb ischemia had similar but smaller effects compared to PNU-282987, and inhibition of cardiac NF-κBp65 signaling via GSK-3β was implicated for both. While it is not expected that PNU-282987 would affect hearts rate, cardiac function was not evaluated. These findings agree with earlier work that found similar beneficial effects in the same model after pretreatment with PNU-120596, a positive allosteric modulator of α7nAChRs [38]. Effects of PNU-120596, which require some source of endogenous ACh, were blocked by α-BTX. Unfortunately, effects of PNU-120596 on heart rate were not evaluated.

Recent experiments with another mouse model of MI support the idea that intrinsic activation of α7nAChRs can have a cardioprotective influence. These investigators found that permanent occlusion of the LAD coronary artery in α7nAChR deficient mice produced larger infarcts and impaired cardiac function more than occurred in WT control mice when evaluated at 24 h post-occlusion [128]. Likewise, MI caused larger systemic inflammatory responses in

knockouts compared to controls. The latter was evidenced by serum pro-inflammatory cytokine levels and the expression of genes for these cytokines in the spleen.

5.3.2 α7nAChRs and Heart Failure

Inflammation also plays a major role in adverse remodeling associated with heart failure (HF), so targeting this pathology through cholinergic anti-inflammatory mechanisms makes sense. Furthermore, autonomic imbalance, with enhanced sympathetic and reduced vagal efferent tone, occurs in HF, as in other cardiovascular diseases, providing another rationale for VNS. Several studies have evaluated effects of cholinergic pharmacotherapy and VNS in experimental models of HF.

Daily treatment of rats with PNU-282987 for 4 weeks after ligation of the LAD, reduced adverse cardiac remodeling, caused a modest improvement of ventricular function, and reduced inducibility of ventricular arrhythmias [129]. Treatment with PNU-282987 also reduced proinflammatory cytokine levels in the peri-infarct region. Beneficial effects were attenuated by an AMPK inhibitor in this model and in isolated LPS stimulated macrophages, which favor the M2 phenotype in the presence of PNU-282987 alone.

Several animal studies have demonstrated that chronic activation of cholinergic efferent pathways by treatment with cholinesterase inhibitors or by VNS can have beneficial effects in HF models. Many of the pharmacological studies have utilized cholinesterase inhibitors like donepezil, which are known to activate the cholinergic anti-inflammatory pathway by a centrally mediated effect [58,130–132]. Recent work, with a rat model of MI-induced chronic heart failure, provided convincing evidence that cardioprotective effects of donepezil are mediated by peripheral α7nAChRs [130]. For this work, donepezil was administered in drinking water, beginning 2 weeks after MI was induced by permanent ligation of the LAD and continued for 4

weeks, when terminal studies were performed. Despite the delayed initiation of donepezil therapy, it caused significant improvement of all cardiac function parameters, decreased cardiac fibrosis, and increased capillary density without decreasing heart rate. Furthermore, donepezil blunted sympathetic activation, as indicated by substantial drops in plasma catecholamines, decreased plasma BNP, and reduced inflammatory markers in the heart. All these beneficial effects of donepezil were blocked by peripheral but not central infusion of MLA, suggesting a major role of peripheral α7nAChRs. Since donepezil and similar drugs are already widely used in the treatment of Alzheimer's disease, such agents might have therapeutic applications for diseases having inflammatory components such as HF. This concept is supported by results from a retrospective study, which demonstrated that Alzheimer's patients treated with ChEIs had a reduced incidence of new-onset HF and cardiovascular death compared to patients not treated with these drugs [133].

Other studies have shown that peripheral inhibition of cholinesterase with pyridostigmine, given by gavage [134] or included in the drinking water [61], can attenuate the early development of HF when assessed one week after coronary ligation in rat models. In both cases, pyridostigmine also had anti-inflammatory effects in the infarct and peri-infarct region of the left ventricles. MI also increased TNF-α in the spleen, and this response was likewise attenuated by pyridostigmine [61]. Immunohistochemical evidence was presented for increased trafficking of leukocytes to the infarct after pyridostigmine treatment, but there was also a switch to higher proportion of the M2 anti-inflammatory macrophage phenotype.

Vagal stimulation is another approach being explored as a potential therapy for HF in animal studies as well as clinical studies [135–137]. Efficacy has been evaluated in HF with reduced ejection fraction (HFrEF) and HF with preserved EF (HFpEF), however, only a few

studies have examined the role of α7nAChRs. While most studies of VNS have used direct stimulation of the cervical vagus, a few recent investigations used tVNS, which has the advantage of not requiring surgery.

In the Dahl salt-sensitive model, rats treated with high salt diet develop hypertension and HFpEF. Four weeks of tVNS lowered blood pressure, improved cardiac function, and decreased inflammatory cytokines in serum, macrophage infiltration into the left ventricle, and cardiac fibrosis [138]. Survival was likewise improved, and all these beneficial effects of tVNS were blocked by MLA, suggesting mediation by α7nAChRs. Interestingly, treatment with the AT1 receptor blocker, olmesartan, also lowered blood pressure but did not improve cardiac function or reduce inflammation. Heart rate variability was reduced in this model of HFpEF, and tVNS normalized this parameter. However, this effect of tVNS was not blocked by MLA, suggesting mediation by direct neural mechanisms. Transcriptomic analysis showed that HFpEF caused differential expression of over 500 genes, primarily related to inflammation and fibrosis, and tVNS reversed most of these changes. Such effects were largely blocked by MLA, suggesting a primary involvement of α7nAChRs. Recently, the same group has reported encouraging results in a pilot clinical study of tVNS in patients with HFpEF [139]. Here, VNS was applied to the tragus, which is innervated by the auricular branch of the vagus, and sham control stimulation was applied to the earlobe. Patients with co-morbidities indicative of a pro-inflammatory state were selected to enhance efficacy based on the known anti-inflammatory effects of VNS. After 3 months of tVNS, patients had reduced global longitudinal strain and levels of serum TNF-α. Furthermore, better quality of life was determined based on a standard questionnaire.

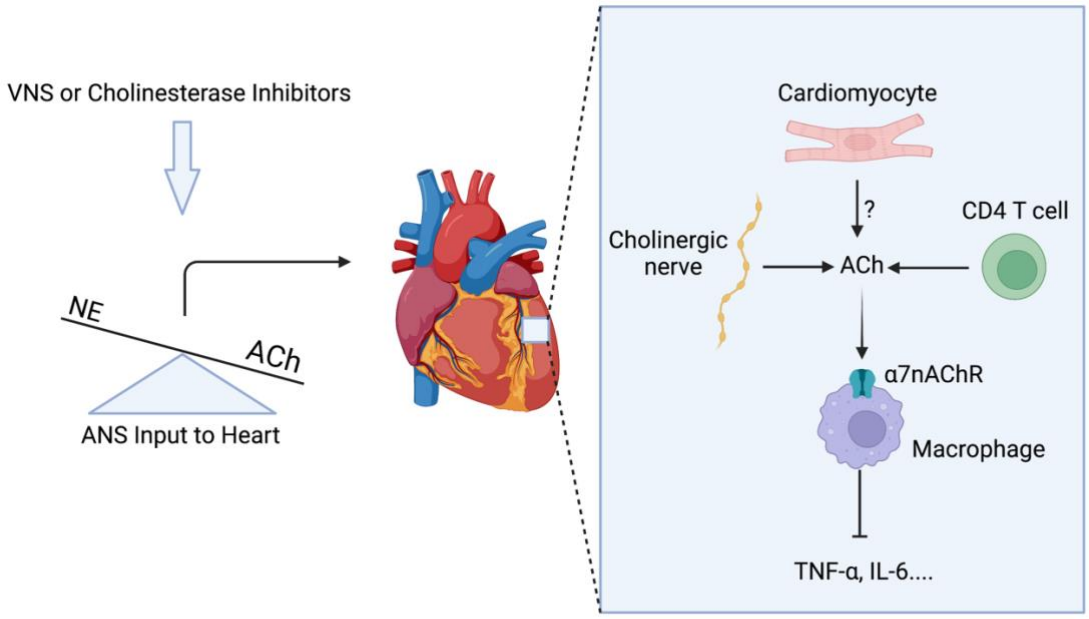

Figure 2.3: Potential beneficial cholinergic mechanisms during MI.

Diagram depicting potential mechanisms for beneficial effect of vagal nerve stimulation (VNS), cholinesterase inhibitors, and α7 agonists in myocardial infarction (MI) and heart failure. Left side: The balance of autonomic nervous system (ANS) input to the heart is disrupted in cardiovascular disease, with cholinergic input decreased and noradrenergic input increased. This balance is shifter toward normal by VNS and cholinesterase inhibitors. Right side: Proinflammatory macrophages, which contribute to disease progression, are present in the heart after MI and during heart failure. Activation of α7 receptors on these macrophages by endogenous ACh or selective agonists is expected to cause a beneficial anti-inflammatory response. Possible sources of endogenous ACh include postganglionic cholinergic nerves, cholinergic T cells recruited to the region of pathology, and possible from cardiac myocytes (based on animal experiments). VNS would increase neuronal release of ACh, and cholinesterase inhibitors would prolong the survival of ACh and, therefore, its duration of action.

To summarize, a growing body of literature supports targeting cholinergic mechanisms for treatment of cardiac disease. Such effects can be mediated two distinct cholinergic systems, which can be activated by pharmacological or bioelectronic approaches (Fig. 4). One approach is restoring balanced autonomic regulation of the heart such that vagal efferent effects mediated by muscarinic receptors are returned toward normal. The other mechanism involves attenuation or reversal of adverse cardiac remodeling by activating α7nAChRs on macrophages that mediate cholinergic anti-inflammatory responses. Macrophages in the heart are most likely the dominant effectors for therapeutic approaches targeting the α7nAChR in cardiac disease, but VNS and treatment with ChEIs also work by improving cholinergic transmission to myocytes. Some benefit might also derive from cholinergic anti-inflammatory effects at the spleen in cardiac diseases (e.g., reduced output of inflammatory cytokines), but the full impact of α7 based therapies on spleen-heart interactions requires much more study.

5.4 α7nAChRs and Atherosclerosis

Lipid uptake and deposition by macrophages in the artery intima leads directly to the formation of atherosclerotic plaques. The formation of these plaques not only decreases the size of the artery lumen, but their rupture can have catastrophic consequences such as heart attack and stroke. Among the anti-inflammatory effects of α7nAChR, it has been demonstrated to attenuate the development of atherosclerosis.

Multiple mouse studies, in both Ldlr-/- and ApoE-/- models, have demonstrated that the activation of α7nAChRs with a specific agonist attenuates atherosclerosis development. Infusion of AZ6983 to ApoE-/- mice through osmotic mini-pumps decreased lesion area by 37% in the aortic root and lipid accumulation in plaques by 48% when compared with controls [91].

Decreased plaque size and lipid content is corroborated using other agonists: namely AR-R17779 [140] and GTS-21 [92].

As a critical addition to this experimental data in mice, it has been demonstrated that α7nAChR is expressed in human atherosclerotic plaques. Human carotid endarterectomy specimens were obtained and analyzed for α7nAChR expression. Most lesions, 7 out of 10 samples, were positive for α7nAChR immunoperoxidase staining. Additionally, α7nAChR staining coincided with staining for CD68 and CD163 positive macrophages [141]. In the same study, α7nAChR deficiency was evaluated in atherosclerosis using Ldlr-/- mice reconstituted with α7nAChR-/- or WT bone marrow. The Ldlr-/- mice receiving α7nAChR-/- bone marrow showed a 72% increase in atherosclerosis at the aortic root and more advanced plaques with larger lipid deposits than those that received WT bone marrow [141].

Activation of α7nAChR is a potential approach to accompany current treatments of atherosclerotic disease. Not only does the activation decrease inflammatory cytokines, but it has also been reported to reduce the lipid content of atherosclerotic lesions. Conversely, α7nAChR deficiency increases atherosclerosis severity and was reported to increase cholesterol accumulation in macrophages. Deficiency of α7nAChR, regardless of ApoE status, increases lipid uptake. In ApoE+/+/α7nAChR-/- mice, oxidized LDL uptake by peritoneal macrophages increased by 35% when compared to ApoE+/+/α7nAChR+/+ control mice. In ApoE/α7nAChR double knockouts, cholesterol mass of peritoneal macrophages increased by 29% compared to the control mice [142]. The same study also examined mRNA levels of CD36, a class B scavenger receptor participating in lipid uptake by macrophages. They found that CD36 mRNA levels were significantly increased in ApoE+/+/α7nAChR-/- mice when compared to control

mice, suggesting that α7nAChRs may regulate macrophage lipid uptake, and therefore plaque formation, through altering expression of scavenger receptors [142].

While most published studies support an anti-atherogenic role for α7nAChR, there are some controversial data showing α7nAChR exacerbates atherosclerosis. In Ldlr-/- mice reconstituted with either control or α7nAChR-/- bone marrow, advanced lesions in the mice with α7nAChR deficient bone marrow had reduced size and macrophage content [143]. It was also reported that a common smoking cessation drug, varenicline, exacerbated atherosclerosis by activating α7nAChR on macrophages. Plaques in the whole aorta of ApoE-/- mice were increased 1.5-fold after daily treatment with 0.5 mg/kg varenicline for three weeks. This effect was validated by using an antagonist, MLA, in conjunction with varenicline [144]. The same research group published data demonstrating that varenicline also increases lipid uptake of macrophages by up regulating expression of CD36 and LOX-1, two scavenger receptors heavily involved in the progression of atherosclerosis [145]. While MLA was used to confirm the involvement of α7nAChR in the macrophage response to varenicline, it is crucial to note that varenicline was designed to be a high specificity α4ß2 nAChR antagonist [146]. Nonetheless, studies to clarify the effects of α7nAChR stimulation in atherosclerotic disease are invaluable to the advancement of patient treatment options.

5.5 Metabolic Disease

Metabolic dysfunction is a complex process that depends on many body functions including pancreatic β-cells, adipocytes, and macrophages in fat tissue. The important role of macrophages in this process points to a potential mechanism, which resembles other chronic inflammatory diseases like atherosclerosis. Accumulation of pro-inflammatory macrophages and secretion of pro-inflammatory cytokines contributes to disease progression. This common

element makes it possible that activation of α7nAChR anti-inflammatory pathway has a protective mechanism for the regulation of insulin secretion and blood glucose during metabolic dysfunction.

Indeed, oral administration of the α7nAChR agonist TC-7020 to homozygous leptin-resistant diabetic (db/db) obese mice reduced elevated glucose and glycated hemoglobin levels. In addition, it decreased weight gain, food intake, and lowered serum TNF-α. Changes were reversed by the α7nAChR-selective antagonist MLA, verifying α7nAChR-mediated effects [147]. Unfortunately, only fasting glucose was evaluated, and a glucose tolerance test may provide much more detailed information.

This problem was resolved in the following papers that showed that α7nAChR stimulation in diet-induced obese mice by the agonist ICH3 improved glucose tolerance and insulin sensitivity [148]. In agreement with this result, GTS-21 or PNU-282987 stimulation of db/db mice lower levels of blood glucose in an oral glucose tolerance test [149].

Critical evidence was obtained by Gausseres and co-authors who tested metabolic parameters in α7nAChR-knockout mice fed a standard chow diet. 12 weeks old α7nAChR -/- mice display chronic mild hyperglycemia combined with impaired glucose tolerance and a marked deficit in β-cell mass. Moreover, 24-week-old mice demonstrated both glucose intolerance and insulin resistance, as well as adipose tissue inflammation and late-onset excessive gain in body weight due to increased fat mass [150]. These results clearly demonstrate the physiological protective effect of α7nAChR in the development of type 2 diabetes. Notably, the mice were kept on a normal chow diet. It is tempting to speculate that a similar experiment set up on a high-fat diet would demonstrate a much stronger difference in metabolic parameters of α7nAChR-/- and WT mice.

Interestingly, the development of diabetes affects α7nAChR expression in different cells. It has been shown that streptozotocin-induced diabetes in Wistar rats upregulates α7nAChR gene expression in the cerebellum [151,152]. In contrast, the expression of α7nAChR in obese diabetic human subjects demonstrated reduced α7nAChR expression by adipocytes compared to normal weight controls. The expression of α7nAChR was partially re-established by diet and physical activity. In addition, in vitro treatment of human adipocytes in the presence of α7nAChR agonists PNU282987 or genistein increases α7nAChR expression [153].

In contrast to these data, another model of diabetes, non-obese type 1 insulin-dependent diabetes (NOD) mice demonstrated different results. Treatment of mice with AR-R17779 or nicotine did not affect overall inflammation and development of diabetes [154]. Probably, it can be explained by the different metabolic mechanism in type 1 diabetes; namely, a reduced level of adipose tissue inflammation and related signaling in NOD model.

The protective mechanism of α7nAChR function during metabolic disorder is activated via different cell types. In addition to adipocytes and adipose macrophages, it has been shown that VNS suppresses hepatic IL-6/STAT3 signaling via α7-nAChRs on Kupffer cells. Hepatic IL-6 expression was suppressed by PNU-282987 administration in high-fat diet-induced obese mice, suggesting that cholinergic action suppresses IL-6 expression even in obese and insulin-resistant mice [155].

In agreement with the protective effect of α7nAChR in metabolic regulation, several independent reports demonstrated that activation of α7nAChR by PNU282987 [156] or nicotine [157] promotes diabetic wound healing. The effect is mediated via suppression of TNF-α production in streptozotocin (STZ)-induced diabetic mice [156] and by blocking TLR2 signaling in leptin-resistant db/db obese mice [157].

