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Studies on the extra-neuronal cholinergic system in HIV-1 infection

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Résumé

L'acétylcholine (ACh) est un important neurotransmetteur qui est produit dans le système nerveux. Cependant, cette molécule est aussi produite par d'autres cellules non-neuronales du corps humain. Cette dernière est produite en abondance par les lymphocytes T CD4+, qui sont la cible principale du virus de l'immunodéficience humaine (VIH). ACh exerce ses effets sur les cellules par l'intermédiaire de ses récepteurs nicotiniques (n) et muscariniques (m) qui sont exprimés à la fois sur les cellules immunitaires et non immunitaires dans le corps. Il est bien connu que l'ACh a des effets anti inflammatoires sur les cellules immunitaires, et c'est le récepteur nicotinique qui est un joueur indispensable de cet effet. SLURP-1 (Secreted Ly6/uPAR-related Protein-1), est une autre molécule secrétée par les cellules T activées et d'autres cellules. Elle agit comme un ligand allostérique pour le récepteur α7, et module les effets de l'ACh sur les lymphocytes T. Il est peu connu comment ce système cholinergique extra-neuronal (ENCS) est régulé chez les individus infectés par le VIH.

Nos résultats démontrent que le taux d'ACh et de SLURP-1 en circulation ne change pas significativement chez les sujets infectés par le VIH comparé aux témoins sains. Cependant, le niveau de ces médiateurs est plus élevé chez les sujets infectés à long termes non progresseur (LTNP), qui contrôlaient la réplication virale, depuis plus que sept ans, sans aucune thérapie. Il est tentant de spéculer que le niveau élevé de ces deux composantes de l'ENCS peut jouer un rôle dans leur capacité à contrôler la réplication du VIH. Les résultats de cette étude montrent que l'agoniste du récepteur α7 diminue, et que l'antagoniste de ce même récepteur augmente la réplication virale *in vitro*, dans les cellules activées par le phytohaemagglutinin (PHA). En outre, l'hémicholinium (HC-3), un composé qui inhibe la capacité des cellules à produire ACh en compétition avec leur absorption de choline, augmente la réplication virale. L'expression du récepteur α7 sur les lymphocytes T CD4 + provenant du sang périphérique, mais pas sur les monocytes, était significativement réduite ($p < 0{,}01$) chez les individus infectés par le VIH, et elle n'a pas été entièrement restaurée par le traitement antirétrovirale (TAR). Tandis que l'expression du récepteur adrénergique β-2 a diminué significativement ($p < 0{,}01$) sur les monocytes et les lymphocytes T CD4 + chez des individus

infectés par le VIH. Ces cellules répondent à la norépinephrine via ce récepteur et l'ACh secrété.

Dans l'ensemble, les résultats cette étude suggèrent que le VIH provoque une modulation significative des différentes composantes de l'ENCS chez les individus infectés par le virus. Ce système pourrait être manipulé pour réduire la réplication virale et l'inflammation chez ces patients.

Mots-clés : Acétylcholine, Récepteur nicotinique α7, SLURP-1, VIH

Abstract

Acetylcholine (ACh) is an important neurotransmitter produced in the nervous system. However, the molecule is also produced by non-neuronal cells in the body. CD4+ T cells, the main targets of HIV-1, produce it abundantly. ACh exerts its effects on cells via its nicotinic (n) and muscarinic (m) receptors that are expressed on both immune and non-immune cells in the body. ACh is well known to exert anti-inflammatory effects on immune cells. The main receptor that is indispensable for the anti-inflammatory effects of ACh is the α7 nicotinic receptor. Another molecule, secreted by activated T cells and by other cells is SLURP-1 (Secreted Ly6/uPAR-related Protein-1), which acts as an allosteric ligand for α7 and fine tunes the effects of ACh on T cells. Little is known as to how this extra-neuronal cholinergic system (ENCS) is regulated in HIV-infected individuals.

Our results show that the circulating levels of ACh and SLURP-1 do not change significantly in HIV-infected individuals, as compared to the circulating levels in healthy controls. Interestingly, higher levels of these soluble mediators were detected in HIV-infected long-term non-progressors (LTNP) who control the viral replication for more than seven years without any chemotherapy. It is tempting to speculate that the increase in levels of these two soluble mediators of the ENCS present in HIV-infected LTNPs may play a role in their ability to control HIV replication. The results from this study show that an α7 agonist decreased HIV replication, whereas a receptor antagonist increased its replication *in vitro* in human PHA blasts. Furthermore, hemicholinium (HC-3), a compound that inhibits the ability of the cells to produce ACh, by competing with their uptake of choline, increases the viral replication. The expression of the α7 receptor on peripheral blood CD4+ T cells, but not on monocytes, was significantly reduced ($p<0.01$) in HIV-infected individuals, and it was not fully restored by antiretroviral therapy (ART). Interestingly, the expression of the β2 adrenergic receptor was decreased significantly ($p<0.01$) on both monocytes and CD4+ T cells in HIV-infected individuals. These cells respond to norepinephrine via this receptor and secrete ACh.

Overall, the results of this study suggest that HIV causes significant modulation of different components of the ENCS in virus-infected individuals. This system could be manipulated to reduce viral replication and inflammation in these patients.

Keywords: HIV, Acetylcholine, α7 nicotinic receptor, SLURP-1

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List of Abbreviations

3TC: Lamivudine, an antiretroviral medication used to prevent and treat HIV/AIDS

ABC: Abacavir

AcCo-A: Acetyl coenzyme A

ACh: Acetylcholine

AChE: Acetylcholinesterase

AKT: AK (mouse) strain Thymoma (other name: Protein kinase B/PKB)

ANS: Autonomic Nervous System

ART: Antiretroviral therapy

AZT: Azidothymidine (Zidovudine)

BuChE: Butyrylcholinesterse

Ca++/Ca2+: Calcium ions

CaM Kinase-II: Ca2+/calmodulin-dependent protein kinase II

CAP: Cholinergic anti-inflammatory pathway

cART: Combination anti-retroviral therapy

CCR5: C-C chemokine receptor type 5

cDNA: Complementary DNA

ChAT: Choline acetyltranferase

CHRFAM7A: CHRNA7-FAM7A fused gene

CHRM1: Cholinergic receptor M1

CHRM5: Cholinergic receptor M5

CHRNA7: Cholinergic receptor nicotinic α-7 (α7)

ChT: Choline transporter

CI: Chronically infected

CNS: Central nervous system

CREB: cAMP response element-binding protein

CTL: CD8+ T lymphocytes

CXCL: chemokine (C-X-C motif) ligand

d4T: Stavudine

DC: Dendritic cells

ddC: Zalcitabine (2′-3′-dideoxycytidine), also called dideoxycytidine, a nucleoside analog reverse transcriptase inhibitor (NRTI) sold under the trade name Hivid

ddI: Didanosine, marketed under trade name Videx, used to treat HIV/AIDS

ddN: 2',3'dideoxynucleoside

DMN: Dorsal motor nucleus

Dup-α7: Duplicated α7 (another name for CHRFAM-7α)

ENCS: Extraneuronal cholinergic system

ENV: HIV envelope glycoprotein

FAM-7A: Family with sequence similarity-7α

Gag: Group-specific antigen, coding for structural proteins

GALT: Gut-associated lymphoid tissue

GI tract: Gastro-intestinal tract

GPCR: G-protein coupled receptor

GPI: Glycophosphatidylinositol

GSK-3: Glycogen synthase kinase-3

HAART: Highly aggressive anti-retroviral therapy

HC: Healthy controls

HC-3: Hemicholinium 3

HIV-1: Human immunodeficiency virus type 1

HIV-2: Human immunodeficiency virus type 2

HMGB-1: High mobility group protein B-1

HMOX1: Heme oxygenase-1; also abbreviated as HO-1

HPA: Hypothalamus-pituitary-adrenal axis

IBD: Inflammatory bowel disease

IgG1: Immunoglobulin G1

IL: Interleukin

INI: Integrate inhibitors

IR: Inflammatory reflex

JAK-2: Janus kinase-2

K+: Potassium ion

LAG-3: Lymphocyte-activation gene-3

LPS: Lipopolysaccharide

LTNP: Long-term non progressor

mAChRs: Muscarinic acetylcholine receptor

MDM: Mal de Meleda

Na+: Sodium ion

nAChRs: nicotinic acetylcholine receptor

ND: Non-detectable

NEF: Negative Regulatory Factor

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NFE2L-2: Nuclear factor erythroid-derived-2-like-2

NLR: Nod-like receptors

NNTRI: Non-nucleotide reverse transcriptase inhibitors

NOD: nucleotide-binding oligomerization domain-like receptors

NOR: Noradrenaline

Nrf2: Another abbreviation for NFE2L-2

NRTI: Nucleotide reverse transcriptase inhibitors

NTS: Nucleus tractus solitaries

PBMC: Peripheral blood mononuclear cells

PD-1: Programmed cell death-1

pDC: Plasmacytoid dendritic cells

PHA: Phytohaemagglutinin

PHI: Primary HIV infection

PI: Protease inhibitor

PKA: Protein kinase A

PKC: Protein kinase C

PNS: Parasympathetic nervous system

Pol: DNA polymerase

PR: Protease

PRR: Pattern recognition receptors

REV: Regulator of expression of viral proteins

RLH: RIG-like helicases

RT: Reverse-transcriptase

RTI: Reverse-transcriptase inhibitor

SIV: Simian immunodeficiency virus

SLC5A7: Solute carrier family 5 member 7

SLURP-1: Secreted mammalian Ly6/uPAR-related peptide-1

SNS: Sympathetic nervous system

STAT-3: Signal transducer and activator of transcription-3

SU: Surface unit

TAT: Trans-activator protein

TH: T helper

TIM-3: T cell immunoglobulin and mucin-domain-3

TLR: Toll-like receptors

TM: Transmembrane

TNF-α: Tumor necrosis factor alpha

ULK-4: Unc51-like kinase-4

uPAR: Urokinase type plasminogen activator receptor

Vif: Viral infectivity factor

VIH: Virus de l’immunodéficience humaine

Vpr: Viral protein R

Vpu: Viral protein unique

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1 Introduction & Review of Literature

The project for my Masters' thesis was aimed at investigating modulation of the extra neuronal cholinergic system (ENCS) in HIV-1 (hereafter referred to as HIV) infections.

Therefore, a brief introduction of the ENCS and HIV infection is provided.

1.1 Extra-Neuronal Cholinergic System

It is a part of the cholinergic system, which works within and outside the nervous system. The system is based upon the production and release of a neuro-endocrine transmitter called acetylcholine (ACh), a low molecular weight chemical (see Figure 1 for structure of ACh) and the first identified neurotransmitter in the brain (Gando et al 2001, Kawashima et al 2015). The transmitter is needed to conduct nerve impulses in the central and peripheral nervous tissues. The ACh released by the neuronal cholinergic system controls autonomic, cognitive, and motor functions in the body.

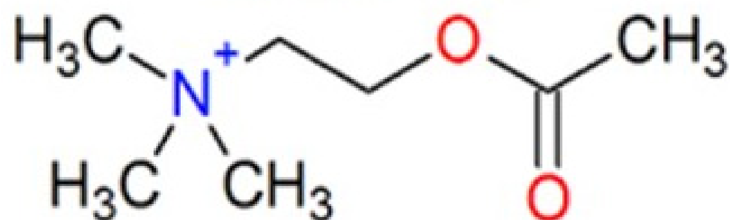


Figure 1. Chemical structure of Acetylcholine (ACh)

Acetylcholine (2-Acetoxy-N,N,N-trimethylethanaminium) is an organic compound that functions as a neurotransmitter, using chemicals released by nerve cells to send signals to other cells.

The extra-neuronal cholinergic system comprises ACh produced by non-neuronal cells and regulates a wide variety of cell functions outside the nervous system. The non-neuronal cholinergic system plays an important role in modulating inflammation and immune response in the body (Kawashima et al 2012). Large varieties of non-neuronal cell types in the body produce ACh and express ACh-specific receptors. ACh acts as a cytotransmitter and acts in both autocrine and paracrine manners. Interestingly, human and mouse CD4+ T cells abundantly produce ACh upon activation (Rinner et al 1998, Rosas-Ballina et al 2011; reviewed in Kawashima & Fujii 2004).

1.1.1 Synthesis of Acetylcholine

ACh is an evolutionarily conserved molecule and its existence predates the development of the nervous system in living organisms (Zoheir et al 2012). Almost every cell in the body of all living organisms (including plants) produces ACh to a variable extent. ACh is synthesized in body cells from choline and acetyl coenzyme A (AcCo-A). The enzyme choline acetyltransferase (ChAT) catalyzes this synthesis (Figure 2). Choline is mainly produced in the liver and is also absorbed by the gut from dietary constituents. Cells uptake choline free and phospholipid (phosphocholine) forms from the circulation, via a high-affinity choline-specific cell surface expressed transporter, Choline Transporter (ChT), which is also known as the solute carrier family 5 member 7 (SLC5A7; Kawashima et al 2004). Hemicholinium-3 (HC-3) is a competitive inhibitor of choline uptake by ChT. HC-3 is used in experiments to deplete ACh in cells and in tissues. Phosphocholine is hydrolysed inside cells before being used for synthesis of ACh. AcCo-A is produced in the cytosol of the mitochondria during the Kreb's cycle and is transported across the mitochondrial membranes into the cytoplasm where it is used for production of ACh (Tucek 1990).