To summarize, the protective role of α7nAChRs in metabolic diseases was demonstrated in several independent studies. The mechanism depends on the reduced expression of pro-inflammatory cytokines and improved function of adipocytes and pancreatic β-cells, but the direct role of macrophages in the regulation of diabetes by α7nAChR was not shown yet.

The convincing results in animal studies were supported by evidence of vagus nerve contribution to the regulation of metabolic parameters in humans. The studies were performed for different pathophysiological conditions but have interesting implications regarding metabolic regulation.

The Netherlands Study of Depression and Anxiety performed on 1,883 individuals demonstrated that a decreased vagus nerve activity and increased sympathetic activity were associated with metabolic syndrome [158]. The application of device-generated VNS in patients with epilepsy is associated with significant weight loss [159]. Cervical VNS treatment of patients with severe treatment-resistant depression also results in significant weight loss without additional dieting or exercising [160].

Specific clinical trials to assess metabolic parameters were performed using galantamine, a centrally acting ChEI with anti-inflammatory properties. The subjects received oral galantamine for 12 weeks or placebo (n=30 individuals per group). Galantamine significantly reduced plasma levels of TNF-α and leptin and increased the level of IL-10 and adiponectin [161]. In the following study with the same parameters (n = 22 individuals per group), galantamine treatment significantly increased antioxidant enzyme activities and decreased lipid peroxidation, which results in reduced oxidative stress [162]. Based on these clinical studies, a low dose of galantamine alleviates inflammation and insulin resistance in metabolic syndrome

subjects. These data demonstrate that stimulation of the CAP has a high therapeutic potential for the treatment of obesity and metabolic syndrome.

5.6 α7nAChRs in Endometriosis

Endometriosis is a chronic inflammatory disease characterized by ectopic endometrial tissue implanting in extra-uterine sites. It affects 176 million women, presenting with chronic pain, dysmenorrhea, and infertility. Available treatments are limited, typically target hormones, and are not consistently tolerated or effective. As not all patients desire treatments that affect hormones, there remains an opportunity to develop treatments for reducing inflammation underlying endometriosis symptoms. Recent studies demonstrate that targeting α7nAChR may be promising for reducing inflammation, therefore pain, in endometriosis.

Multiple changes occur in the endometrial cells that implant at distal sites, including increased NF-κB expression and activation. In a study utilizing endometriosis patient biopsies, NF-κB activity was increased in highly inflammatory ("red") lesions. Red lesions also had a higher expression of ICAM-1 [163]. Peritoneal macrophages isolated from endometriosis patients were also shown to have increased NF-κB expression, a key observation, as the condition of the peritoneal environment is hypothesized to be involved in the pathogenesis of the disease [164]. NF-κB is known to activate many pro-inflammatory genes, such as cytokines associated with inflammation like TNF-α, therefore its increased expression and activity in endometriosis lesions supports interest in α7nAChR as a putative treatment target.

Crucially, Hao et al [165] reported that human endometrial tissue is positive for α7nAChR. Endometrial tissue samples were collected from female patients categorized as having either ovarian or deep (peritoneal) lesions, and age-matched controls were recruited from women undergoing colposcopy or loop electrosurgical excision procedures. In these samples,

endometriosis patients in either category had significantly reduced α7nAChR staining in the glandular epithelium when compared to endometrium of control patients, which was associated with a higher extent of fibrosis and greater severity of dysmenorrhea. Cholinergic agonists were next tested in an endometriosis mouse model, using Balb/C mice, where PNU-282987 significantly reduced total endometriosis lesion weight and fibrosis when compared to controls. It was verified by treatment using the α7 antagonist MLA, which ablated any beneficial effects of PNU-282987 treatment [165]. The same research group evaluated VNS in the Balb/C endometriosis model, which also reduced overall lesion weight [166]. In a separate study, the same endometriosis mouse model demonstrated significantly reduced lesion weight when treated with the specific agonist PHA-543,613 [167]. Overall, these studies demonstrate that activation of the CAP by VNS may be utilized to reduce lesion size and combat inflammation associated with endometriosis, although they lack detail on specific mechanisms. As of writing this review, there is an ongoing clinical trial of tVNS for endometriosis pain at Hospital Foch, Suresnes, France [168].

5.7 α7nAChRs in Arthrits

Arthritis, in general, is simultaneously a disease of chronic inflammation and autoimmunity that results in degeneration of joints such as the hips, fingers, and knees. There are multiple clinical classifications under the umbrella of arthritis: osteoarthritis, rheumatoid, and psoriatic arthritis. All arthritic conditions result in greatly reduced quality of life. Treatment focuses on mitigation of symptoms by decreasing inflammation, relieving pain, and preventing further loss of joint mobility. However, these treatment strategies do not always mitigate disease progression for all patients, especially in osteoarthritis cases. Additional safe and effective early treatments are desperately needed.

5.7.1 α7nAChR Suppresses Iimmune Cell Infiltration in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is characterized by autoantibodies to IgG FcR bound by rheumatoid factor and anti-citrullinated protein antibodies. The pathophysiology of rheumatoid arthritis involves, among other factors, infiltration of activated leukocytes and inflammatory cytokine production within the synovial tissues [169]. In a collagen-induced RA model using DBA/1 mice, activation of α7nAChRs was reported to modulate the infiltration of macrophages into the synovium. Mice given intraperitoneal treatment with nicotine had significant decreases in CD11b positive cells in the synovium compared with control mice and the group receiving vagotomy. In addition, mice receiving nicotine treatment had reduced arthritis scores, synovial inflammation, and expression of ICAM-1 and CCR2 in synovial tissue. While this study relied heavily on immunohistochemistry in the absence of other methods, it illustrates the impact of leukocyte infiltration, and the CAP, on RA severity [170].

Dendritic cells (DCs) are among the activated leukocytes infiltrating the synovium and contributing to disease progression in RA, thus targeting α7nAChR on DCs may be effective in treatment. Using the collagen-induced arthritis model, treatment with GTS-21 was reported to significantly decrease arthritis scores and DC infiltration into the synovium when compared to controls [74]. Overall, reduction of leukocyte infiltration and pro-inflammatory mediators within the synovium via α7nAChR activation could be a strategy to mitigate disease.

5.7.2 Activation of α7nAChR Inhibits NF-kß/NLRP3 in Osteoarthritis

Osteoarthritis is primarily a disease of articular cartilage degradation and remodeling of synovial joints. Specific mechanisms underlying the pathology of osteoarthritis are not well understood, but it is suggested that synovial chondrocytes may overexpress inflammatory mediators as well as matrix metalloproteinases. In a monosodium iodoacetate-induced model of

osteoarthritis using Wistar rats, treatment with the α7nAChR agonist PNU-282987 reduced cartilage degeneration and MMP-1 and MMP-13 expression. Additionally, activating α7nAChRs in this osteoarthritis model promoted autophagy in chondrocytes and mitigated disease progression [171]. While this result is promising, further study is desperately needed to confirm α7nAChR as a treatment target in osteoarthritis.

5.7.3 α7nAChR is Expressed in Affected Joints of Psoriatic Arthritis Patients

Psoriatic arthritis typically features not only synovial inflammation and damage, but the same skin lesions associated with psoriasis, an autoimmune disease resulting in a high amount of inflammation and over-proliferation of skin cells. Using synovial joint samples from nine patients with a confirmed diagnosis of psoriatic arthritis, expression of α7nAChR was confirmed within these joints using immunoperoxidase staining. Double immunofluorescence staining revealed that α7nAChR staining coincided with CD68/CD163+ macrophages and fibroblasts [172]. While it is crucial to report that α7nAChR is expressed in human joints, translational studies in mice examining the effects of its activation are lacking.

5.8 Inflammatory Diseases of the Gastrointestinal Tract

Inflammatory bowel diseases, namely Crohn's disease and ulcerative colitis, have a complex etiology that combines genetic, immunologic, and environmental factors. Patients with inflammatory bowel diseases have a wide range of gastrointestinal symptoms, stemming from aberrant inflammation in the intestines. Paradoxically, it was noted by clinicians that cigarette smoking reduced symptoms in ulcerative colitis patients but exacerbated disease in Crohn's disease patients. Subsequently, the CAP became of major interest in inflammatory bowel diseases, as nicotine is an agonist of the α7nAChR. Decreasing pain, ulceration, and other

debilitating symptoms by ameliorating inflammation through activating the α7nAChR is a promising therapeutic strategy.

5.8.1 Severity of Crohn's Disease is Ameliorated by VNS

Crohn's disease can occur anywhere along the digestive tract, presenting with swelling, abdominal pain, fatigue, and malnutrition. Triggers for the onset of disease are being heavily studied, and leading hypotheses suggest an initial viral or bacterial infection [173]. Management plans for patients typically include drugs like corticosteroids, immunosuppressants, and biologics, which must be taken long term. Because treatments often extend throughout a patient's lifespan, non-drug alternatives are sought out by many. The vagus nerve is a crucial part of the axis connecting the gut, brain, and intestinal microbiota. Vagotomy in patients is associated with the development of inflammatory bowel disease, particularly Crohn's disease [174]. For these reasons, VNS has been evaluated clinically to activate the CAP and elicit a therapeutic response.

Nine Crohn's disease patients with moderate active disease were selected to participate in a year-long study on the efficacy and safety of VNS for Crohn's disease. Vagal nerve stimulators were surgically implanted around the left vagus nerve. After one year of consistent vagus nerve stimulation, five patients were in clinical remission and six were in endoscopic remission. Patients also had restored vagal tone, reduced digestive pain scores, and lowered serum levels of inflammatory cytokines [175]. Immune cells within the gastrointestinal tract are thought to be influential in this effect of the vagus nerve on Crohn's disease, namely macrophages, dendritic cells, and mast cells. Confirmation for this hypothesized mechanism behind the success of VNS in Crohn's disease patients is needed.

5.8.2 α7nAChR Stimulation Reduces Ulcerative Colitis

Ulcerative colitis (UC) is a primarily colorectal inflammatory bowel disease, which not only causes intestinal sores, pain, and bloody stool in patients but also increases their risk of developing colon cancer. Colitis can be induced in BALB/c mice using dextran sulfate sodium (DSS), resulting in not only bloody stools but also colon shortening. In this colitis model, treatment with GTS-21 reduced colon shortening and immune cell infiltration in mice [176]. Paracellular invasion of intestinal bacteria into the mucosa is another concern with UC, usually resulting from the destruction of tight junctions due to inflammation and NF-kB activation. It was demonstrated that the number of tight junctions in the mucosa was improved by GTS-21 treatment, with increases in zonula occludens-1, claudin-1, occludin, and JAM-1. Leakage of FITC-dextran from the intestinal pouch was reduced in GTS-21 treated mice with DSS induced colitis, which was further confirmed by using the probe EUB338 to track bacterial translocation. GTS-21 treated mice showed attenuated bacterial translocation [176]. Treatment with PNU-282987, another α7nAChR agonist, combined with an SHP2 (a tyrosine phosphatase) inhibitor, gave similar results: reducing colon shortening and histological changes in the colon such as damage to intestinal crypts [177].

6. Conclusions and Perspectives

We have presented basic information on the presence and function of α7nAChRs in the immune system and reviewed evidence showing that they are relevant targets for treating a wide range of diseases that have inflammation as a key contributor to pathophysiology. The preclinical worked presented in this review was selected to be representative rather than all inclusive, so potential applications for α7 based therapy are enormous. Much of this review has focused on the CAP and its anti-inflammatory effect on splenic monocytes/macrophages. While this is

clearly an important therapeutic target, there is still much to learn about α7 contributions to other aspects of immune regulation. For example, what effect does α7 activation have on trafficking of leukocytes, hematopoiesis, and the functions of B and T lymphocytes? Recent work suggests a major role for α7nAChRs in the bone marrow where they affect hematopoiesis and trafficking of inflammatory myeloid cells to other tissues [4]. Importantly, activation of α7nAChRs in bone marrow occurs by a unique mechanism that is independent of the CAP. To broaden our understanding of α7 functions in immune regulation, future studies will need to consider the broad cellular expression of α7nAChRs, the localization and mobility of these cells, and regional sources of ACh (neurons and/or non-neuronal).

While a wealth of preclinical work has established the potential value of α7 based therapies (drugs and VNS) in a wide range of inflammatory disease models, translation of this work to patients has been limited. Neuromodulation therapy with VNS has gained the most traction, especially for the treatment of heart failure, but this approach is limited by the need for surgery. Recent success with tVNS suggests that this non-invasive approach could have broader applications. Pharmacological activation of the CAP has been limited to clinical studies using ChEIs approved for other indications. Despite intensive work by pharmaceutical companies to develop new drugs that activate α7nAChRs, this work has targeted central disorder. While some of these drugs have been used to activate the CAP in preclinical studies, this work has not been moved to the clinics. Perhaps broader use and success of tVNS and ChEIs for treating inflammatory disorders will be a catalyst for similar clinical investigation of α7 active drugs.

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CHAPTER 3. CHOLINERGIC SIGNALING VIA THE α7 NICOTINIC ACETYLCHOLINE RECEPTOR REGULATES THE MIGRATION OF MONOCYTE-DERIVED MACROPHAGES DURING ACUTE INFLAMMATION

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Abstract

Background: The involvement of the autonomic nervous system in the regulation of inflammation is an emerging concept with significant potential for clinical applications. Recent studies demonstrate that stimulating the vagus nerve activates the cholinergic anti-inflammatory pathway that inhibits pro-inflammatory cytokines and controls inflammation. The α7 nicotinic acetylcholine receptor (α7nAChR) on macrophages plays a key role in mediating cholinergic anti-inflammatory effects through a downstream intracellular mechanism involving inhibition of NF-κB signaling, which results in suppression of pro-inflammatory cytokine production. However, the role of the α7nAChR in the regulation of other aspects of the immune response, including the recruitment of monocytes/macrophages to the site of inflammation remained poorly understood.

Results: We observed an increased mortality in α7nAChR-deficient mice (compared with wild type controls) in mice with endotoxemia, which was paralleled with a significant reduction in the number of monocyte-derived macrophages in the lungs. Corroborating these results, fluorescently-labeled α7nAChR-deficient monocytes adoptively transferred to WT mice showed significantly diminished recruitment to the inflamed tissue. α7nAChR deficiency did not affect monocyte 2D transmigration across an endothelial monolayer, but it significantly decreased the migration of macrophages in a 3D fibrin matrix. In vitro analysis of major adhesive receptors (L-selectin, β1 and β2 integrins) and chemokine receptors (CCR2 and CCR5) revealed reduced expression of integrin αM and αX on α7nAChR-deficient macrophages. Decreased expression of αMβ2 was confirmed on fluorescently-labeled, adoptively transferred α7nAChR-deficient macrophages in the lungs of endotoxemic mice, indicating a potential mechanism for α7nAChR-mediated migration.

Conclusion: We demonstrate a novel role for the α7nAChR in mediating macrophage recruitment to inflamed tissue, which indicates an important new aspect of the cholinergic regulation of immune responses and inflammation.

Introduction

Active research during the last 20 years has revealed the important role of the vagus nerve in the regulation of immunity and inflammation in a physiological mechanism termed the inflammatory reflex (V. A. Pavlov, Chavan, & Tracey, 2018; Tracey, 2002). In the inflammatory reflex, sensory (afferent) vagus nerve signaling is activated by cytokines and other inflammatory molecules in response to pathogens, injury, or other pathophysiological events (Chavan, Pavlov, & Tracey, 2017; V. A. Pavlov et al., 2018). This signaling is integrated in the brainstem with motor (efferent) vagus nerve cholinergic signaling, which controls pro-inflammatory cytokine levels and inflammation (V. A. Pavlov & Tracey, 2012; Tracey, 2002). This efferent arm of the inflammatory reflex was termed the cholinergic anti-inflammatory pathway (V. A. Pavlov, Wang, Czura, Friedman, & Tracey, 2003; Tracey, 2002). The α7 nicotinic acetylcholine receptor (α7nAChR) expressed on macrophages and other immune cells has been identified as a key mediator of cholinergic anti-inflammatory signaling (Keever, Yakubenko, & Hoover, 2023; H. Wang et al., 2003). Stimulation of the α7nAChR on macrophages activates downstream intracellular mechanisms, including suppression of NF-κB activation and results in decreased production of TNF-α and other pro-inflammatory cytokines (Li, Zhou, Kolosov, & Perelman, 2011; Parrish et al., 2008; Rosas-Ballina et al., 2009; Rosas-Ballina et al., 2008). These discoveries opened an avenue of preclinical research revealing the anti-inflammatory efficacy of vagus nerve stimulation (VNS) and α7nAChR agonists in endotoxemia, sepsis and many other inflammatory conditions (Borovikova et al., 2000; J M Huston et al., 2006). This research paved

the way to recent successful clinical trials with VNS in patients with inflammatory disorders (V. A. Pavlov & Tracey, 2022).

Murine endotoxemia and cecal ligation and puncture (CLP) have been widely used in studying the role of the α7nAChR in the cholinergic regulation of inflammation. Endotoxemia, associated with robust systemic cytokine release and inflammation is considered by some as a model of Gram-negative sepsis, while CLP is a clinically relevant model of polymicrobial sepsis (Buras, Holzmann, & Sitkovsky, 2005). Sepsis is a life-threatening condition characterized by organ dysfunction resulting from a dysregulated inflammatory response to infection. This organ system dysfunction is correlated with higher long-term mortality, even if patients recover from their illness in the hospital (Prescott & Angus, 2018). In mice, VNS or pharmacological cholinergic α7nAChR activation suppresses pro-inflammatory cytokine levels and mitigate mortality in mice with endotoxemia and CLP (J M Huston et al., 2007; J M Huston et al., 2006; Kim, Kim, & Lee, 2014; Nullens et al., 2016; V A Pavlov et al., 2007). The role of macrophages in endotoxemia and sepsis is complex; some reports characterize macrophages as protective due to their crucial role in efferocytosis of neutrophils, phagocytosis of bacteria, and tissue repair while other reports indicate their detrimental effects (Bailey et al., 2021; Kumar, 2020; Parisi et al., 2018). The role of α7nAChR on macrophages in mediating cholinergic suppression of pro-inflammatory cytokine production in the cholinergic anti-inflammatory pathway has been characterized as a major mechanism underlying the neural control of immune responses. However, other potential mechanisms, such as modulating the recruitment of monocytes/macrophages to damaged tissue, remain unclear.

In this study, we investigated the broader role of the α7nAChR in inflammation by examining the migration and accumulation of macrophages during endotoxemia. We utilized

genetically deficient α7nAChR-/- (knockout, KO) mice and pharmacological cholinergic stimulation of macrophages with PNU-282987, a specific α7nAChR agonist during endotoxemia. We found that α7nAChR deficiency resulted in significantly reduced survival of mice during endotoxemia, which clearly demonstrated the key protective role of the α7nAChR in the regulation of inflammation. We also observed a correlation between increased lethality and a decrease in the number of macrophages in the lungs, accompanied by an increased number of neutrophils. Furthermore, we demonstrated that the α7nAChR deficiency impaired macrophage migration both in vivo and in a 3D migration assay in vitro. Finally, we showed that the α7nAChR deficiency was associated with reduced expression of a major adhesive receptor integrin αMβ2 (CD11b/CD18) at both the transcript level and on the macrophage surface, thus indicating an important molecular link to reduced macrophage recruitment. These results provide important insights into the role of α7nAChR in macrophage migration during inflammation and reveal a novel protective mechanism of the cholinergic anti-inflammatory pathway.

Materials/Methods

Reagents and Antibodies

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), BioRad (Hercules, CA, USA), BioLegend (San Diego, CA, USA), and Thermo Fisher Scientific (Waltham, MA, USA). Lipopolysaccharide (LPS, endotoxin) derived from *E. coli* O55:B5, and PNU-282987 were purchased through Sigma-Aldrich. Antibodies against cell surface markers Ly6-G (clone 1AB), Ly6-C (clone HK1.4), αM (clone M1/70), αL (clone M17/4), F4/80 (clone BM8), αX (clone N418), and α4 (clone R1-2) are from eBioscience. Antibodies against Siglec F (clone 1RNM44N) and L-selectin (clone MEL-14) are from Invitrogen. Antibodies against CCR2 (clone SA203G11) and CCR5 (clone HM-CCR5) are from BioLegend.

Animals

Wild type (WT; C57BL/6J, stock #000664) and α7nAChR-deficient (α7nAChR-/-; B6.129S7-Chrna7tm1Bay/J, stock #003232) mouse colonies were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The α7nAChR deficient strain was backcrossed to C57BL/6 for eight generations. Mice aged between 8 to 14 weeks were used for the study. Similar age WT and α7nAChR-/- mice were employed for each experiment. All animal procedures were performed according to animal protocols approved by East Tennessee State University IACUC.

Endotoxemia

In survival experiments, male or female WT and α7nAChR-/- mice were intraperitoneally injected with a sublethal dose of LPS. The doses ranged from 7-8μg/g in males and 9-12μg/g in females, calculated based on body weight. All lots of LPS from the manufacturer were tested to determine optimal dosing before the start of the experiment. In all endotoxemia experiments, body temperature was monitored twice daily using a rectal probe connected to a ThermoWorks (American Fork, UT, USA) MicroTherma meter.

To examine macrophage accumulation in the lungs, male or female WT and α7nAChR-/- mice were given an intraperitoneal injection of LPS as described above. Mice were euthanized and perfused after 48h, and lungs were removed. Lungs were digested using collagenase II as described below (section 8) and prepared for flow cytometry. In an additional experiment, male and female WT mice were treated with PNU-282987, 15 minutes before injection of LPS (doses as above), to examine the effect of α7nAChR stimulation on macrophage accumulation. Control mice received DMSO (vehicle) 15 minutes before LPS. Samples were incubated with anti-αM/PE-Cy7, anti-CCR2/APC, anti-CCR5/PE-Cy7, anti-Siglec F/FITC, anti-Ly6-G/PE, anti-F4/80/PE, anti-F4/80/APC, and anti-αX/APC across multiple samples.

Isolation of Peritoneal Macrophages

Peritoneal macrophages from 8- to 12-week-old WT and α7nAChR-/- mice were collected via peritoneal lavage with 5mL of sterile PBS 72 h after intraperitoneal injection of 1mL 4% thioglycolate. The cells were counted and plated in petri dishes for 2 h in RPMI 1640 (Corning, Corning, NY) with 10% FBS and 1% P/S, after which non-adherent cells were removed.

Isolation of Monocytes from Bone Marrow

Monocytes were isolated from the femoral and tibial bone marrow of WT and α7nAChR-/- mice by first flushing out bone marrow with RPMI 1640, followed by lysis of red blood cells. Magnetic assisted cell sorting (MACS) was then used to purify monocytes via a negative separation kit, following the manufacturer's protocol (Miltenyi Biotec, Gaithersburg, MD, USA). Purity of the isolated monocytes was analyzed by flow cytometry using antibodies to αM/PE-Cy7, Ly6-G/PE, and Ly6-C./FITC. In all experiments, the purity was between 87-92%.

Adoptive Transfer of Monocytes in LPS-induced Endotoxemia

Monocytes were isolated from the bone marrow of WT and α7nAChR-/- mice, as above, and labeled with red PKH26 (WT), or green PKH67 (α7nAChR-/-) fluorescent dyes. A total of $1X10^6$ red and $1X10^6$ green monocytes were mixed equally and injected into the tail veins of WT mice or α7nAChR-/- mice. These mice received a sub-lethal dose of LPS (males 7-8μg/g, females 9-12μg/g) intraperitoneally within 5 minutes after injection of cells. After 48 h, the mice were sacrificed and perfused with PBS. Lungs, liver, and spleen were isolated and digested with 2mg/mL collagenase II (Sigma Aldrich, St Louis, MO, USA). Digested cell suspension was filtered through a 70μm cell strainer and any remaining red blood cells were lysed. Cell filtrate was incubated with a viability dye and analyzed using flow cytometry (Fortessa X-20, Becton

Dickson, Franklin Lakes, NJ, USA) and imaging flow cytometry (ImageStream Mark II, Amnis, Seattle, WA, USA) for the detection of fluorescently labeled cells. The dye colors were used with only WT cells in a separate experiment to confirm that dye color does not influence the result.

Adoptive Transfer rescue in LPS-induced Endotoxemia

WT or α7nAChR-/- monocytes were isolated from bone marrow as described above. Freshly isolated cells, either WT or α7nAChR-/-, were injected into the tail veins of WT or α7nAChR-/- recipient mice. Cell injection was immediately followed by a sub-lethal dose of LPS (males 7-8μg/g, females 9-12μg/g). In all adoptive transfer rescue experiments, body temperature and survival were monitored twice daily. Survival rate was analyzed using the Kaplan-Meier method.

Flow Cytometry and Imaging Flow Cytometry Analsyses

Flow cytometry analysis was used to assess cell surface expression of integrin subunit αM, as well as determine the number of PHK26 and PKH67 positive cells in the lungs, liver, and spleen during adoptive transfer. For the analysis of cell surface markers, harvested cells were first incubated with FcR-Blocking solution (eBioscience) for 15 min on ice. Next, samples of $2x10^6$ cells were incubated with appropriate antibody panels for 30 minutes on ice. Cells were then washed and analyzed using the Fortessa X-20 (Becton Dickson) and FACSDiva software.

To detect labeled macrophages in tissue, the lungs, liver, and spleen were digested using 2mg/mL collagenase II (Sigma-Aldrich, St Louis, MO, USA). Cell suspension was next pre-cleaned via filtering through a 70μm cell strainer. Cells were incubated with live/dead viability dye for 30 minutes on ice (Thermo Fisher, Waltham, MA, USA). PKH26 and PKH67 labeled macrophages within the digested organs were analyzed with flow cytometry (Fortessa X-20) and

imaging flow cytometry (Image Stream Mark II, Amnis). For analysis of αM on labeled macrophages, preparation was carried out as above.

Imaging flow cytometry analysis results were analyzed using IDEAS 6.2 software. The PKH26 and PKH67 labeled cells were captured on channels 2 and 3, respectively.

Macrophage 3-D Migration Assay

WT and α7nAChR-/- peritoneal macrophages were labeled with PKH26 red fluorescent dye or PKH67 green fluorescent dye. An equal number of WT and α7nAChR-/- were mixed and plated on the membranes of 6.5mm transwell inserts with 8μm pores (Costar, Corning, NY) pre-coated with 4μg/mL fibrinogen for 3 h. A 3-D fibrin gel was made by mixing 0.75mg/mL fibrinogen containing 1% FBS and 1% P/S with 0.5 U/mL thrombin, at a total volume of 100uL per transwell. MCP-1 (30nM) or RANTES (12.8nM) were added to 100uL of HBSS containing 1% FBS and 1% P/S the top of the gel to initiate migration. Transwells were incubated for 48h at 37°C in 5% CO2 in a 24-well plate. In each well, 650uL of HBSS containing 1% FBS and 1% P/S was added beneath the transwell insert to prevent drying of the gel during incubation. Migrating cells were detected using confocal microscopy (Leica TCS SP8), and results were analyzed with IMARIS 8.0 software.

qRT-PCR

Prior to RNA isolation, peritoneal macrophages were incubated overnight with LPS (10ng/mL) and PNU-282897 (30μM). Total RNA was extracted from thioglycolate-induced mouse peritoneal macrophages using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio Rad, Hercules, CA, USA). Roughly 0.8-1.0μg of cDNA was synthesized in a 20μL reaction volume, per the kit instructions. Real-time PCR reactions were set up in a 96-well qPCR plate using IQ

SYBR Green Supermix (Biorad, Hercules, CA, USA) and run using the CFX96 Real Time Thermal Cycler fitted with a C1000 lid (BioRad). Each sample was plated in duplicate. Specific primers for each target were designed and are listed in Table 1 (Integrated DNA Technologies, Coralville, IA). Fold changes were normalized to GAPDH. Relative expression of each target was calculated using the Livak Method.

Table 3.1 Primers used in qRT-PCR analysis.

Table 1

Primer	Sequence (5'-3')
αX forward	CTGGATAGCCTTTCTTCTGCT
αX reverse	GCACACTGTGTCCGAACTCA
αM forward	TCCGGTAGCATCAACAACAT
αM reverse	GGTGAAGTGAATCCGGAACT
αD forward	GGAACCGAATCAAGGTCAAGT
αD reverse	ATCCATTGAGAGAGCTGAGCTG
CCR2 forward	ACAGCTCAGGATTAACAGGGACTTG
CCR2 reverse	ACCACTTGCATGCACACATGAC
CCR5 forward	TCCGTTCCCCCTACAAGAGA
CCR5 reverse	TTGGCAGGGTGCTGACATAC
MCP-1 forward	TGGAGCATCCACGTGTTGGC
MCP-1 reverse	ACTACAGCTTCTTTGGGACA
RANTES forward	GCTTCCCTGTCATTGCTTGCTC
RANTES reverse	AGATGCCCATTTTCCCAGGACC
GAPDH forward	AAGGTCATCCCAGAGCTGAA
GAPDH reverse	CTGCTTCACCACCTTCTTGA

Trans-endothelial Migration Assay

Endothelial cells (HUVECS) were labeled using CellVue Claret (Sigma-Aldrich, St-Louis, MO) and incubated overnight on the membranes of 6.5mm transwell inserts with 8μm pores (Costar, Corning, NY) to form a monolayer. Non-adhered endothelial cells were gently

washed out. WT and α7nAChR-/- monocytes were isolated from bone marrow using magnetic assisted cell sorting as described above in methods section 5. Isolated monocytes were labeled using either PKH67 (green) or PKH26 (red), with colors switched to confirm that dye color does not influence the result. Stained monocytes were added on top of the endothelial cells. MCP-1 (30nM) or RANTES (12.8nM) were added to the bottom chamber to start migration, along with media containing 650μL of HBSS with 1% FBS and 2% P/S to prevent drying. After 3 hours, the migration was evaluated by confocal microscopy (Leica TCS SP8). Results were analyzed using IMARIS 8.0.

Isolation of Peripheral Blood Monocytes

Mice were injected with 8μg/g LPS. After 3 hours, mice were euthanized, and blood was collected in EDTA (200mM) coated syringes through cardiac puncture. Each mouse yielded 500-700μL of blood, which was diluted using an equal volume of balanced salt solution, prepared as instructed in the Cytiva Ficoll-Pacuqe protocol. The monocytes were separated from whole blood using Ficoll-Pacque 1.084 (Cytiva) according to manufacturer instructions. Isolated monocytes were then prepared for flow cytometry using anti-Ly6-C/FITC, anti-CD11b/PE-Cy7, anti-CD11a/APC, anti-CD62L/PE, and anti-CD49d/PE across multiple samples.

Statistical Analysis

Experimental data were analyzed using student's t-test. Results are given as mean ± SEM. Survival experiments were analyzed using the Kaplan Meier Method and Log Rank Test. Quantitative Real-Time PCR data was analyzed using the Livak Method. Values of $p<0.05$ were considered to be statistically significant.

Results

α7nAChR Deficiency Results in Reduced Survival and Decreased Macrophage Accumulation in the Lungs, While Activation of α7nAChR Increases Macrophage Accumulation During Endotoxemia

The anti-inflammatory effects of α7nAChR activation using small molecule agonists have been extensively studied in mice with endotoxemia and CLP (Nullens et al. 2016; Parrish et al. 2008; Pavlov et al. 2007). For instance, administering a partially specific α7nAChR agonist, GTS-21, or choline - a product of acetylcholine degradation and a selective endogenous α7nAChR agonist suppresses circulating pro-inflammatory cytokine levels, which is linked to reduced NF-κB activation and increase survival in endotoxic mice and rats (Pavlov et al. 2007; Rosas-Ballina et al. 2008). However, the role of α7nAChR deficiency on survival in endotoxemia and macrophage migration into tissues was not previously investigated.

We first evaluated the impact of α7nAChR deficiency on survival during endotoxemia. Wild type (WT) and α7nAChR-/- mice (n=10/strain), both male and female, were injected with LPS (males 7-8ug/g, females 9-12ug/g) and survival was monitored for four days. α7nAChR deficiency significantly reduced the survival rates, exhibiting a similar pattern in both male ($p<0.01$) and female ($p<0.05$) mice (Fig. 2.1. A, C). This reduction in survival correlated with a decrease in body temperature that indicated the severity of induced endotoxemia (Fig. 2.1. B, D).

The recruitment of monocytes/macrophages into the lungs can have a protective outcome during endotoxemia (24-27). Therefore, we reasoned that decreased macrophage accumulation in lungs could be responsible for the increased mortality of α7nAChR-/- mice. To provide insight, age-matched WT and α7nAChR-/- mice were injected with LPS (n=5/group) and the severity of endotoxemia was verified by a drop in body temperature after 48 h (Fig. 2.2B). After 48 hours,

the lungs were collected and digested to analyze leukocyte populations using flow cytometry (Fig. 2.2A). Live cells were selected using a viability dye, and specific markers were used to evaluate different leukocyte subsets. Neutrophils were analyzed as CD11b+, Ly6-G+, and F4/80-. Monocyte-derived macrophages were identified as CD11b+, F4/80+, and Ly6-G-, while alveolar macrophages were selected as F4/80+, CD11b-, and Siglec F+. The gating strategies for monocyte-derived and alveolar macrophages are depicted in Supplementary Figure 1 and Supplementary Figure 2, respectively. We observed that the percentage of monocyte-derived macrophages in the lungs of α7nAChR-/- mice was significantly lower. The number of resident alveolar macrophages and neutrophils was not markedly different (Fig. 2.2B).

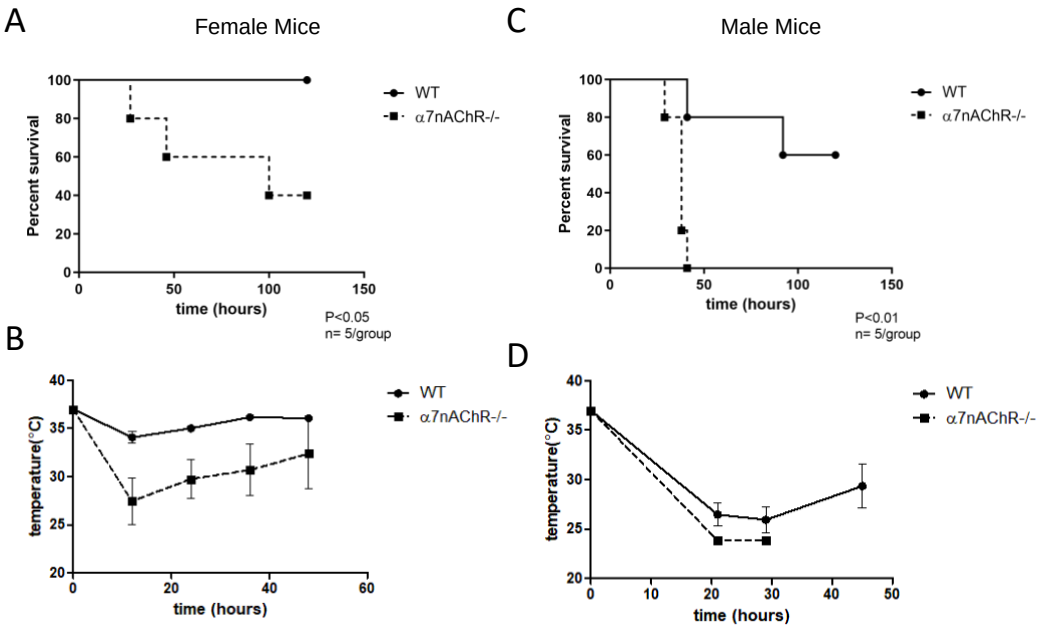


Figure 3.1: α7nAChR is protective during endotoxemia.