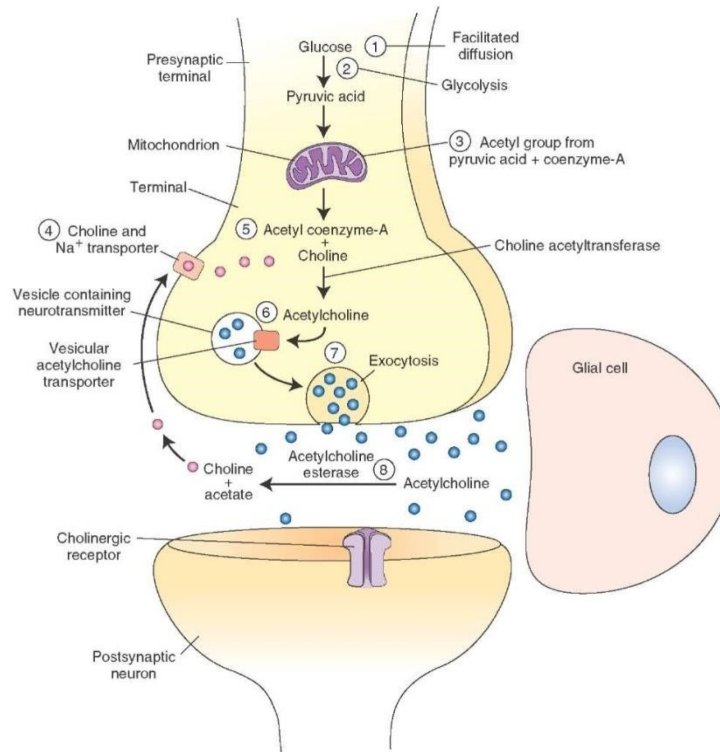


Figure 2. Synthesis of Acetylcholine in neuronal cells

This figure depicts how acetylcholine is synthesized within neuronal cells from choline and acetyl coenzyme A, and is degraded in the extracellular synaptic cleft by the enzyme Acetycholinesterase. (Accessed on 14 June 2016; Permission to use granted from: http://what-when-how.com/ neuroscience/ neurotransmitters-the-neuron-part-2/).

The bioavailability of choline and AcCo-A limits the synthesis of ACh. ACh, as a negative feedback mechanism, binds and inhibits ChAT. Once synthesized inside cells, ACh is packaged into vesicles. These vesicles are derived from the Golgi apparatus and store ACh. Each vesicle may store up to 10,000 molecules of ACh. The vesicles are stored in the axonal termini of neuronal cells (Tucek 1990). Upon a proper stimulus, ACh is rapidly released from the nerve termini into the synaptic clefts. The released ACh binds specific receptors on post-synaptic neuronal dendrites and propagates the nerve impulse across neuronal junctions. ACh also conducts impulses across neuro-muscular junctions. The release of ACh is rapidly metabolized into choline and acetate by the enzyme acetylcholinesterase (AcChE) in neuronal tissues and by butyrylcholinerase (BuChE) in non-neuronal tissues (Picciotto et al 2012). The

BuChE enzyme is mainly produced in the liver. Choline released from the action of these enzymes is transported back into the cells and is re-used. The activity of both of these enzymes is markedly increased during inflammation and these enzymes have often been used as markers for chronic inflammatory conditions such as Alzheimers' disease, type II diabetes and obesity (Das 2007). The levels of acetylcholine in the circulation are normally decreased in chronic inflammatory diseases. Not surprisingly, reversible AChE inhibitors are used for the treatment of some brain diseases such as Alzheimer's (Ohta et al 2017). Non-reversible AcChE inhibitors are fatal and are used as insecticides, pesticides and warfare agents (Colovic et al 2013).

1.1.2 ACh Receptors

ACh exerts its biological effects via two groups of receptors: nicotinic (n) and muscarinic (m). These receptors differ from each other with respect to their structures and functions (Kawashima et al 2012, Kruse et al 2014, Hurst et al 2013). The nicotinic acetylcholine receptors (nAChRs) are ionotropic and act as ligand-gated ion channels and the nAChRs are designed to transmit impulses across synaptic clefts fast (in milliseconds). Upon binding of ACh or nicotine, the receptors cause an influx of calcium ions (Ca++) and sodium ions (Na+) and an efflux of potassium ions (K+), leading to activation of protein kinase C (PKC) and Ca2+/calmodulin-dependent protein kinase II (CaM Kinase-II). In parallel, they also activate Janus kinase II (JAK-2) independent of the Ca++ and Na+ influx. The two pathways induce activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Chernyavsky et al 2010). The nicotinic receptors in vertebrates could be of neuronal or of muscular type. The neuronal types are built from nine α (α2-10) and three β (β2-4) subunits (Figure 3). They could be homo- or hetero-oligomers. For example, α7 nicotinic AChR is made from five α7 subunits, and α4β2 is a hetero-pentamer made from three α4 and two β2 subunits (Gotti & Clementi 2004). The nAChRs are present in the central and peripheral nervous tissues and transmit motor signals from the presynaptic to the postsynaptic cells within autonomic nervous system. The muscle type nAChRs in adults are made from α1, β1, δ, and ε subunits in a 2:1:1:1 ratio. They are located in the neuromuscular junctions and cause contractions of skeletal muscles. Several drugs such as curare, hexamethonium, and α-bungarotoxin specifically block these receptors. Unlike nicotinic receptors, mAChRs are metabotropic and associate with G-

proteins, thus are G protein-coupled receptors (GPCR). The mAChRs mediate many of the effects of acetylcholine in the central and peripheral nervous systems.

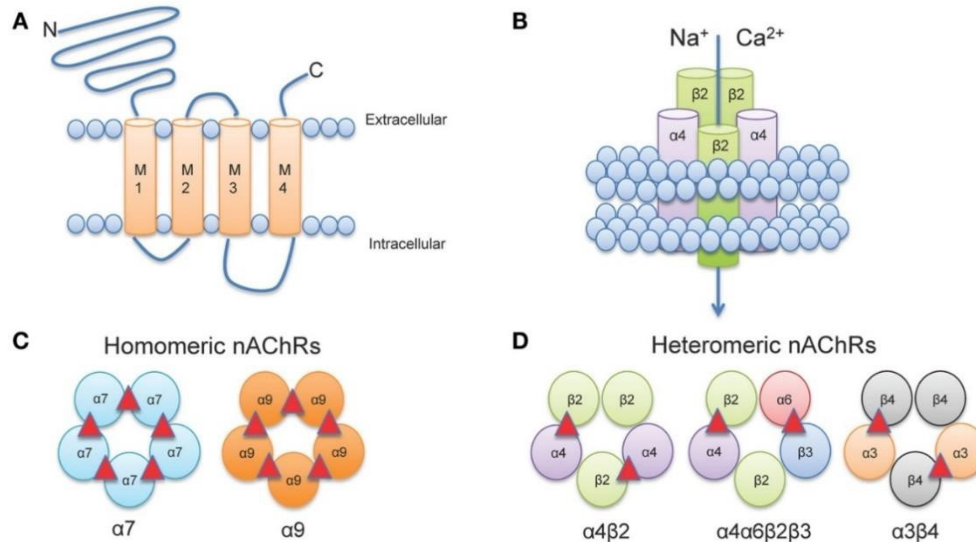


Figure 3. The Structure of different nicotinic Acetylcholine receptors

(A) Schematic representation of a neuronal nAChR subunit in the plasma membrane. Each nAChR subunit comprises four transmembrane domains (designated as M1-M4) and extracellular amino- and carboxy-termini with M3-M4 intracellular loops of variable length. (B) Assembly of five subunits forming a functional receptor. (C) Functional homomeric receptors comprised of five α7, α9, or α10 (not shown) subunits. (D) Most high affinity nAChRs are heteromeric and are made up of a combination of α and β subunits and usually show high affinity for ACh. Heteromeric receptors may comprise more than two types of sub-units, e.g. the α4α6β3β2 receptor shown here. The red triangles represent ACh binding sites (Permission to use granted by Hendrickson et al 2013).