A. Survival curves after intraperitoneal administration of LPS (9-12μg/g) to induce endotoxemia in female mice. (WT, n=5; α7nAChR-/-, n=5). p<0.05. **B.** After injection of LPS, a decrease in body temperature to 21-27°C confirmed the development of endotoxemia. **C.** Survival curves after LPS-induced endotoxemia in male mice (7-

8μg/g). $p<0.01$. **D.** Body temperature drop in male mice. WT (n=5) and α7nAChR-/- (n=5). Statistical significance was assessed by the Kaplan-Meier method.

To assess the impact of α7nAChR activation on macrophage accumulation, we treated WT mice with either LPS and DMSO (vehicle) or LPS with α7nAChR agonist PNU-282987 (n=7/group) (Fig.2.2C). 3mg/kg PNU-282987 dissolved in DMSO or DMSO alone were administered 30 minutes prior to LPS injection as previously described (26). Body temperature of mice receiving treatment with PNU-282987 was significantly higher at 48 hours when compared to LPS only control. Using the same gating strategy for leukocyte subtypes, we revealed that pre-treatment with PNU-282987 significantly increased the percentage of viable macrophages in the lungs and decreased the percentage of viable neutrophils (Fig. 2.2C,D). Interestingly, the proportion of viable resident alveolar macrophages in the lungs did not change with PNU-282987 treatment (Fig. 2.2D), but the absolute cell count was increased in PNU-282987-treated mice (Supplementary Fig. 2B).

Multiple mechanisms can contribute to the reduced accumulation of macrophages in the lungs during endotoxemia, including decreased monocyte/macrophage infiltration, increased apoptosis and promoted necrosis. Analysis with Annexin V staining did not reveal a significant difference in the amount of apoptotic or necrotic (late apoptotic) macrophages (Suppl. Fig. 3A-C). Notably, the number of necrotic neutrophils was also similar (Suppl. Fig. 3D). Together, these findings suggest that α7nAChR-dependent macrophage accumulation is reliant on monocyte/macrophage migration.

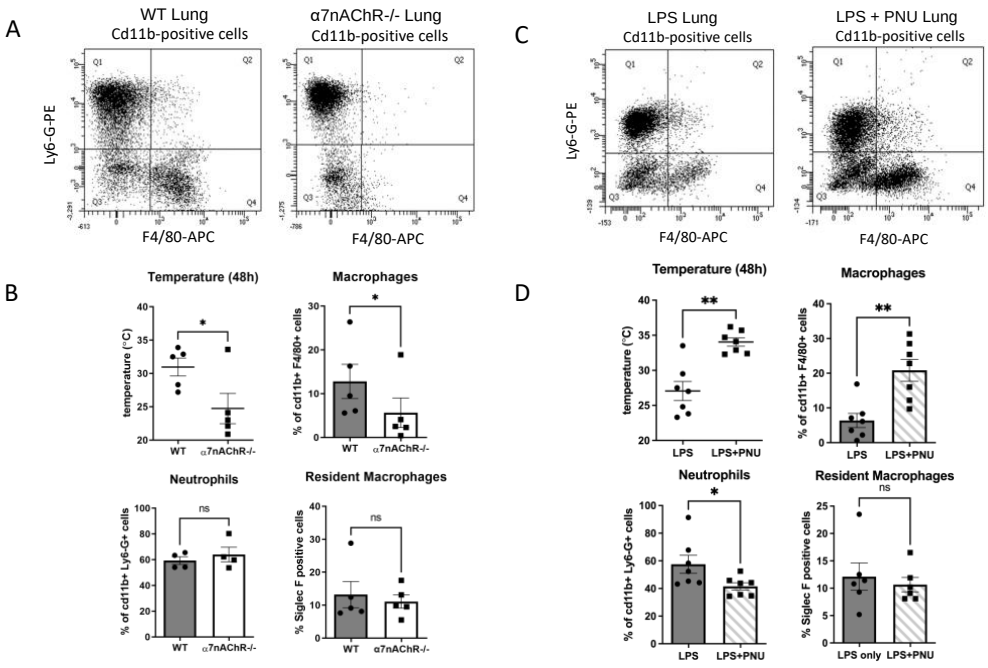


Figure 3.2: Macrophage accumulation in the lungs is affected by α7nAChR signaling.

A. WT and α7nAChR-/- mice were injected with a sublethal dose of LPS. After 48h lungs were removed, digested, and analyzed using flow cytometry. CD11b–positive cells were selected and tested with antibodies against Ly6-G and F4/80 to identify neutrophils and macrophages, respectively. Results were analyzed and calculated using FACSDiva software and GraphPad Prism. **B.** Plots representing the number of WT and α7nAChR-/- macrophages (top right, n=5), neutrophils (bottom left, n=4), and resident macrophages (bottom right, n=5) in digested lungs. Residents were identified as CD11b- F4/80+ Siglec F+. Body temperature at 48h is shown at top right (n=7). **C.** WT mice were injected with either a sublethal dose of LPS (males 8-9ug/g, females 10-12ug/g) or 3mg/kg PNU-282987 followed by LPS fifteen minutes later. The dose of LPS used was higher than for part A to generate sublethal conditions for WT mice. After 48h lungs were removed, digested and analyzed using flow cytometry. CD11b positive cells and residents were selected and analyzed as above. Results were analyzed with FACSDiva software and calculated with GraphPad Prism. **D.** Plots depicting the number of macrophages (top right, n=7), neutrophils (bottom left, n=7), and resident macrophages (bottom right, n=6). Body temperature at 48h is shown at top right (n=7). Statistical analysis was performed using a paired t-test. * $P<0.05$, ** $P<0.01$.

α7nAChR Deficiency Reduces the Recruitment of Monocyte-derived Macrophages to the Lungs During Endotoxemia

To further assess the role of α7nAChR in monocyte/macrophage migration, we conducted an in vivo adoptive transfer tracking experiment in the same model of endotoxemia to examine monocyte recruitment to the lungs, liver, and spleen. Fluorescently labeled WT and α7nAChR-/- monocytes were injected intravenously to recipient mice as depicted in Fig. 2.3A. Monocytes were isolated from bone marrow by negative selection (87-92% purity, Fig. 3B) and labeled with either green PKH67 (α7nAChR-/-) or red PKH26 (WT) fluorescent dyes. WT and α7nAChR-/- monocytes were mixed in equal proportions and injected into recipient mice immediately followed by a sub-lethal dose of LPS. The cell mixture proportion was validated by fluorescent microscopy of a Cytospin slide (Suppl. Fig. 4A). After 48 hours, the lungs, spleen and liver were digested for flow cytometry analysis to identify labeled, migrated macrophages (Fig. 2.3C). Flow cytometry data for the liver and spleen are shown in Supplementary Figure 5. Additionally, Imaging Flow Cytometry (Amnis) was employed to verify macrophage integrity, morphology and labeling (Fig. 2.3D). Our analysis revealed that lungs and other organs of WT recipient mice accumulate a significantly lower number of donor α7nAChR-/- macrophages compared to donor WT macrophages (Fig. 2.3E).

An overall similar trend was observed in the adoptive transfer of WT and α7nAChR-/- monocytes to α7nAChR-/- recipient mice, (Fig. 2.4), that indicates that other cell types expressing α7nAChR do not significantly influence the migration of monocytes/macrophages to tissue. Therefore, the expression of α7nAChR on the monocyte/macrophage surface is critical for effective cell migration.

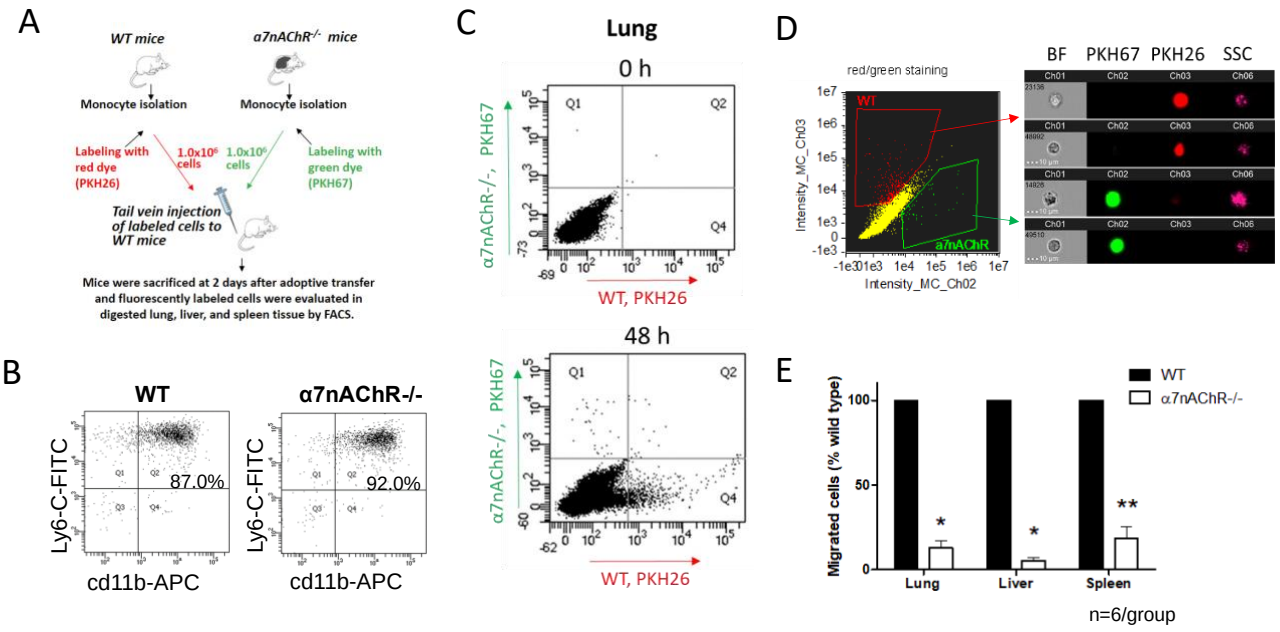


Figure 3.3: α7nAChR deficiency impedes the migration of macrophages to organs during LPS-induced endotoxemia.

A. Schematic representation of the experimental design. Monocytes were isolated from bone marrow of male or female WT and α7nAChR-/- mice via MACS. Cells were labeled with red (WT) or green (α7nAChR-/-) fluorescent dyes, mixed in equal proportion and injected in tail vein of WT recipient mice. After 48 hours, the lung, liver and spleen were isolated, digested and analyzed using flow cytometry. **B.** Representative dot plots of monocyte purity analysis. Isolated monocytes were labeled with anti-CD11b (APC) and anti-Ly6-G (FITC). Monocyte population is visible in Q2. **C**. Representative results of flow cytometry analysis are shown. Data was analyzed using FACSDiva software. Migrated WT macrophages (red) were detected in Quadrant 4; α7nAChR-/- macrophages (green) were detected in Quadrant 1. **D.** Imaging flow cytometry of labeled macrophages. **E.** Bar graphs representing the amount of WT and α7nAChR-deficient macrophages detected in lungs, liver, and spleen by flow cytometry, * $P<0.05$, ** $P<0.01$. Statistical analysis was performed using Student's t-test.

It is worth noting that PKH67 and PKH26 are widely used membrane stains and that we have previously demonstrated that switching red and green dye colors does not impact the results of our adoptive transfer experiments (28-30). In an additional experiment, the adoptive transfer approach was performed by using only WT monocytes labeled with PKH26 or PKH67 dyes, and

no significant difference was observed in the number of PKH26- or PKH-67-labeled WT macrophages in the inflamed tissue (Suppl. Fig. 2.4C,D).

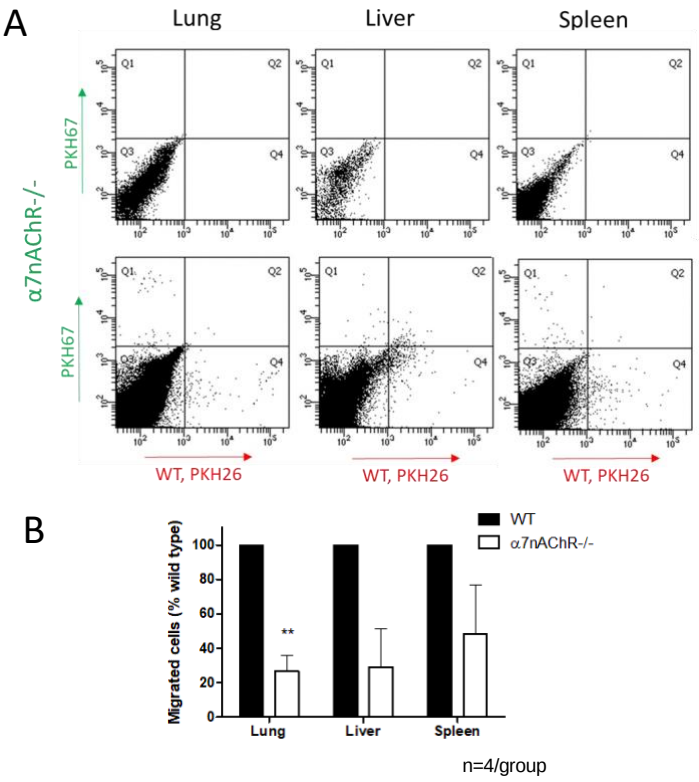


Figure 3.4: The effect of α7nAChR deficiency on migration does not depend on other cell types.

A. Representative dot plots of flow cytometry showing migrated red (WT) and green (α7nAChR-/-) monocytes in α7nAChR-/- recipient mice. **B.** Bar graphs representing the amount of WT and α7nAChR-deficient macrophages detected in organs by flow cytometry. The experimental setup is same as depicted in Fig. 3A, using 8-week-old male and female α7nAChR-/- recipients instead of WT. Statistical analysis was performed using Student's t-test, ** $P<0.01$.

Trans-endothelial Migration of Monocytes is not Affected by α7nAChR Deficiency

Trans-endothelial migration is a critical step in the recruitment of monocytes from the blood into inflamed tissues. We tested whether α7nAChR deficiency affects this process. A diagram depicting the experimental setup is shown in Supplementary Figure 6A. After a 3-hour

incubation, cells that had crossed the endothelial cell monolayer were visualized using confocal microscopy, and images were reconstructed using IMARIS 8.0 software (Supplementary Figure 6B). Notably, we observed no significant difference in the number of translocated WT and α7nAChR-/- monocytes in response to MCP-1 or RANTES (Supplementary Figure 6C).

Because αM, αL, α4, and L-selectin are adhesive receptors and crucial in the trans-endothelial migration process, we examined their expression on the cell surface of mouse peripheral blood monocytes using flow cytometry. Monocytes were gated based on Ly6-C positivity before assessing the receptors relative expression in mean fluorescence intensity (MFI). We found no significant difference in MFI or the percentage of αL+, L-selectin+, α4+, or αM+ cells between WT and α7nAChR-/- mice (Supplementary Figure 6D). These findings confirm that α7nAChR deficiency does not impact the ability of isolated monocytes to migrate across an endothelial monolayer.

α7nAChR Deficiency Reduces the Migratory Ability of Macrophages in a 3D Fibrin Matrix along a Chemokine Gradient

To validate our *in vivo* findings, we conducted an *in vitro* experiment to investigate the impact of α7nAChR on macrophage migration within a 3D matrix. WT and α7nAChR-/- macrophages were allowed to migrate through a 3D fibrin matrix within a transwell insert in response to a gradient of either MCP-1 or RANTES. A schematic diagram of the experimental setup is illustrated in Fig. 2.5A. Equal proportions ($7.5x10^5$) of fluorescently labeled WT (PKH26, red) and α7nAChR-/- (PKH67, green) macrophages were placed on the membrane of the insert before the addition of the matrix. MCP-1 (30nM) or RANTES (12.8nM) were then added to the top of the fibrin matrix to initiate migration. After 48 hours of incubation at 37°C

and 5% CO_2, migrated cells were visualized using confocal microscopy (Fig. 2.5B,C) and images were reconstructed using IMARIS 8.0 software.