Muscarinic acetylcholine receptors (mAChRs) are mainly located in the sympathetic nervous system and at the neuromuscular junctions of cardiac and smooth muscles. Five different mAChR, named M1-M5, have been described. Although these receptors show significant sequence homologies, they differ in their preferences for associations with different G-proteins. M1, M3, and M5 associate with Gq/11, whereas M2 and M4 associate with Gi/o

proteins (Kawashima et al 2012, Kruse et al 2014). Atropine and scopolamine are used for blocking these receptors (Lochner & Thompson 2016). Several small chemically defined molecules have been discovered that act as specific agonists or antagonists for different n- or m-type AChRs. They have been used in a variety of human diseases such as Alzheimer's, Sjogren's syndrome, cancer, and psychosocial disorders, wherein they work by improving neurotransmission and by reducing inflammation (Mudo et al 2007, Greig et al 2013).

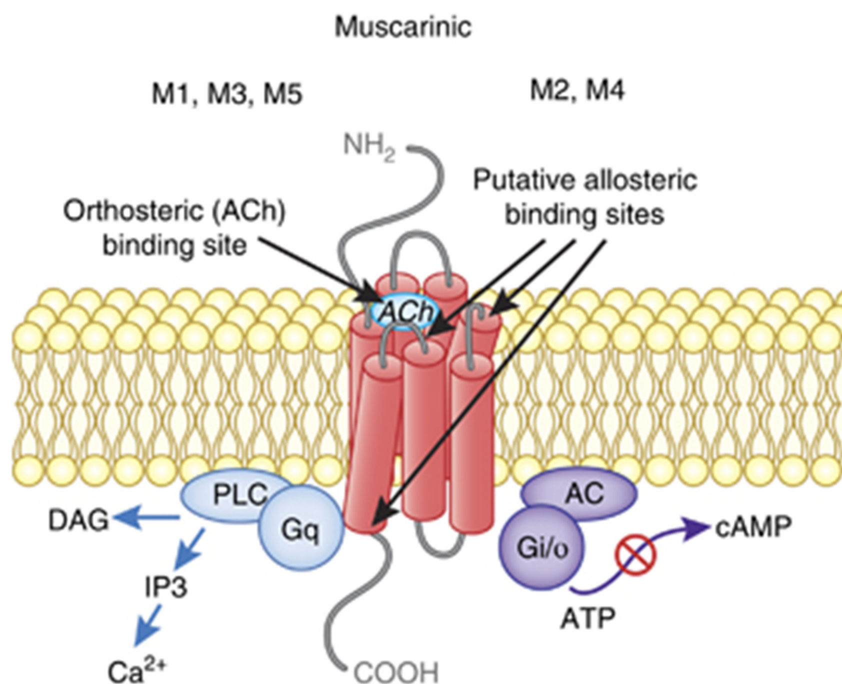


Figure 4. Structures of different muscarinic Acetylcholine receptors

This figure shows structures of M1-M5 muscarinic acetylcholine receptors, and their associated G-proteins. These receptors mediate the many actions of ACh in the central nervous system, as well as throughout non-nervous tissues. (Permission to use granted by Nature Publishing Group, Jones et al 2011).

1.1.3 Acetylcholine as a mediator of Anti-Inflammatory Reflex

The immune and the neuro-endocrine systems cross talk with each other and maintain homeostasis via the neuro-endocrine-immune axis. Several neural circuits function reflexively and achieve this homeostasis (Rosas-Ballina & Tracey 2009, Anderson et al 2012, Olofsson et al 2012). The best understood of these circuits is the anti-inflammatory reflex. It is also called the cholinergic anti-inflammatory pathway (CAP), named so as ACh plays the role of an essential effector molecule in the function of this pathway. The pathway works reflexively in response to increased concentrations of pro-inflammatory mediators (Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α) in the circulation. The reflex works as a component of the autonomic nervous system.

1.1.4 The Autonomic Nervous System

The CAP functions as a component of the Autonomic Nervous System (ANS), which maintains internal homeostasis of the body by regulating involuntary activities such as cardiovascular, urogenital, gastrointestinal, thermoregulatory and exocrine, systems (Vinik et al 2011, Schwartz & De Ferrari 2011, Vinik 2012). The ANS comprises two distinct functional and anatomical components: Sympathetic Nervous System (SNS) and Parasympathetic Nervous Systems (PNS; Figure 5). The two systems function synchronously, as well as independently from each other. The activities of the two systems often, but not always, oppose and cross regulate one another.

The SNS becomes active during fear, stress, and life threatening situations resulting in an increased heart rate, a rise in blood pressure, sweating, bronchodilation and piloerection. The PNS opposes these actions and tends to maintain the "rest and digest" functions. For example, it slows heart rate, increases peristaltic movements and gastric secretions, induces broncho-constriction and causes relaxation of the urinary bladder and GI tract sphincters.