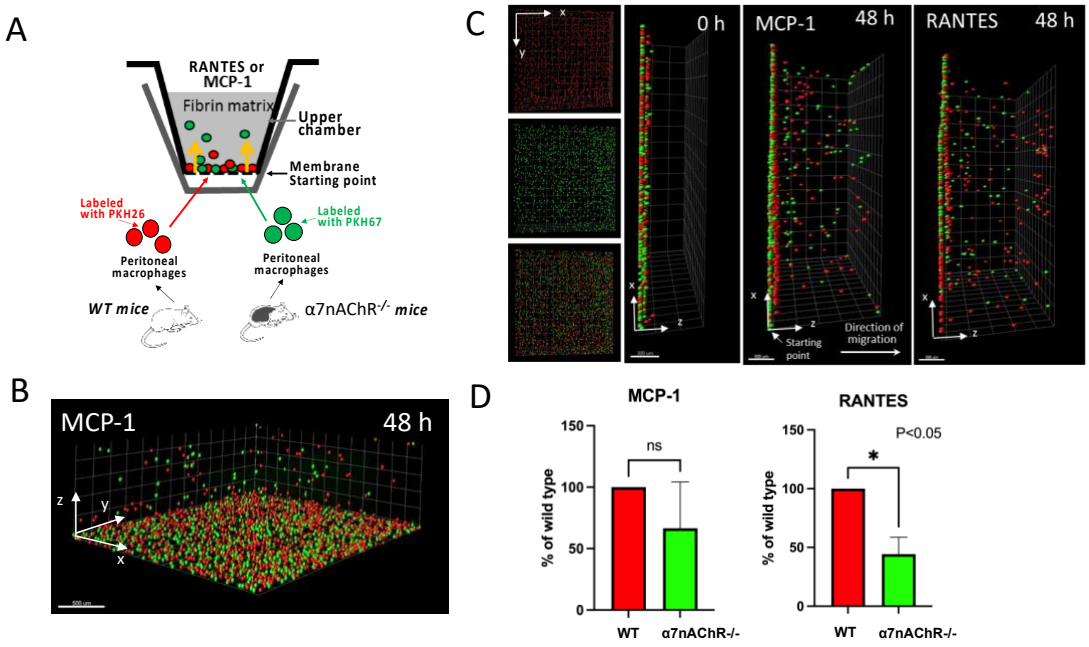


Figure 3.5: 3-D migration of peritoneal macrophages along MCP-1 and RANTES gradients.

A. Schematic drawing of experimental setup within a Corning transwell insert, with yellow arrows indicating the direction of macrophage migration. WT macrophages were labeled red (PKH26) and α7nAChR-/- macrophages were labeled green (PKH67) before being added to the membrane. Migration was initiated using RANTES (12.8nM) or MCP-1 (30nm) in medium added to the top of the fibrin gel. **B.** 3-D view of labeled macrophages migrating inside the fibrin gel after 48 hours. **C.** IMARIS 8.0 reconstruction of WT (red) and α7nAChR-/-(green) macrophages before the initiation of migration and after 48 hour incubation. Left shows the top view of individual and combined channels. Center, side view showing starting point at 0h. Right, side view showing macrophages migrating along MCP-1 or RANTES gradients. **D.** The number of macrophages migrating greater than 80μm was analyzed as a percentage of WT. * P<0.05. Statistical analysis was carried out using a student's t-test.

α7nAChR-/- macrophages exhibited significantly reduced migration in response to a RANTES gradient compared to WT macrophages (Fig. 2.5D). Although the response to an MCP-1 gradient showed a similar pattern, it did not reach statistical significance. These findings

complement our in vivo results and support the suggestion that α7nAChR deficiency may alter the expression of chemokine receptors and/or adhesive receptors.

α7nAChR Deficiency Markedly Reduces Relative mRNA Levels of Integrins αM and αX

Based on the results obtained from our 3D migration assay and tracked adoptive transfer experiment, we hypothesized that the expression of chemokine receptors and adhesive receptors may be altered in α7nAChR-deficient macrophages, leading to a decrease in their migration. We investigated the expression levels of adhesion receptors from β2 integrin family: αM, αD, αX and directly from β1 integrin (which forms the complex with several α subunits, including α2, α3, α4, α5) as these receptors play a role in macrophage migration by interacting with extracellular matrix proteins. Chemokine receptors CCR2 and CCR5 were also examined, as they are major receptors in macrophage chemotaxis, as well as the respective receptors for MCP-1 and RANTES, the chemokines used in our 3-D migration assay. To assess the mRNA levels of these selected receptors, we performed quantitative real-time polymerase chain reaction (qRT-PCR) using thioglycolate-induced peritoneal macrophages that were incubated overnight with LPS (10ng/mL) and PNU-282987 (30μm). Total RNA was extracted from the macrophage lysate and used for qRT-PCR analysis. The specific primers used for detecting αM, αD, αX, CCR2, and CCR5 are listed in Table 1 of the methods section.

α7nAChR-deficient macrophages exhibited similar relative mRNA levels of CCR2 and CCR5 compared to WT controls (Fig. 2.6A). However, the relative mRNA levels of integrins αM and αX in α7nAChR-deficient macrophages showed a statistically significant decrease compared to WT controls (Fig. 2.6B). Integrin subunit β1 did not show any significant changes in α7nAChR deficient mice (Fig. 2.6B). Furthermore, we examined the relative mRNA levels of the corresponding chemokines, MCP-1 and RANTES, which are secreted by macrophages to

attract additional leukocytes to the site of inflammation. The transcription of RANTES and MCP-1 remained relatively unchanged by α7nAChR deficiency (Fig. 2.6A).

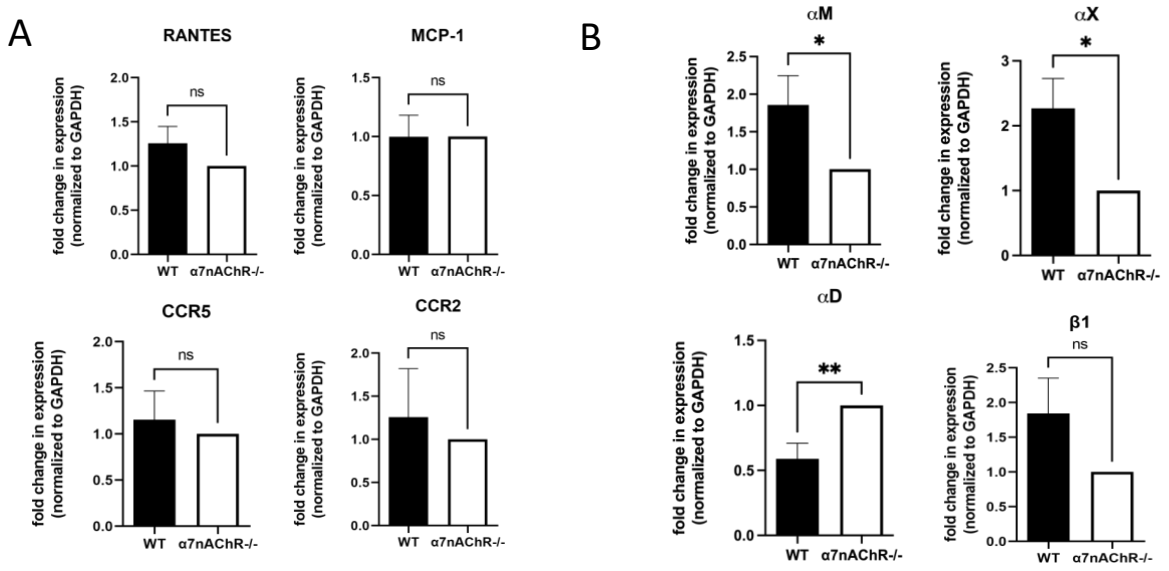


Figure 3.6: Quantitative real-time PCR of chemokines and surface receptors.

A. Peritoneal macrophages were isolated from WT and α7nAChR-/- mice and subsequently incubated with LPS (10ng/mL) and PNU (30μM) overnight before isolation of RNA and production of cDNA. Plots show relative mRNA levels of CCR2 and its ligand MCP-1, and CCR5 and its ligand RANTES. **B.** Cells were prepared for qRT-PCR identically to part A. Plots showing relative mRNA levels of integrin subunits αM, αX, αD, and β1. Experiment had eight independent replicates. *P<0.05, **P<0.01. Data was analyzed using the Livak Method. Statistical analysis was performed with a student's t-test.

These findings indicate that the deficiency of α7nAChR leads to significant decreases in the relative mRNA levels of αM and αX in macrophages. These alterations in receptor expression may contribute to the impaired migration observed in α7nAChR-deficient macrophages. Additionally, the similar relative mRNA levels of CCR2, CCR5, RANTES, and MCP-1 rule out the possibility of an altered chemotactic response in a7nAChR deficient macrophages via these pathways.

α7nAChR Deficiency Reduces Expression of αM at the Cell Surface of Adoptively Transferred Monocytes

The macrophage expression of integrin αX is dramatically lower when compared with the level of integrin αM. Due to its lower expression, αX is unlikely to significantly contribute to macrophage migration. Therefore, we focused primarily on αM. To verify our qPCR results, we assessed the expression levels of αM on adoptively transferred fluorescently-labeled WT (PKH26) and α7nAChR-/- (PKH67) macrophages in lungs at 48 hours after intraperitoneal injection of LPS (Fig. 2.7A-C). The expression of αM on adoptively transferred α7nAChR-deficient (green) monocytes was significantly reduced compared to WT (red) monocytes (Fig. 2.7C). This finding suggests that α7nAChR deficiency may affect the mesenchymal mode of macrophage migration, as αM plays a crucial role in the movement and adhesion of macrophages in the extracellular matrix.

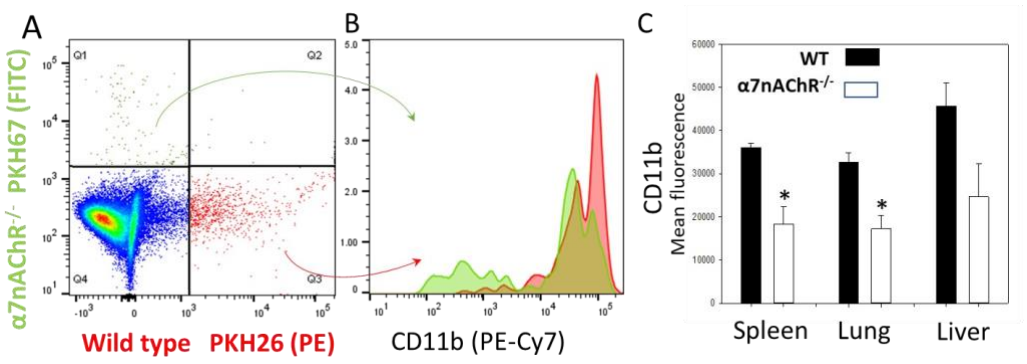


Figure 3.7: Surface expression of integrin CD11b on migrating WT (red) and α7nAChR-/- (green) macrophages. **A.** Representative flow cytometry dot plot showing migrated WT (red) and α7nAChR-/- (green) macrophages, in quadrant 3 and quadrant 1, respectively. **B**. Histogram overlay of CD11b fluorescence, colors correspond to cell staining in part A. Data was analyzed using FACSDiva software. Migrated WT macrophages (red) were detected in Quadrant 3; α7nAChR-/- macrophages (green) were detected in Quadrant 1. **C.** Bar graphs representing the amount of WT and α7nAChR-deficient macrophages detected in organs by flow cytometry. Statistical analysis was performed using Student's t-test. *, $P<0.05$.

Adoptive Transfer of WT Macrophages to α7nAChR-deficient Recipients Leads to the Partial Rescue of Phenotype

Since α7nAChR expression on macrophages has a protective effect during endotoxemia, we reasoned that injecting WT monocytes into α7nAChR-deficient mice could potentially rescue the protective phenotype of α7nAChR and improve survival. Conversely, we also investigated whether injecting α7nAChR-deficient monocytes to WT recipients would adversely affect survival.

First, we evaluated the potential effect of α7nAChR-deficient monocytes injected to WT recipient mice. WT mice were divided into two groups (n=6/group) and injected with WT or α7nAChR-/- monocytes intravenously 5 minutes prior the induction of endotoxemia. In addition, a third group was injected with the same concentration of LPS without adoptively-transferred monocytes. A schematic diagram illustrating the experimental setup is shown in Fig. 2.8A. Body temperature and survival of the mice were monitored twice daily for four days. We did not find the significant differences in survival between the three groups (Figure 2.8B).

To test a potential protective mechanism of WT monocytes, we divided α7nAChR-/- recipient mice into two groups (n=12/group) and administered unlabeled WT or α7nAChR-/- monocytes intravenously prior to inducing endotoxemia. The mice were monitored for four days for changes in body temperature and survival (Fig. 2.8C). All α7nAChR-deficient recipient mice injected with α7nAChR-/- monocytes died within 60 hours, while the same strain injected with the WT monocytes demonstrated a modest improvement in survival (25%) (Figure 2.8C). These results demonstrate a partially protective effect of WT monocyte transfer to α7nAChR-deficient recipients.

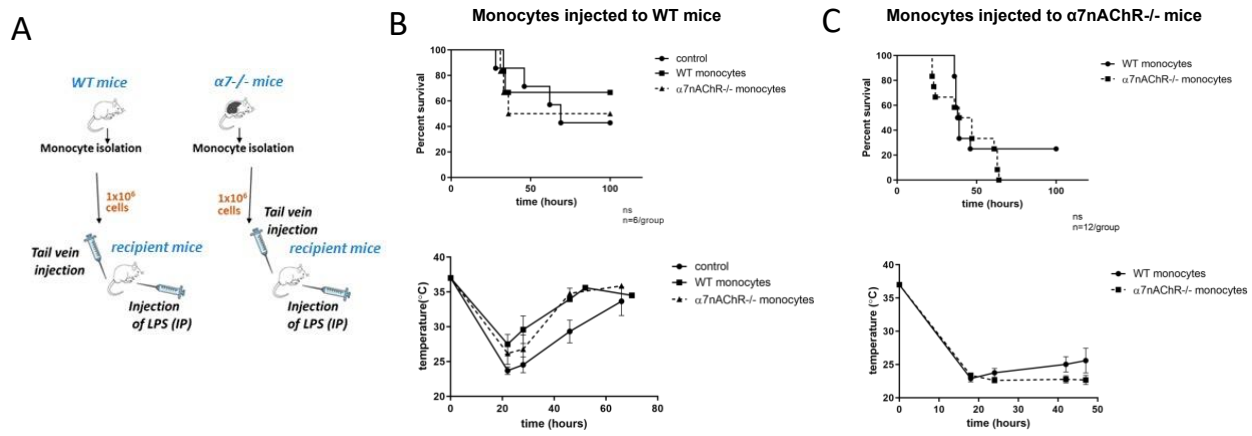


Figure 3.8: Survival of WT and α7nAChR-/- mice injected with monocytes during LPS-induced endotoxemia.

A. Graphical representation of experimental setup. Recipient mice are either WT (part B) or α7nAChR-/- (part C). Control mice were given LPS only, with no monocytes. **B.** Survival curve and temperature graph of WT recipients receiving WT, α7nAChR-/-, or no monocytes (control) intravenously before a sub-lethal intraperitoneal dose of LPS. 8-12 week old WT male and female mice were used as recipients (n=6/treatment group). **C.** Survival curve and temperature graph of α7nAChR-/- recipients receiving either WT or α7nAChR-/- monocytes intravenously before a sub-lethal intraperitoneal dose of LPS. 8-12 week old α7nAChR-/- male and female mice were used as recipients (n=9/treatment group). For survival curves, statistical significance was assessed by the Kaplan-Meier method. Temperature graphs report mean temperature and standard error.

Discussion

Here we show that genetic α7nAChR deficiency is associated with reduced macrophage migration to the lungs during murine endotoxemia and specific pharmacological activation of this receptor results in increased macrophage migration. These observations indicate a previously unrecognized role for the α7nAChR - a key peripheral component of the cholinergic anti-inflammatory pathway in mediating macrophage migration during acute inflammation. In parallel, α7nAChR deficiency results in worsened survival of mice during endotoxemia, which indicates a tonic protective function of the α7nAChR in inflammation. These findings identify

macrophage migration as an important mechanism contributing to the physiological cholinergic regulation of inflammation. Additional mechanistic insight substantiates this notion, revealing that the expression of integrin αMβ2 is reduced on α7nAChR-deficient monocyte-derived macrophages, indicating its potential role in α7nAChR-mediated macrophage migration.

Macrophages are essential players in innate immunity that may have a protective or pathological contribution to the development of inflammatory diseases (23). Macrophage phenotype, tissue distribution, molecular environment, and disease stage define the outcomes of macrophage function. Inhibition of pro-inflammatory cytokine secretion by macrophages was the major anti-inflammatory function reported for α7nAChR (10, 31, 32). Pioneering work from Kevin Tracey's group revealed that α7nAChR activation blocks the nuclear translocation of NF-κB, a master transcription factor for multiple pro-inflammatory genes that generate inflammatory responses (6, 12, 33-35). However, other potential mechanisms may have a significant contribution to the α7nAChR-mediated macrophage response.

Despite a well-characterized protective role of α7nAChR in endotoxemia and CLP sepsis, the direct effect of α7nAChR-deficiency to survival during endotoxemia was not investigated previously. Our results of reduced survival of α7nAChR-/- mice compared with WT clearly demonstrate the detrimental impact of α7nAChR deficiency on survival during murine endotoxemia. The significant drop of body temperature at 24 hours after LPS-injection is an important pathophysiological effect during endotoxemia. Thus, body temperature analysis indicating a significant decrease was implemented as a reliable additional verification of endotoxemia severity and progression in our in vivo experiments.

Our results demonstrating the effect of α7nAChR deficiency on survival complement previous observations that administration of the α7nAChR agonists GTS-21 or choline improve

the survival of mice during endotoxemia and CLP sepsis (9, 19). Of note, a similar protective role for the α7nAChR was demonstrated in models of acute lung injury induced by *E. coli* intratracheal administration utilizing α7nAChR-/- mice and α7nAChR agonist treatments (36).