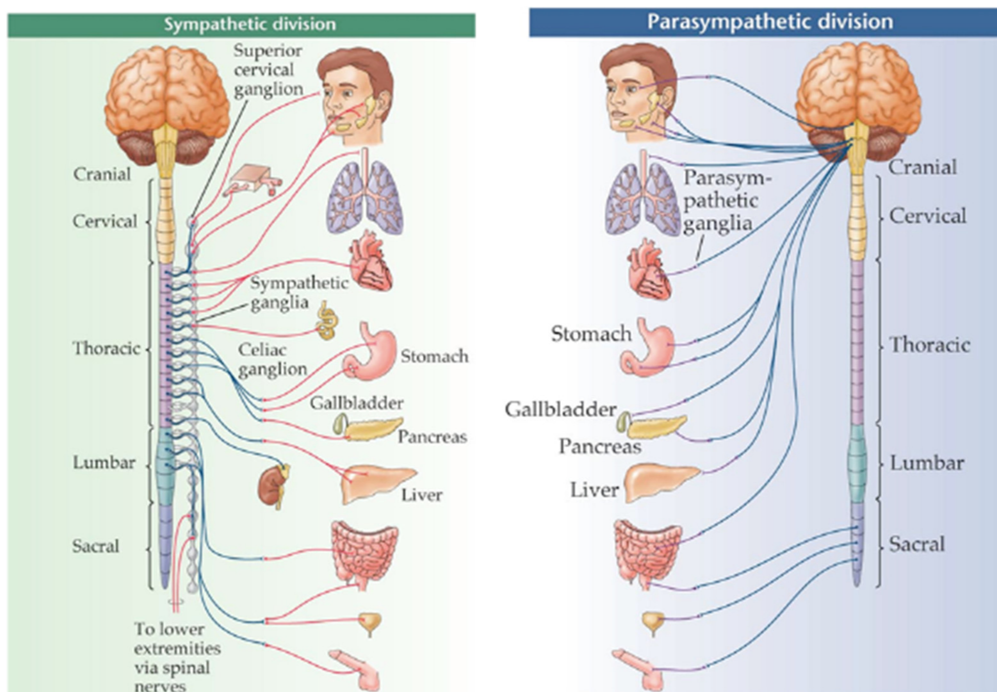


Figure 5. Structural anatomy of the Autonomic Nervous System

This figure shows the sympathetic and parasympathetic components of the autonomic nervous system. The Sympathetic Nervous System is a short-acting system that originates from the thoracic and lumber spinal cord. It redirects blood flow to improve performance. The Parasympathetic Nervous System stems from the cranial nerves and the sacral spinal cord. It is responsible for maintaining the stability of normal body functions and handling energy acquisition and storage. (Permission to use granted by Bethopedia, Wikidot Inc. 2016)

The pre-ganglionic nerve fibers of the SNS originate from thoracic and lumbar regions of the spinal cord (Figure 5). They travel to para- and pre-vertebral ganglia, a collection of neurons outside the CNS (Elenkov et al 2000, Schwartz & De Ferrari 2011, Vinik 2011). They have relatively smaller pre-ganglionic fibers and longer post-ganglionic fibers, which supply the organs (Figure 6). They mainly secrete norepinephrine (NOR) and/or dopamine, with the exception of sweat glands and adrenal medulla, where they secrete ACh.

The PNS preganglionic fibers originate from cranial nuclei in the brain stem and in the sacral regions of the spinal cord (Figure 5). They are relatively long and travel within cranial and spinal nerves to the ganglia. The ganglia of the PNS are located close to or within the innervated organs. As a result, postganglionic fibers are relatively short compared with those of

the SNS (Figure 6; Vinik et al 2011, Olofsson et al 2012, Vinik 2012). The pre-ganglionic fibers of both the PNS and SNS secrete ACh at their termini, while the post-ganglionic fibers of the PNS and SNS secrete ACh and NOR respectively. Based upon the secreted neuro-transmitter, the nerve fibers are also called cholinergic (ACh) or adrenergic (NOR).

The main nerve of PNS is the vagus nerve, also known as the 10th cranial nerve (Figure 5). It supplies parasympathetic innervation to visceral organs present in the neck, thorax, and abdomen. The organs responding to the vagus nerve include the larynx, lungs, heart, esophagus, stomach, small intestine, colon, pancreas, gall bladder and blood vessels. The nerve provides structural and functional bases of the CAP (Van Der Zanden et al 2009, Pavlov et al 2012). For this reason, the words parasympathetic and vagal activities are used interchangeably.

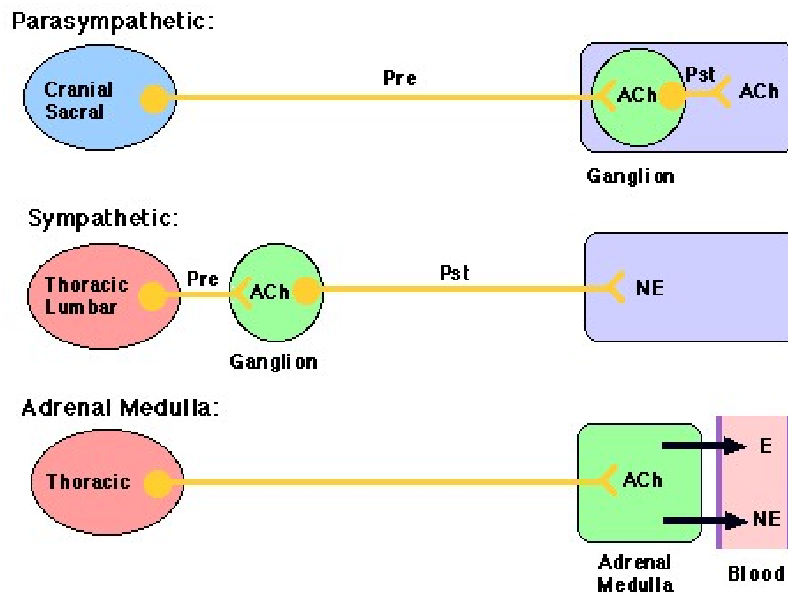


Figure 6. Preganglionic and postganglionic nerve fibers and secreted neurotransmitters of the Autonomic Nervous System

This figure illustrates various secreted neurotransmitters in the parasympathetic, sympathetic, and adrenal pathways. Pre and Pst refer to preganglionic and postganglionic fibers, whereas E and NE refer to epinepharine and norepinepharine respectively. (Permission to use granted by Nature Publishing Group, Tracey 2002).

1.1.5 Activation of the Cholinergic Anti-Inflammatory Pathway (CAP)

CAP reflex occurs in response to increased concentrations of pro-inflammatory mediators in the circulation (Huston 2012, Pavlov & Tracey 2015). These mediators include IL-1β, TNF-α, IL-6, High mobility group protein B (HMGB)-1, prostaglandins and microbial products (e.g. lipopolysaccharide (LPS)). The sensory (afferent) nerve fibers of the vagus nerve arise from neurons whose cell bodies lie in the nodose and jugular ganglia. The neurons are bipolar with one projection ending in the targeted organs and the other in the Nucleus Tractus Solitarius (NTS) in the brain stem (Valentin et al 2012; Figure 7). The sensory vagal fibers express receptors for proinflammatory cytokines, different metabolites and microbial products. Furthermore, vagal nerves endings in organs and tissues lie in close proximity to dendritic cells (DCs) and macrophages. When these cells become activated, they secrete proinflammatory cytokines and stimulate sensory fibers of the vagus nerve (Goehler et al 1999; Olofsson et al 2012). The sensory vagal nerves transmit signals to the nuclei present in the brain stem (e.g. the NTS), which is connected to the Dorsal Motor Nucleus (DMN) of the vagus. The motor vagal fibers originate from the DMN of the vagus. They innervate end organs and project to the hypothalamus and other nuclei in the CNS nuclei, and regulate activity of the Hypothalamus-Pituitary-Adrenal (HPA) axis. Thus, the activation of sensory vagal fibers results in activation of the vagal motor nerves as well as of the HPA axis reflexively.

Activation of the HPA results in the release of glucocorticoids from the adrenal glands. The activation of vagal motor fibers activates CAP, which prevents the production of proinflammatory cytokines from macrophages and DCs. The mechanism of action of the CAP was discovered by in 2000 by Tracey' group (Borovikova et al 2000). Studies have demonstrated that vagal stimulation attenuates systemic proinflammatory response to LPS in mice. In this work, the authors proposed the existence of an inflammatory reflex (IR), also known as CAP (Tracey 2002). Studies have revealed an essential role played by the spleen in the functioning of the inflammatory reflex, as vagal stimulation fails to inhibit the production of TNF-α in splenectomized mice (Huston et al 2006). This posed a conundrum, as the spleen is not directly innervated by any vagal motor nerve fibers. Rather, the spleen is innervated by the noradrenergic splenic nerve, which arises from the celiac plexus (Figure 8). Previous works showed that the activation of the vagus activates noradrenergic neurons present in the celiac ganglion and

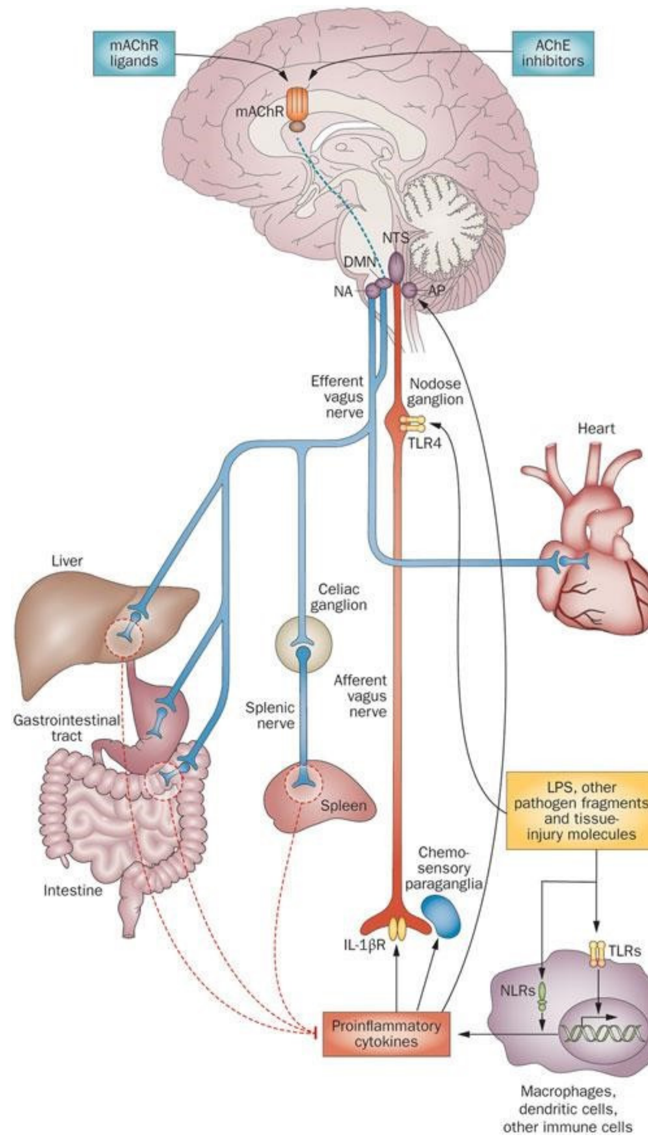


Figure 7. Anatomy of the Inflammatory Reflex or Cholinergic Anti-Inflammatory Pathway (CAP)

This figure illustrates the neural and humoral pathways in which cells release cytokines that modulate the immune system. Immunomodulation of the central nervous system is achieved by the cholinergic anti-inflammatory pathway, HPA axis, and sympathetic nervous system. Activation of the sensory vagal fibres results in activation of the vagal motor nerves, as well as of the HPA axis. (Permission to use granted by Nature Publishing Group, Pavlov and Tracey 2012).

induces the release of NOR in the spleen (Rosas-Ballina et al 2011). In addition, the release of NOR in the spleen activates a population of β2 adrenergic receptor-expressing CD4+CD25-CD44 high and CD62L low T cells. These receptors lie in close proximity to

splenic nerve endings in the T cell-rich zones of the organ (Rosas-Ballina et al 2011). Upon activation by NOR, CD4+ T cells synthesize ACh, which inhibits the production of TNF-α and other proinflammatory mediators from neighboring monocytes, macrophages, B cells and DCs. The cells responding to ACh express a nicotinic AChR called α-7. These findings were supported by another group (Vida et al 2011). The ACh-producing CD4+CD25- T cells represent a population of regulatory T cells (Tregs) that are different from classical Tregs. Unlike ChAT+ T cells, classical Tregs express FoxP3 and CD25 (Vida et al 2011, Peña et al 2011). The ChAT+ CD4+ CD25- T cells, that function as effectors for CAP, also express high levels of choline transporter (ChT)-1 encoded by the solute carrier family 5 member 7 (SLC5A7) gene (Kawashima & Fujii, 2004). However, they do not store ACh. During vagal stimulation, ACh is released from postganglionic nerve endings (Figure 5 and 6), which are located within or very close to the innervated organs, e.g., intestines. The released ACh acts on neighboring immune and non-immune cells to regulate their functions.

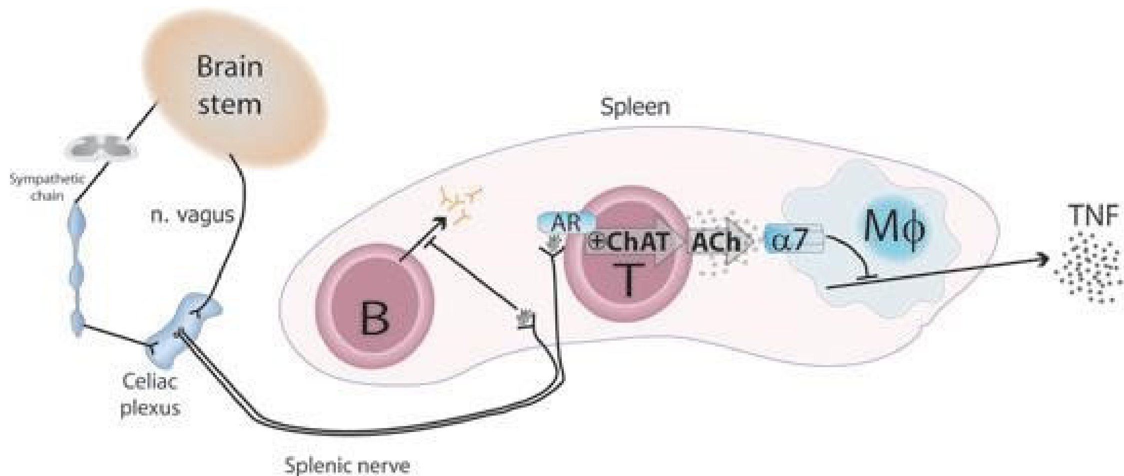


Figure 8. A model of functioning of the Cholinergic Anti-Inflammatory Pathway

The model of the cholinergic anti-inflammatory pathway, proposed by the Tracey's group, postulates that sensory or afferent signals from the brain stem travel through the vagus nerve to different nuclei in brain. The efferent vagus reflexively activates the splenic nerve arising in the celiac plexus and innervates the spleen. Splenic nerve endings release nor-adrenaline, which activates closely lying choline acetyltransferase (ChAT)-positive T and B cells. Upon activation, these cells produce and release ACh. Released ACh signals macrophages and other immune cells through the α7 nicotinic acetylcholine receptor and suppresses the production of TNF-α and other proinflammatory cytokines (Permission to use granted by PubMed Central, Olofsson et al 2012).