Previous studies have reported that the accumulation of macrophages in the lungs during sepsis can have a protective function (21, 22). In contrast, the accumulation of neutrophils is a characteristic feature of sepsis-induced acute lung injury and is associated with poor outcomes (25, 37). One of the mechanisms by which macrophages provide protection is through the control of inflammation via efferocytosis of activated neutrophils (Bailey et al., 2021). Here we evaluated the effect of α7nAChR activation using a specific agonist on macrophage accumulation in lungs. Previous studies have demonstrated the potent anti-inflammatory effects of α7nAChR activation by agonists such as GTS-21 and PNU-282987 in murine models of systemic inflammation and sepsis (18, 19, 26, 32, 38). We observed that wild-type mice treated with the agonist PNU-282987 exhibited a significant increase in the number of monocyte-derived macrophages and body temperature, along with a decrease in neutrophil numbers when compared to untreated mice. This finding is consistent with data reported by Huston et al (2009), where nicotine treatment decreased the number of neutrophils accumulated in carrageenan-filled air pouches, as compared to controls (39).

Consistent with our α7nAChR activation approach, we observed a significant reduction in the number of macrophages in the lungs of α7nAChR-deficient mice during endotoxemia. These data were supported by the decrease in body temperature in α7nAChR-deficient mice which indicates the greater severity of systemic inflammation in these animals.

Notably, there was no significant difference in the proportion of macrophages in any stage of cell death measured by annexin V and a viability dye and in the number of apoptotic

neutrophils in the lungs. Activation of α7nAChR in non-neuronal cells has been previously shown to promote cell survival and protect against apoptosis (40, 41). Nicotine plays a role in promoting cellular survival during *Mycobacterium avium paratuberculosis* infection by increasing the expression of anti-apoptotic protein Bcl2 (42). Vagus nerve stimulation reduces the infarct size and inflammation in an ischemia-reperfusion injury model through a nicotinic antiapoptotic effect (43). This cell survival mechanism is linked to the STAT3 pro-survival pathway, activated via α7nAChR, which protects macrophages from ER stress-induced apoptosis. Interestingly, it specifically affects M2 macrophages while the apoptosis of M1 macrophages was not significantly changed (41). Apparently, the time frame of our experiments (48 hours after stimulation of endotoxemia) is not sufficient to generate strong M1-like/M2-like polarization that can affect macrophage apoptosis. In our study cell death was not significantly linked to accumulation of neutrophils and macrophages in tissue and, therefore, indicates the potential role of α7nAChR in macrophage migration.

To provide additional insights in our study we applied in vivo migration assay by monitoring of fluorescently labeled, adoptively transferred monocyte/macrophage in the model of endotoxemia (28-30, 44). We employed an internal control within each recipient mouse by injecting an equal number of monocytes from both WT and α7nAChR-/- donors, thus facilitating direct comparison between the two monocyte types. An additional adoptive transfer tracking experiment was performed using α7nAChR-deficient recipients. Both experiments revealed the same pattern: more WT monocytes were detected in the lungs, liver, and spleen when compared to α7nAChR-deficient monocytes. In addition, to confirming the outcome of the experiment with WT recipients, the repetition with α7nAChR-deficient recipients suggests that the enhanced migration of WT monocytes does not depend on the expression of α7nAChR on other cell types.

In both setups, the quality of the isolated donor monocytes was validated using flow cytometry, where a purity of 87-92% was confirmed. The migrated cells within the lungs were also examined using imaging flow cytometry (Amnis), verifying their morphology, and ruling out double-staining.

To address any potential influence of fluorescent dyes on macrophage migration in vivo, we conducted a separate experiment comparing the migration of equal numbers of WT monocytes labeled with either PKH26 (red) or PKH67 (green) and found that both red and green-labeled WT macrophages exhibited similar motility when migrating towards inflamed tissue. These results provided evidence that the fluorescent dyes themselves do not significantly affect macrophage migration.

To demonstrate the direct involvement of α7nAChR in macrophage migration, we conducted in vitro 3D migration assays, a well-developed technique, we have previously used (28-30). Namely, our experimental setup provided a comprehensive assessment of macrophage migration, wherein monocyte-derived WT and α7nAChR-/- macrophages, labeled with different fluorescent dyes, migrated through a fibrin matrix against gravity in the presence of a chemokine gradient. By including two types of fluorescently-labeled cells (WT and α7nAChR-/-) within the same matrix, we were able to reduce data variability and calculate the migration ratio between control and knockout macrophages in each sample. Within a fibrin matrix, we observed that α7nAChR-deficient macrophages exhibited reduced effectiveness in migrating along both RANTES and MCP-1 gradients compared to WT macrophages.

In comparison to our experimental protocol, previous studies that attempted to evaluate the contribution of α7nAChR to macrophage migration utilized macrophage-like cell lines, wild-type cell phenotype (no α7nAChR-knockout), and the most importantly, a simplified 2D

transmigration setup without chemokine gradients or protein coatings. For example, the ability of the α7nAChR agonists (PHA-543613 and varenicline) to decrease migration of RAW264.7 cells was demonstrated by testing cell transmigration through uncoated trans-well membranes (Boyden chambers) without a chemokine gradient (45, 46). Similar results were obtained by others who showed that acetylcholine can inhibit LPS-induced RAW264.7 cell migration. This study suggested that the inhibition of migration was attributed to the blocking of MMP-9 expression (47). MMPs play a role in 3D macrophage migration through the extracellular matrix (ECM) by degrading ECM proteins and creating space for cell movement. However, it should be noted that the presented experiments do not directly verify this hypothesis, as the proposed model using un-coated transwells does not involve MMP-mediated ECM degradation. In this model, macrophages transmigrate via an 8-μm pore-size membrane without immobilized ligands and chemokine gradients, where cell motility is mostly regulated by gravity and diffusion. Therefore, evaluating the role of α7nAChR in macrophage migration remained incomplete. In this study, we provide advanced characterization by implementing an improved experimental design and methodology.

In addition to migration through the extracellular matrix, trans-endothelial migration is another crucial step in the recruitment of leukocytes during inflammation. Our findings reveal no significant difference in the transmigration of WT and α7nAChR-/- monocytes across an endothelial monolayer in response to either MCP-1 or RANTES. This data is supported by the similar expression of integrins αL, αM, α4, and L-selectin on WT and α7nAChR-/- mouse peripheral blood monocytes. These molecules are key adhesion receptors in the process of adhering to, and migrating across, the endothelial wall. Based on these results, we can conclude

that trans-endothelial migration does not contribute to the differential migration observed between WT and α7nAChR-/- monocytes/macrophages.

Reduced expression of integrin αX and integrin αM at the transcriptional level in α7nAChR-/- macrophages provides a potential mechanistic explanation for their reduced migration. Integrin αMβ2 is a crucial adhesive receptor for the recruitment of monocytes and migration of macrophages through the extracellular matrix. Integrin αXβ2 possesses multiple regulatory functions on macrophages (48, 49), but has a limited effect on macrophage migration due to relatively low level of expression on macrophage subsets. Therefore, the decreased αM level in α7nAChR-/- macrophages on mRNA and protein levels suggest that its impaired migration may be due to an altered adhesion. Since we observed reduced αM in fully differentiated tissue macrophages but not in peripheral blood monocytes, it's important to emphasize that these cells are functionally different populations. Unlike macrophages, monocytes do not carry out effector functions within inflamed or infected tissues. Monocytes are myeloid progenitors that circulate in the blood and differentiate into macrophages after extravasation.

In contrast to our observations with αM and αX, we observed an increased expression of integrin αDβ2 on α7nAChR-/- macrophages. Integrin αDβ2 is significantly upregulated on pro-inflammatory (M1-like) macrophages in vivo and in vitro and contributes to the development of various chronic inflammatory diseases, such as atherosclerosis and diabetes (28, 30). Importantly, previous studies have shown that activation of α7nAChR leads to macrophage polarization toward the M2 phenotype (26, 27, 50), therefore α7nAChR deficiency should be associated with M1 phenotype, where integrin αDβ2 is upregulated. Based on the levels of αMβ2 and αDβ2 expression on different macrophage subsets, it has been suggested that αMβ2 is

involved in macrophage migration to and from the sites of inflammation, while αDβ2 plays a role in the retention of pro-inflammatory, M1-polarized macrophages at the sites of chronic inflammation (29). Therefore, the modest upregulation of the low-expressed αDβ2 on monocyte-derived macrophages may have a limited impact on macrophage migration but highlights the potential pathological role of integrin αDβ2 in α7nAChR-deficient mice during the development of atherosclerosis and diabetes (51-53).

Because macrophages also secrete RANTES and MCP-1, perpetuating leukocyte recruitment, we also examined the relative mRNA levels of these two chemokines and reported a marked, but not significant, decrease in RANTES with no change in MCP-1. The expression of corresponding chemokine receptors, CCR5 and CCR2, respectively, were also unaltered by α7nAChR deficiency, showing that reduced migratory ability of these macrophages is not due to differences in the perpetuation or perception of chemotactic signals.

Interestingly, the injection of WT monocytes does not completely rescue the phenotype of α7nAChR-deficient mice, resulting in only partial improvement in survival. Considering that 4% of blood leukocytes in mice are monocytes, this incomplete phenotype rescue could be attributed to the already overwhelming NF-κB activation present in α7nAChR-/- mice; thus, adding a population of $1X10^6$ WT monocytes may not be sufficient to reverse the deleterious effects caused by α7nAChR deficiency.

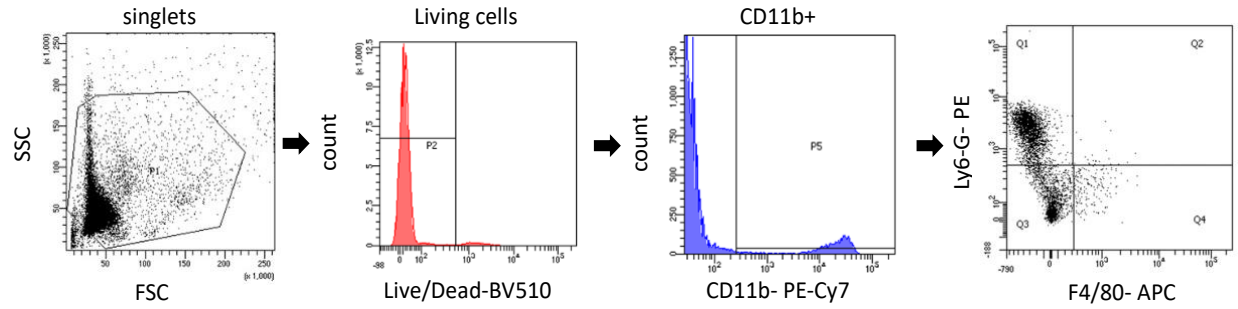
Based on our results, we propose that α7nAChR deficiency leads to reduced migration of macrophages to the lungs and other inflamed organs, thereby impairing the clearance of recruited neutrophils through efferocytosis. Consequently, neutrophils present in the tissues of α7nAChR-/- mice secrete pro-inflammatory cytokines. Furthermore, the failed recruitment of α7nAChR-deficint monocytes results in their accumulation in the bloodstream and the secretion of pro-

inflammatory cytokines via NF-κB-related mechanisms (54, 55). Collectively, these processes contribute to an increased cytokine storm and higher mortality rate.

Conclusions

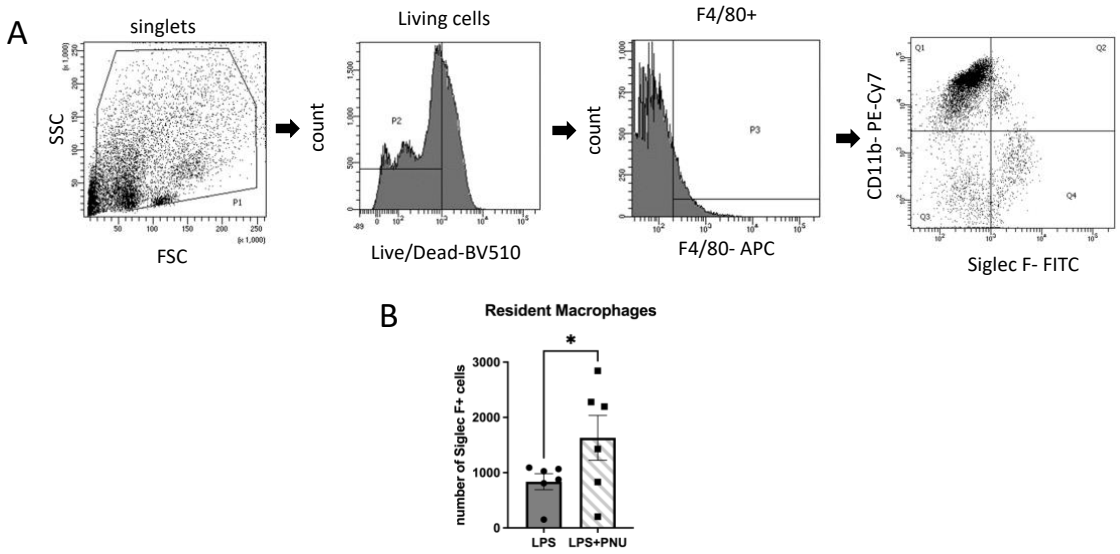
Our findings indicate that the cholinergic anti-inflammatory pathway, specifically the α7nAChR, plays a crucial role in regulating the migration and accumulation of macrophages at inflammation sites. We present evidence that α7nAChR is not only protective during endotoxemia but also essential for efficient monocyte/macrophage trafficking. By using α7nAChR-deficient mice or stimulating WT mice with PNU-282987, we demonstrate that the α7nAChR supports the recruitment of monocyte-derived macrophages to the lungs in vivo and their migration in a 3D matrix in vitro. α7nAChR deficiency adversely affects migration, which is associated with reduced the levels of integrin αMβ2, a critical integrin involved in various stages of the leukocyte migration process. In summary, our findings provide novel insights into α7nAChR-mediated monocyte/macrophage migration to inflamed tissues, expanding the clinical possibilities for septic patients.

Supplemental Figures



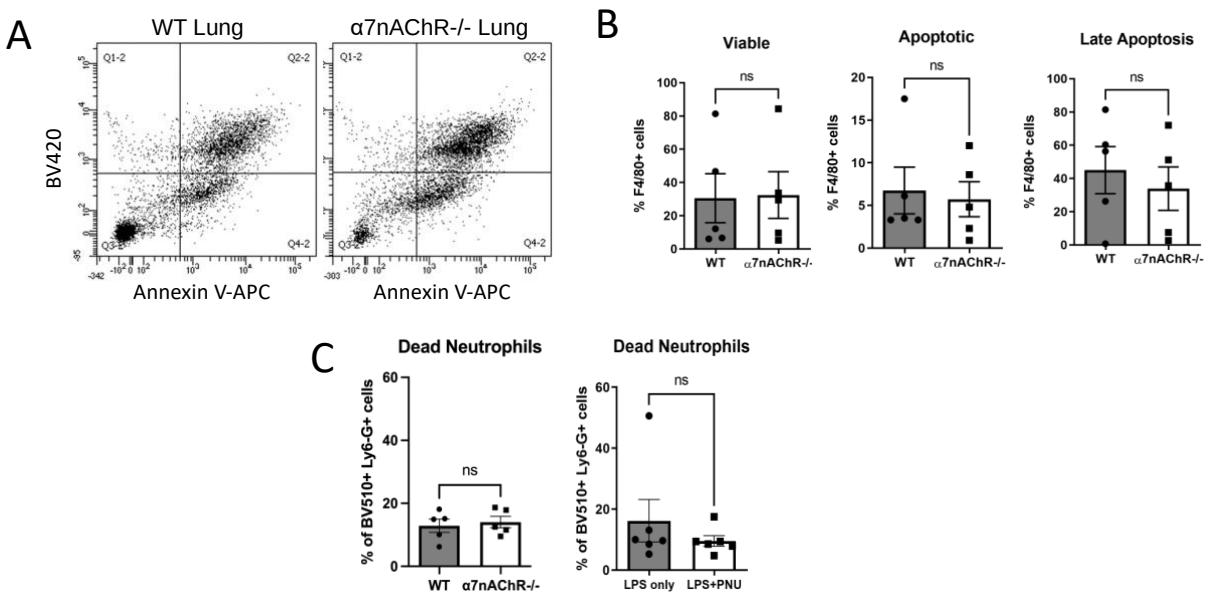
Supplemental Figure 1: Gating strategies for Fig. 3.2.

A. To analyze alive macrophages and neutrophils, cells were gated by size to select singlets from all events (P1). Next, living cells were selected by gating cells negative for yellow fixable dye, BV510 (P2). From living cells, all CD11b+ events were selected (P5). Lastly, CD11b+ alive cells were analyzed using a dot plot, Ly6-G (neutrophil marker) VS F4/80 (macrophage marker). A quadrant gate was placed in reference to the negative control, so that neutrophils could be quantified in Q1 (Ly6-G-PE) and macrophages in Q4 (F4/80-APC). **B.** In order to examine resident macrophage populations, cells were first gated by size to select singlets (P1), then gated for live cells using BV510 yellow viability dye. From living cells, all F4/80+ events were selected (P3) and analyzed using a dot plot, CD11b (integrin αM) VS Siglec F (resident marker). Lastly, a quadrant gate was placed in reference to the negative control, and residents were quantified in Q4 as CD11b- (PE-Cy7) F4/80+ (APC) Siglec F+ (FITC).



Supplemental Figure 2: PNU-282987 treatment increased absolute cell counts of alveolar macrophages.