The model of CAP proposed by Tracey's group (Pavlov & Tracey 2015) has been challenged, as no synaptic connections could be found between postganglionic parasympathetic (vagal) nerve fibers and preganglionic sympathetic neurons that project to spleen (Martelli et al 2016). A newly proposed model suggests that the efferent arm of the anti-inflammatory reflex travels in greater splanchnic sympathetic nerves, and not via vagal efferent nerves. The postganglionic sympathetic nerve fibers release NOR that inhibits the production of proinflammatory cytokines from macrophages via β2 adrenergic receptors. The released NOR also induces ACh from T cells. Furthermore, a direct stimulation of the vagus also has an anti-inflammatory effect. It does so by mobilizing ACh-producing T cells from intestinal lymphoid tissues. The mobilized ACh-producing T cells migrate to the spleen and probably to other lymphoid tissues to reduce inflammation. In this model, ACh released from T cells can also stimulate the sympathetic nerve terminals via the α7AChR (Martelli et al 2016).

1.1.6 Extra-Neuronal Acetylcholine and its biological effects

As mentioned above, ACh is an evolutionarily conserved molecule that is also produced by non-neuronal cells in the body. Morris was the first researcher to report the synthesis of ACh in the human placenta (Morris 1966). Since then, several studies have reported the production or the presence of ACh in various cell types, including vascular endothelial cells (Parnavelas et al 1985), keratinocytes (Zia et al 2000), epithelial cells (Nguyen et al 2000), T lymphocytes (Fujii et al 1996) and B cells (Arredondo et al 2009). There is overwhelming evidence suggesting that extraneuronal ACh is a ubiquitous cell signalling molecule that plays an important role in the homeostasis of a variety of cell functions, such as proliferation, differentiation, cell-to-cell contact, secretion and absorption in non-neuronal cells (Grando et al 2003).

The extraneuronal cholinergic system plays an important role in the regulation of immune responses, both innate and adaptive. ACh is produced by several types of immune cells including T cells, DCs, and B cells (Fujii & Kawashima 2001, Zdanowski et al 2015). However, the role of ACh has been investigated more extensively in lymphocytes, especially in CD4+ T cells. CD4+ T cells express all components of the cholinergic nervous system,

including ChAT, ACh, as well as various types of cholinergic receptors. The ACh synthesized in lymphocytes is not stored inside the cell but rather is continuously secreted via a special transporter called mediatophore (Fujii et al 2012; Figure 9). ACh acts as an immune modulator and regulates immune cell functions independently of the neuronal cholinergic system. It can function both in an autocrine and a paracrine fashion.

The biological effects of ACh depend on the expression of AChR on target cells. As mentioned above, there exist muscarinic and nicotinic AChR. Several studies have demonstrated the existence of both muscarinic and nicotinic type AChR on human and murine immune cells, including lymphocytes (Costa et al 1995, Fujino et al 1997 and Sato et al 1999).

Sato et al (1999) demonstrated the expression of mRNAs encoding α2, α5, and α7 subunits in human mononuclear cells, while there was no expression of α1, β1, and ε (the skeletal muscle type subunits) in these cells. On the other hand, the expression of α1 subunits was reported in human thymocytes (Wakkach et al 1999). Non-neuronal immune cells also express different types of muscarinic receptors. The stimulation of these receptors induces cell proliferation and the production of proinflammatory cytokines like IL-6 (Wessler & Kirkpatrick 2008). The production of proinflammatory cytokines and antigen-specific immunoglobulin G1 (IgG1) is markedly reduced in mice lacking the Cholinergic Receptor M-1 (CHRM1) and CHRM5 genes, which encode receptors M1 and M5 respectively (Fujii et al 2007b).

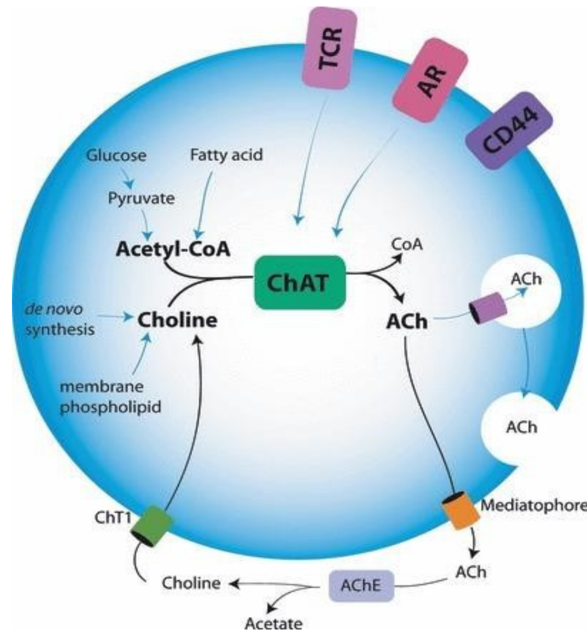


Figure 9. Synthesis and release of acetylcholine in lymphocytes

Lymphocytes express the enzyme ChAT, which catalyzes synthesis ACh from Acetyl-CoA and choline. Acetyl-CoA is produced from glucose and fatty acids through glycolysis and beta-oxidation respectively. Choline comes from different sources. It can be taken up by cells through ChT1 or is synthesized de novo inside cells. T cells, unlike neurons, do not store ACh in vesicles. It is continuously released from the cells upon their activation through mediatophore by as yet unknown mechanism. Black and blue arrows indicate known and unknown processes respectively. (Permission to use granted by PubMed Central, Olofsson et al 2012).

The most significant of the nicotinic acetylcholine receptors studied with regard to the immune system is the α7 nicotinic acetylcholine receptor (α7nAChR). It is a homo-oligomeric receptor made up of five α7 subunits. In addition to T cells, the receptor is also expressed by monocytes, macrophages, DCs, B cells, mast cells, endothelial cells, epithelial cells and keratinocytes. When this receptor is stimulated, it causes an influx of Ca++ and an efflux of K+ from the cells. The Ca++ influx leads to activation of CaM Kinase II and PKC. Recent studies have shown that the receptor activation also leads to metabotropic effects. This involves activation of JAK-2 and signal transducer and activator of transcription-3 (STAT-3); the inhibition of cAMP response element-binding protein (CREB) phosphorylation and glycogen synthase kinase-3 (GSK-3); and the nuclear translocation of nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) induced by AK (mouse) strain thymoma (AKT) activation (Figure 10; Kalkman & Feuerbach 2016).