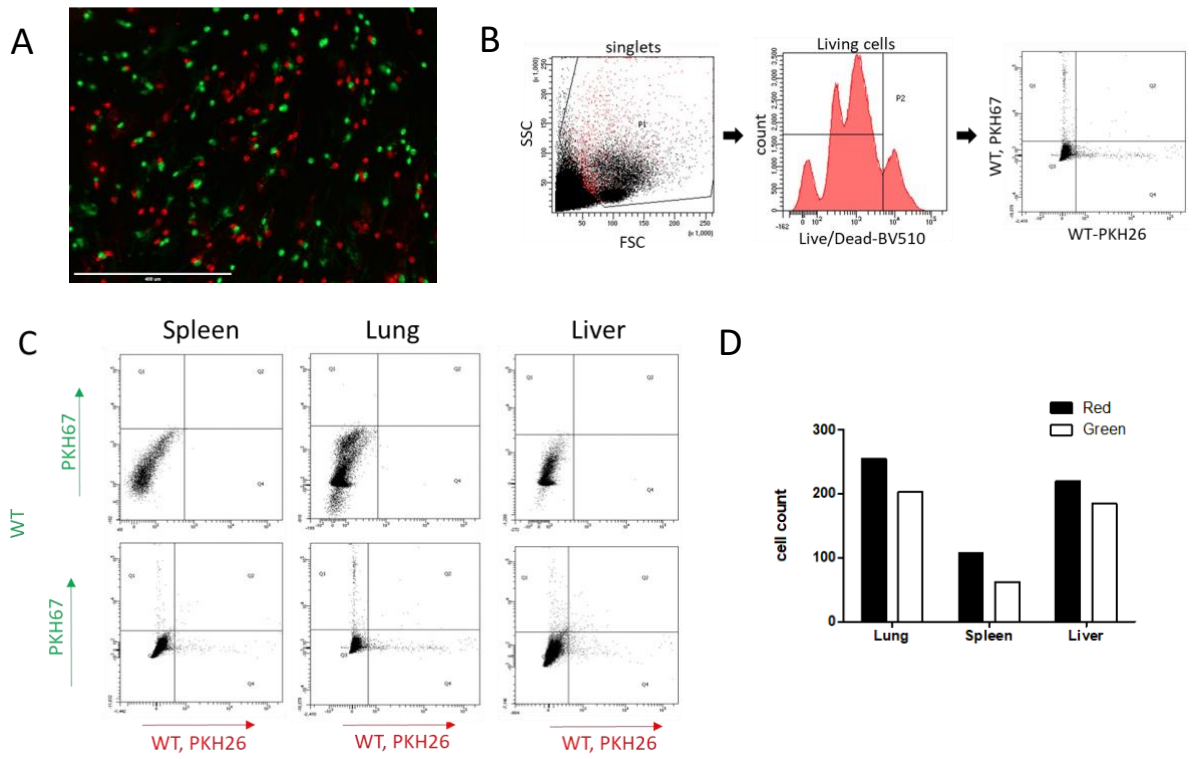
A. In order to examine resident macrophage populations, cells were first gated by size to select singlets (P1), then gated for live cells using BV510 yellow viability dye. From living cells, all F4/80+ events were selected (P3) and analyzed using a dot plot, CD11b (integrin αM) VS Siglec F (resident marker). Lastly, a quadrant gate was placed in reference to the negative control, and residents were quantified in Q4 as CD11b- (PE-Cy7) F4/80+ (APC?) Siglec F+ (FITC). Same gating strategy was used for Fig. 2. **B.** WT and α7nAChR-/- mice were injected with a sublethal dose of LPS. After 48h lungs were removed, digested and analyzed using flow cytometry. F4/80 positive cells were selected and examined with antibodies against CD11b and Siglec F. Resident macrophages were identified as F4/80+ Siglec F+ CD11b-. Statistical analysis was carried out using a paired t-test. *$p<0.05$.



Supplemental Figure 3: Apoptotic macrophages.

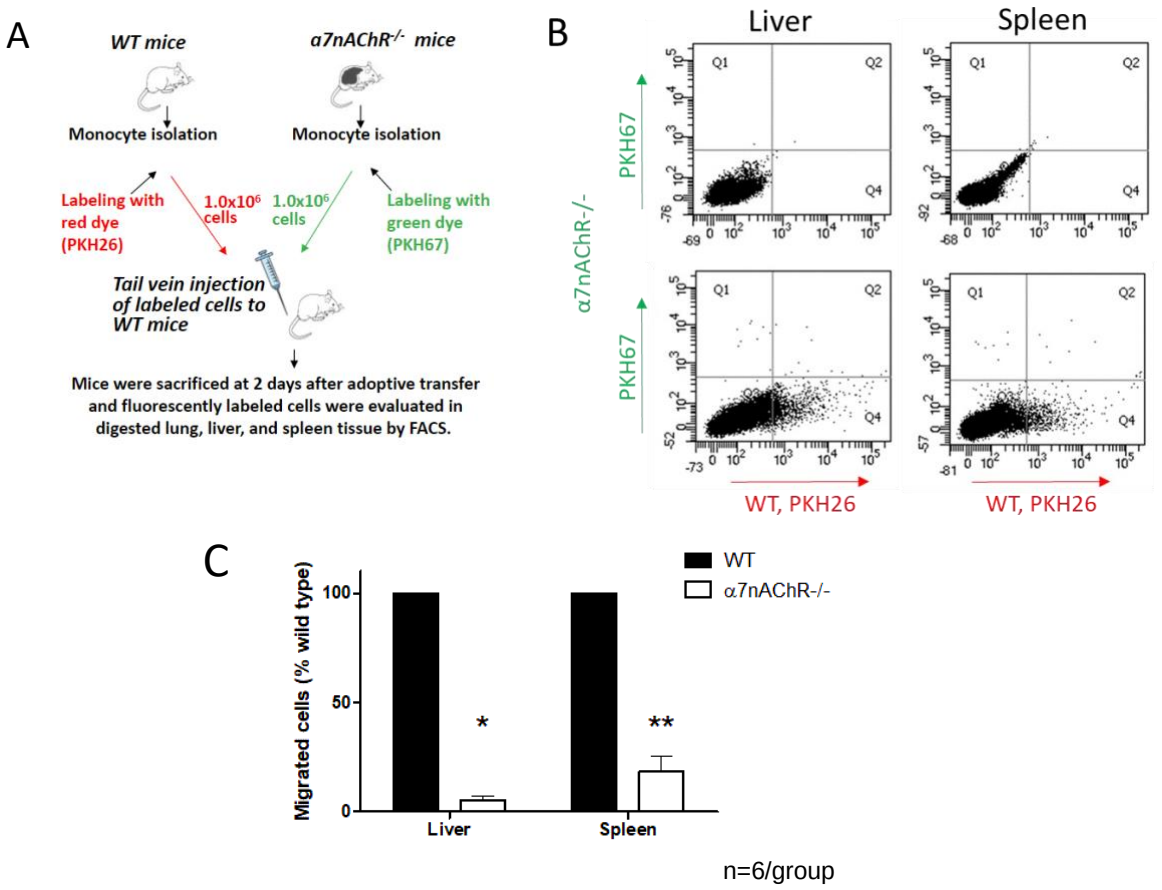
A. WT and α7nAChR-/- mice were injected with a sublethal dose of LPS (n=5/group). After 48 hours the lungs were analyzed for apoptotic macrophages using flow cytometry. Using Annexin V (APC) and violet fixable viability dye (BV420) F4/80+ macrophages were analyzed for viability. Cell viability was determined by positivity for these two stains, with viable cells being those negative for both dyes (Q3-2), Annexin/BV420 double positive considered late-stage apoptosis (Q2-2), and Annexin V+ cells determined as apoptotic (Q4-2). **B.** Graphical representation of the proportion of F4/80+ cells in each viability category. Statistical analysis performed using Student's t-test.

C. Proportions of dead neutrophils within the lungs of mice treated with or without PNU (left) and within the lungs of WT or α7nAChR-/- mice treated with LPS and vehicle (right).



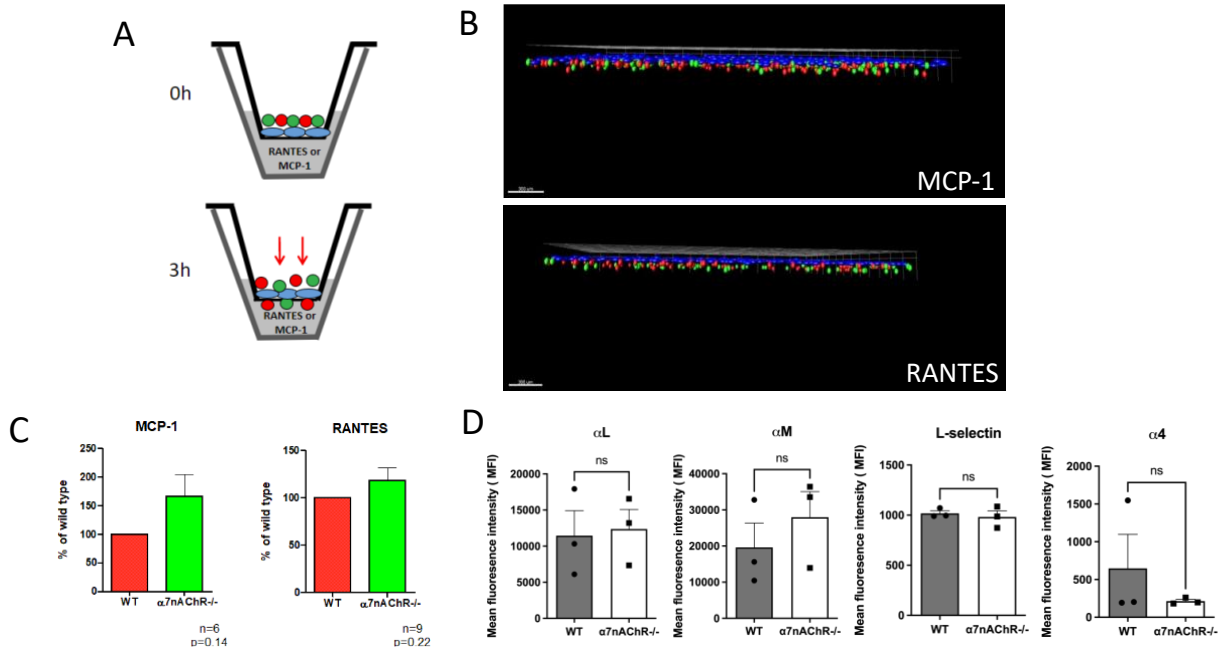
Supplemental Figure 4: PKH dye color does not significantly affect in-vivo migration of injected monocytes.

In this experiment, both dye colors were used to stain WT monocytes, with experimental design and procedure otherwise identical to Fig 3. **A.** Cytospin and flow cytometry analysis checking proportion of cells in mix. Mix proportion was evaluated in all adoptive transfer experiments, representative image is shown. **B.** Gating strategy for the detection of labeled cells in organs. **C.** Representative flow cytometry dot plots of experimental results. **D.** Bar graphs illustrating the number of red and green WT macrophages detected in flow cytometry analysis of digested organs.



Supplemental Figure 5: α7nAChR deficiency impedes the migration of macrophages to organs during LPS-induced endotoxemia.

A. Schematic representation of the experimental design. Monocytes were isolated from bone marrow of WT and α7nAChR-/- mice via MACS. Cell were labeled with red (WT) or green (α7nAChR-/-) fluorescent dyes, mixed in equal proportion and injected in tail vein of WT recipient mice. After 48 hours, the lung, liver and spleen were isolated, digested and analyzed using flow cytometry. **B.** Representative results of flow cytometry analysis are shown. Data was analyzed using FACSDiva software. Migrated WT macrophages (red) were detected in Quadrant 4; α7nAChR-/- macrophages (green) were detected in Quadrant 1. **C.** Bar graphs representing the amount of WT and α7nAChR-deficient macrophages detected in organs by flow cytometry. Statistical analysis was performed using Student's t-test. *, $P<0.05$, ** $P<0.01$.



Supplemental Figure 6: Trans-endothelial migration of monocytes along MCP-1 and RANTES gradients.

A. Schematic representation of experimental setup within a corning transwell insert. **B.** 2-D view of representative transwell image after 3 hours. Top, image showing transmigration in response to MCP-1. Bottom, image showing transmigration to RANTES. **C.** Plots showing the number of monocytes migrating across the endothelial monolayer, as a percentage of WT. **D.** Plot of CD11a, CD11b, and CD62L expression on the surface of mouse peripheral blood monocytes, analyzed using flow cytometry (n=3). Statistical analysis was carried out using a student's t-test. * $P<0.05$.

Declarations

Ethics Approval and Consent to Participate

All animal procedures were performed according to animal protocols approved by East Tennessee State University.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare they have no competing interests.

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Authors' Contributions

KRK, VPY, KC, JC, and SS performed experiments and collected resulting data. VPY, VAP, DH, and DLW provided insight and supervision throughout the course of the project. All authors read and approved the final manuscript.

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CHAPTER 4. MIGRATION OF MACROPHAGES TO ATHEROSCLEROTIC PLAQUES IS INFLUENCED BY α7NACHR.

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Introduction

Since Kevin Tracy's research team first described it, the CAP has been studied extensively in inflammatory diseases, including atherosclerosis. Macrophages are directly responsible for the lipid deposition that leads to the formation of atherosclerosis within arteries. The CAP is widely regarded as beneficial in atherosclerotic mice, with multiple research teams reporting decreased atherosclerosis and lesion macrophage content in response to treatment with α7nAChR specific agonists (Al-Sharea et al. 2017; Hashimoto et al. 2014). Coinciding with these observations, Johansson et al. reported increased atherosclerosis in the aortic root in Ldlr-/- mice receiving α7nAChR-/- bone marrow transplants when compared to controls receiving WT bone marrow (Johansson et al. 2014). The α7nAChR on macrophages may have a role in atherosclerosis pathogenesis by influencing macrophage migration to atherosclerotic plaques. Thus, we designed an adoptive transfer experiment to directly compare the migration of WT and α7nAChR-/- monocytes to aortic plaques of ApoE-/- mice.

Materials and Methods

Reagents

Cell membrane dyes PHK67 and PKH26, DNase I type II, Collagenase I, and Collagenase II were purchased from Sigma Aldrich (St. Louis, MO, USA). Magnetic assisted cell sorting (MACS) kit and LS columns were purchased from Miltenyi Biotec (Gaithersberg, MO, USA).

Animals

Wild type (WT; C57BL/6J, stock #000664), α7nAchR-deficient (B6.12957-Chrna7tm1Bay/J, stock #003232), and ApoE-deficient (B6.129P2-Apoetm1Unc/J) mice colonies were purchased from Jackson Laboratory (Bar Harbor, ME, USA). α7nAChR-deficient mice

were backcrossed to C57BL/6 for eight generations, and ApoE-deficient mice for ten generations. All experimental work involving animals was performed according to protocols approved by East Tennessee State University IACUC.

Induction of Atherosclerosis

Before the start of the experiment, 8 week-old ApoE-/- mice were fed a high fat diet (at least 42% kcal from fat) for twelve weeks. ApoE-/- mice were therefore 20 weeks at the time of the experiment. The diet was continued until sacrifice three days after the adoptive transfer procedure.

Isolation of Bone Marrow Monocytes

WT and α7nAChR-/- mice were euthanized by asphyxiation with CO_2. The tibia and femur of each leg was removed and cleaned of any muscle tissue. The ends of each bone were removed, then the bone marrow was flushed out using RPMI. Bone marrow cells were centrifuged at 2100 RPM for 8 minutes to form a pellet. After removing the supernatant, the red blood cells were lysed using a lysis buffer. Magnetic separation of monocytes then proceeded according to manufacturer instructions given with the MACS kit (Miltenyi Biotec, Gaithersburg, MD, USA).

Adoptive Transfer

Isolated WT monocytes were stained green (PKH67) and α7nAChR-/- monocytes were stained red (PKH26) according to kit instructions. Stained WT and α7nAChR monocytes were mixed together in equal proportions. $1x10^6$ of each cell type was injected into the tail veins of ApoE-/- mice that had already been on a high fat diet for 12 weeks. After 3 days, the ApoE-/- mice were sacrificed for aorta isolation and digestion.

Aorta Digestion and Flow Cytometry Analysis

Mice were euthanized and perfused with 10mL 1x PBS to clear the aorta of blood. Next, the aortas were carefully excised from the abdominal wall and excess fatty tissue. Isolated aorta was minced into small pieces and digested in an enzyme cocktail suspended in HBSS: DNase I type II (120U/mL), Collagenase I (2mg/mL), and Collagenase II (2mg/mL). Digestion samples were incubated at 37°C for 45-50 minutes in a tube rotator. Enzyme activity was stopped by adding 1mL of 100mM EDTA. The digestion suspension was then passed through a 70μm cell strainer and centrifuged at 1200 RPM for 8 minutes. The cell pellet was resuspended and counted. Digested aorta suspension was divided into samples of $2x10^6$ cells and analyzed using flow cytometry to detect red and green labeled cells (Fortessa X-20, Becton Dickson).

Results

To collect preliminary data on the role of α7nAChR on monocyte/macrophage migration in atherosclerosis, an adoptive transfer experiment was performed. An illustration of the experimental setup for the adoptive transfer of fluorescent monocytes is provided in Fig 13A. Briefly, donor monocytes were isolated from WT and α7nAChR-/- mice and stained with PKH67 and PKH 26, respectively. The labeled cells were mixed equally, and $2X10^6$ labeled cells were injected into the tail veins of ApoE-/- mice. After 72 hours, the aortas were digested and analyzed with flow cytometry. Fluorescently labeled cells were detected in all three ApoE-/- recipient mouse aortas. Our analysis revealed significantly more α7nAChR-/- macrophages within the aorta when compared with WT (Fig 13B,C). PKH67 and PKH26 are common membrane labeling dyes, and we have confirmed in previous publications that dye color does not significantly influence the result (Aziz et al. 2017; Cui et al. 2018; Cui et al. 2019)

Discussion

This result is opposite what we obtained from our sepsis adoptive transfer experiments in Chapter 3, where WT macrophages outnumbered α7nAChR-/- macrophages, which is to be expected as sepsis and atherosclerosis have vastly different pathogenesis. Additionally, we found increased expression of αD in α7nAChR-/- macrophages in our qRT-PCR experiments in Chapter 3. Coupling this data with the increased number of α7nAChR-/- macrophages in the aorta and previous publications of our lab, this elevated αD expression suggests that α7nAChR-deficient macrophages may accumulate more in atherosclerotic lesions because their egress is impeded (Aziz et al. 2017). Macrophages deficient in α7nAChR may also be more likely to take on a more M1-like phenotype, as α7nAChR stimulation shifts macrophages to the M2-like phenotype, which has drastic implications for migration that are αDβ2 mediated (Cui et al. 2018; Pinheiro et al. 2017; Wang et al. 2019; Zhang et al. 2017). Our result with ApoE-/- mice agrees with previous reports that α7nAChR-deficiency exacerbates atherosclerosis, because macrophage retention and foam cell formation drive the pathogenesis of atherosclerosis. A key limitation of this study, however, is the lack of further experimentation beyond adoptive transfer tracking. Future investigation is needed to gather evidence regarding the relationship between integrin αD and the increased retention of α7nAChR-/- monocytes within atherosclerotic plaques.

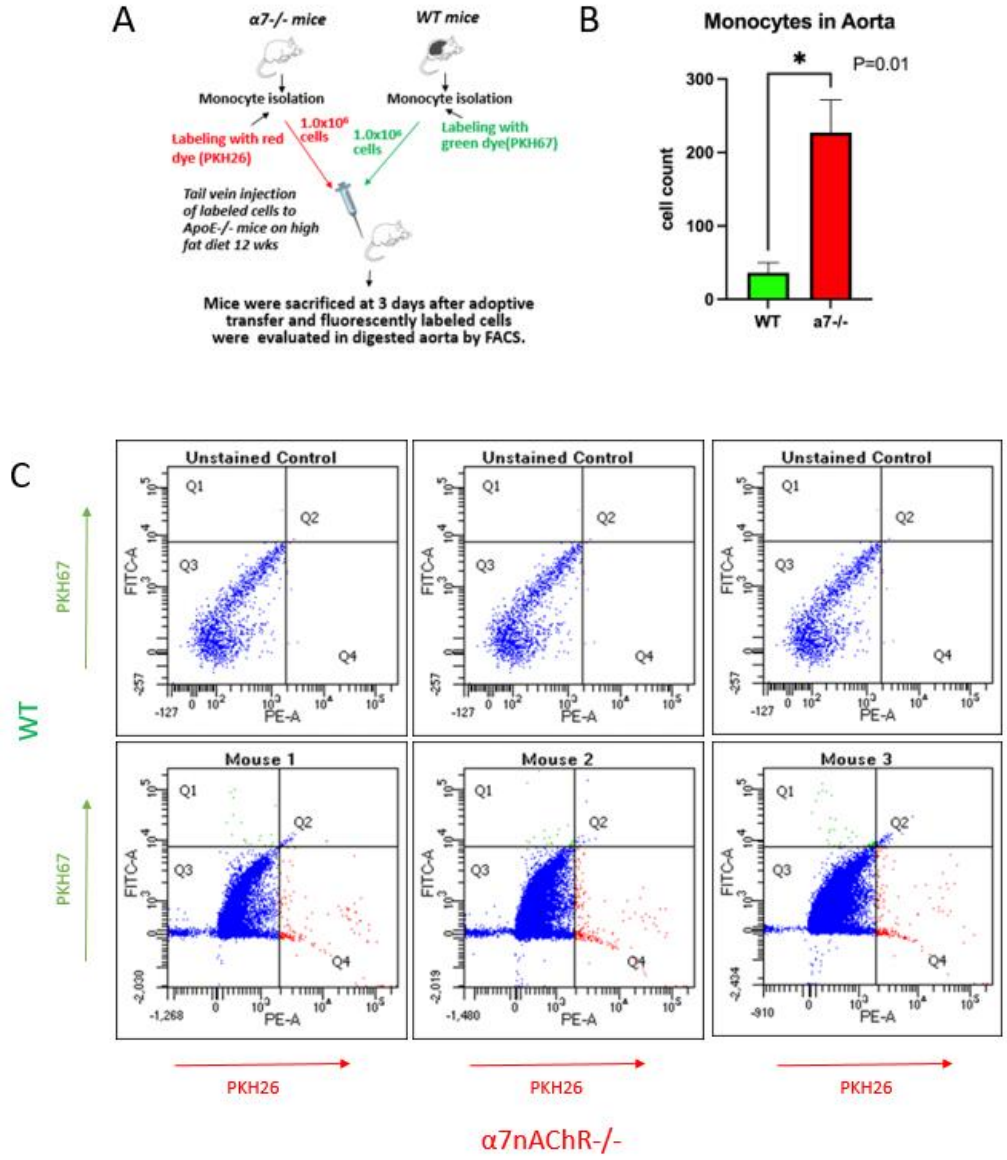

Figure 4.1: a7nAChR-deficiency increases macrophage accumulation in the aorta.

A. Illustration of experimental procedure. Monocytes were isolated from the bone marrow of WT and α7nAChR-/- mice via MACS. WT cells were labeled green (PKH67) and α7nAChR-/- cells were labeled red (PKH26), then mixed in equal proportions and intravenously injected into ApoE-/- mice (n=3). After 3 days, mice were sacrificed and perfused aortas were isolated and digested for flow cytometry. B. Bar graph representing the number of WT and α7nAChR-/- macrophages found in the digested aortas. C. Dot plots of all three ApoE-/- recipient aortas are shown.

WT macrophages (green) were detected in quadrant 1, and α7nAChR-/- macrophages (red) were detected in quadrant 4. Statistical analysis was performed using a student's t-test. *p=0.01.

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CHAPTER 5. CONLCUSIONS

Migration of macrophages into inflamed tissues during sepsis/endotoxemia has a major effect on disease outcome. Previously, we have shown an association between the number of macrophages migrating and accumulating in the lungs and severity of murine endotoxemia. Mice with a higher number of macrophages accumulating in the lungs tend to have higher body temperature and survival rates (Bailey et al. 2021). When compared to wild type mice, α7nAChR-deficient mice have significantly reduced survival during LPS-induced endotoxemia. We also found dramatically decreased numbers of macrophages in the lungs of α7nAChR-deficient mice, coinciding with the reported trend. Pre-treatment for 15 minutes using a selective agonist, PNU-282987, significantly improved the body temperature of endotoxemic WT mice, compared to those receiving LPS and vehicle. The group receiving PNU-282897 also had increased macrophages in their lungs, which was directly related to reduced endotoxemia severity.

Neutrophils also play a crucial role in the inflammatory response during sepsis. A high number of neutrophils migrating to the site of inflammation is detrimental, with studies showing that severity of sepsis associated acute lung injury is worsened in proportion to neutrophil content in the lungs (Grommes and Soehnlein 2011; Su et al. 2010). Our data collected in endotoxemic mice supports this consensus, we reported significantly increased neutrophils in the lungs of α7nAChR-deficient mice. In addition, we observed that treatment with specific agonist PNU-282987 reduced the number of neutrophils in the lungs of septic mice.

In vivo experiments were designed to directly compare the migration of WT and α7nAChR-/- monocytes during endotoxemia. Both WT and α7nAChR-deficient recipients were given a mixture of stained cells intravenously. The mixture was equal parts WT (red/PKH26)

and α7nAChR-/- (green/PKH67) bone marrow monocyte progenitors. For both recipient strains the same pattern was observed: a larger number of WT monocytes migrated compared to α7nAChR-/- monocytes to the lungs, liver, and spleen of septic mice. Using Amnis imaging flow cytometry, we validated the morphology of individual macrophages as well as ruled out dye transfer between PKH67 and PKH26 stained cells. We validated in a separate experiment that dye color does not influence the number of cells migrating to these tissues. This *in vivo* result suggests that α7nAChR is required for efficient trafficking of monocytes/macrophages to their target tissues.

Migration of leukocytes to inflamed issues is a multi-step process requiring the concerted efforts of multiple cell surface receptors and molecules in the extracellular milieu. The process begins in circulation with rolling along the endothelium, followed by firm adhesion at the site of extravasation. After emigration through the vascular endothelium, immune cells will travel through the target tissues and carry out their effector functions. Extravasation of immune cells into target tissues is a crucial step in the migration process and involves cooperation between chemotactic signals and adhesion receptors. Namely, β1 and β2 integrins are the major adhesion receptors in leukocyte migration, with αMβ2, αLβ2, and α4β1 being the most well studied in macrophage migration (Furie et al. 1991; Issekutz and Issekutz 1992; Weber et al. 1996)

In a transmigration assay, there was no difference in the ability of WT and α7nAChR bone marrow monocytes to migrate across an endothelial monolayer in response to either MCP-1 or RANTES. Furthermore, flow cytometry examination of adhesive receptors α4, αM, αL, and L-selectin at the surface of mouse peripheral blood monocytes likewise revealed no differences. We concluded that transmigration is not affected by α7nAChR-deficiency.

Since transendothelial movement is not affected by α7nAChR-deficiency, we next examined 3-D migration within the extracellular matrix. Our experimental setup employed a fibrin matrix polymerized atop stained peritoneal macrophages within a pre-coated transwell insert. Migration was initiated with the addition of either MCP-1 or RANTES to the top of the gel. WT macrophages migrated through the fibrin matrix in higher numbers than α7nAChR-deficieny macrophages, in response to both MCP-1 and RANTES. When compared with previous attempts from other groups, this transwell setup has the advantage of inducing macrophages to migrate through a fibrin matrix instead of an uncoated membrane. In addition, this setup is designed so that macrophages are moving against gravity, excluding the possibility of passive diffusion that may confound results in the simpler 2-D setup used to examine the movement of RAW264.7 macrophages (Yang et al. 2015). Our observations suggest that movement of macrophages through the extracellular matrix is affected by α7nAChR-deficiency. We sought to determine if decreased migration of α7nAChR-deficient macrophages was due to changes in adhesion molecules or perception of chemotactic signals.

Chemokine gradients were used to initiate migration in both our transmigration and 3-D migration experiments. Using qRT-PCR, relative mRNA levels of the same chemokines, MCP-1 and RANTES, as well as their respective receptors CCR2 and CCR5 were examined in peritoneal macrophages. Differences in expression levels for all four targets were not found, suggesting that impaired migration of α7nAChR-/- macrophages is not due to changes in expression of classical macrophage chemotactic molecules.

Adhesion molecules are another critical player in macrophage migration. Of interest are β2 integrins αM, αX, αD, and integrins α1, α2, α4, α5 for their role in adhering to extracellular matrix proteins. In the same experimental setup, the number of mRNA transcripts was examined

using qRT-PCR. There was no difference in the expression of the β1 integrin subunit. In contrast, all three β2 integrins were changed by α7nAChR-deficiency. Both αM and αX were decreased in α7nAChR-/- macrophages, while αD was upregulated. Integrin αX has low expression on macrophages, therefore its role in the differential migration seen in our *in vivo* and *in vitro* work was considered to be minimal. Integrin αD upregulation is associated with chronic inflammation and a pro-inflammatory, M1 polarization (Aziz et al. 2017; Cui et al. 2019). Activating α7nAChR pushes macrophages toward the M2 phenotype, an effect that would not be seen in α7nAChR deficient cells (Pinheiro et al. 2017; Wang et al. 2019; Zhang et al. 2017). Thus, the upregulation of αD transcripts in α7nAChR-/- macrophages agrees with current knowledge. In contrast to αX, integrin αM is a highly expressed molecule on macrophages and is well known to adhere to fibrinogen, the extracellular matrix protein used in our 3-D migration gel (Cao et al. 2005). Consequently, integrin αM was investigated further.

Expression of αM at the cell surface was examined on tracked, adoptively transferred monocytes, with the addition of an incubation with anti-αM/PE-Cy7 before flow cytometry analysis. When mean fluorescence intensity of αM was compared between monocyte types, WT monocytes had significantly more αM at the surface than α7nAChR-/- monocytes in the lung and spleen. A visible, but not significant, reduction was observed on α7nAChR-/- macrophages in the liver. Taken together with the reduced mRNA levels of αM in α7nAChR-/- peritoneal macrophages, this data suggests regulation of αMβ2 as the potential mechanism behind their reduced infiltration into inflamed tissues.

Because α7nAChR has been demonstrated to influence both macrophage migration and endotoxemia severity, we hypothesized that adoptive transfer of WT monocytes to α7nAChR-/-

mice before inducing endotoxemia would have a protective effect. As described previously in Bailey et al. (2022), we performed a phenotype rescue adoptive transfer experiment (William P. Bailey 2021). Contrary to our prediction however, injection of WT monocytes to α7nAChR-/- recipients was unable to rescue the protective phenotype of α7nAChR during LPS-induced endotoxemia. Conversely, injection of α7nAChR-/- monocytes to WT mice did not significantly worsen their condition when compared with controls and mice receiving WT monocytes. To completely rescue the anti-inflammatory phenotype, injecting WT cells may not be adequate to overcome the existing immune response to endotoxin in α7nAChR-/- mice. The experimental design should be altered to include either a larger proportion of injected cells or be carried out with a targeted depletion method added to the experimental protocol.

In addition to LPS-induced endotoxemia, differences in macrophage accumulation have been noted in both Ldlr-/- and ApoE-/- atherosclerotic mouse models. In an experiment designed to directly compare α7nAChR-mediated migration in atherosclerosis, ApoE-/- mice were fed a high fat diet for 12 weeks to induce plaque development. At 12 weeks, an adoptive transfer tracking experiment was conducted. No other injections were given to the ApoE-/- mice. After 72 hours, fluorescently labeled WT and α7nAChR-/- macrophages were tracked to digested aortas. Each mouse was confirmed to have numerous pearly opaque plaques in the aorta at necropsy. Flow cytometry analysis revealed more α7nAChR-/- macrophages within the digested aorta suspension compared to WT. This result is in stark contrast to that of the data collected in endotoxemia experiments, yet it indirectly agrees with reports from the literature. Mice treated with specific agonists had plaques reduced in both size and macrophage content (Al-Sharea et al. 2017; Hashimoto et al. 2014; Ulleryd et al. 2019a; Ulleryd et al. 2019c). Atherosclerotic mouse chimeras with α7nAChR-/- bone marrow transplants had increased atherosclerosis severity

(Johansson et al. 2014). It can be reasoned that an increased number of adoptively-transferred α7nAChR-/- monocytes agrees with these previous observations, because atherosclerosis pathogenesis is directly related to the amount of both living and apoptotic macrophages within atherosclerotic plaques (Tabas 2009). Increased accumulation of α7nAChR-/- macrophages in atherosclerotic plaques may be attributed to their higher expression of αDβ2 when compared to WT. Further investigation of this observation is a crucial future extension to expand the understanding of α7nAChR-mediated macrophage migration in atherosclerosis. This understanding could potentially be applied to future preventative therapeutics for at-risk patients.

While the basic research shows promise for the use of treatments directly targeting α7nAChR, there is another challenge left to overcome. Recently, a human-specific duplicate of the gene for α7nAChR was discovered, called CHRFAM7A, which would require additional research before the use of drugs directly targeting α7nAChRs can be approved for use in humans. The chimeric protein, dupα7, not only forms heteropentamers with α7 subunits, the gene encoding it also occurs in varying copy numbers on chromosome 15 (Di Lascio et al. 2022; Peng et al. 2022). The regulation between CHRFAM7A and CHRNA7 has been suggested as NF-κB dependent. When inflammatory stimuli are present, CHRFAM7A is down regulated and CHRNA7 is upregulated. Such a regulation method likely prevents unwarranted CAP activation at steady state and promotes its activation in the presence of inflammatory insults (Di Lascio et al. 2022). While a significant understanding of CHRFAM7A has already been gained, more studies integrating CHRFAM7A are necessary.

To work toward closing this translational gap, mouse models are being mindfully generated by Costantini et al (Costantini et al. 2019). Their work developing a transgenic model included evaluation of the variations in CHRFAM7A existing in the human population, such as

the 2-bp deletion and the multiple copy numbers. Unfortunately, these mice are not commercially available for purchase at suppliers like JAX yet, although they will undoubtedly become vital for future studies of the cholinergic anti-inflammatory pathway and its effects in immune cells. The inability to incorporate CHRFAM7A in these studies due to novelty of the discovery and the experimental models is a notable limitation of this thesis study.

Nonetheless, modulating the migration of macrophages through the cholinergic anti-inflammatory pathway is a promising strategy in mitigating disease severity in multiple conditions like sepsis and atherosclerosis. We have demonstrated that α7nAChR is necessary for the efficient accumulation of macrophages in the lungs during endotoxemia. Additionally, we observed that stimulating α7nAChRs with a specific agonist before LPS injection increases macrophages in the lungs, and that α7nAChR deficiency is detrimental to survival. Down-regulation of αM in α7nAChR-/- macrophages at the cell surface and at the transcriptional level provides a potential mechanism for their reduced macrophage migration. To summarize, α7nAChR plays a role in the regulation of macrophage recruitment to inflamed tissues by changing the expression of cell adhesion molecule integrin αMβ2. Illuminating the intracellular signaling process connecting α7nAChR signaling to αMβ2 expression is a vital future study.

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