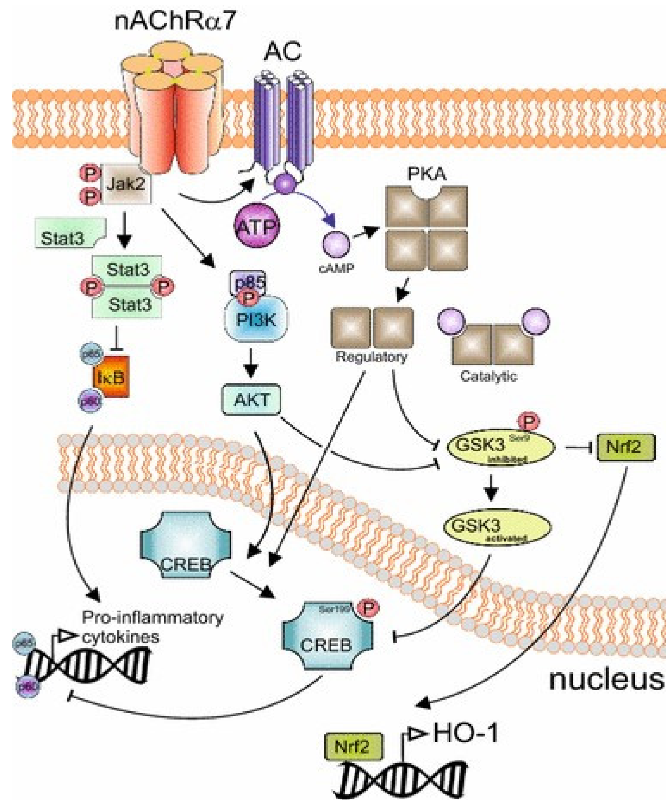


Figure 10. Signaling cascades activated by ligand-induced activation of α7nAChR

This image describes the anti-inflammatory signaling pathways activated by the α7nAChR. Upon binding ACh, the receptor activates Jak2, which leads to the inhibition of NF-kB and GSK3, and to the activation of CREB. (Permission to use granted by http://creativecommons.org/licenses/by/4.0/; originally published in Kalkman & Feuerbach 2016)

The biological effects of α7nAChR depend on the cell type: they are anti-inflammatory on B cells, macrophages, and mast cells; proinflammatory on lymphocytes and epithelial cells; and variable (pro- or antiinflammatory) for monocytes and neutrophils (Hallquist et al 2000, Aicher et al 2003; Arredondo et al 2009, Zdanowski et al 2015). The α7nACh receptor is required to mediate cytokine suppressive effects of the CAP and vagal stimulation. KO mice for the CHRNA7 gene, which encodes α7nAChR, produce abundant amounts of proinflammatory cytokines and IgG1 antibodies (Fujii et al 2007a). These effects are opposite to those observed

in CHRM1 and CHRM5 gene KO mice (Fujii et al 2007b). Another distinct CHRNA7-related gene, CHRFAM7A, has been described in humans and is located on the same chromosome (15q13.3) about 1.6 megabase upstream to CHRNA7 (Riley et al 2002; Costantini et al 2015a). The expression of the two genes is regulated independent from each other. The CHRFAM7A gene comprises exons 5-10 of the CHRNA7 gene with exons A-E from another partially duplicated gene, ULK-4 (Unc51-like kinase-4). The CHRFAM7A gene encodes the α7 subunit (called duplicated or du-α7) that does not bind ACh or nicotine. It acts as a negative regulator of α7. The dup-α7 is expressed relatively more (compared to α7) on non-neuronal cells. The two genes are expressed in different ratios in human leukocytes. The α-7 and dup-α7 subunits may combine in different ratios to form novel nicotinic receptors, which may dampen ACh-mediated anti-inflammatory effects (Costantini et al 2015b; Figure 11). Interestingly, an increase in the ratio between the expressions of CHRFAM7A and CHRNA7 genes occurs in the colons of patients with Inflammatory Bowel Disease (IBD; Baird et al 2016).

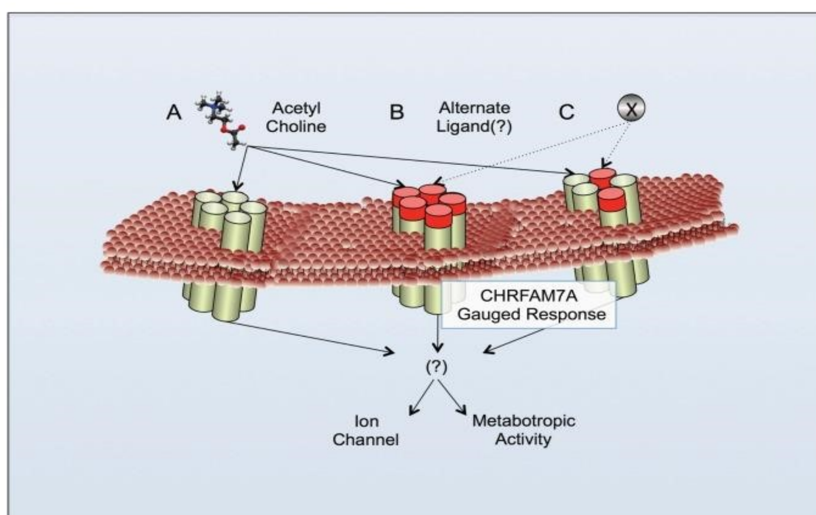


Figure 11. Expression of α7nAChR and its variant Dup-α7nAChR in human cells

A partially duplicated CHRNA7 gene has been discovered in humans. It is named CHRFAM7A and encodes duplicated (dup)-α7. It lacks ACh binding site. The α7 and dup-α7 can each form homopentameric receptors, which are responsive and unresponsive to ACh, respectively. They are shown as A and B in this Figure, respectively. The two subunits can also combine in different proportions to form AChR (C) with reduced responsiveness to ACh. The incorporation of dup-α7 units into the receptor decreases its responsiveness to ACh. The relative expression of CHRNA7 and CHRFAM7 in immune cells can regulate their responses to ACh and hence inflammation. (Permission to use granted by Copyright 2015, The Feinstein Institute for Medical Research; originally published in Costantini et al 2015b).

1.1.7 ACh-mediated regulation of T cell activation: Role of SLURP-1

The release of ACh upon T cell activation is an important aspect of the extra-neuronal cholinergic system. This activation may be polyclonal, T cell receptor (TCR)-mediated, or via another molecule such as CD44 (Kawashima & Fujii 2004, Zdanowski et al 2015). The activation of different T cell lines, as well as of peripheral blood mononuclear cells (PBMCs) with phytohaemagglutinin (PHA), results in the release of ACh from these cells and its detection in the blood (Fujii et al 1998, Fujii & Kawashima 2001). This circulating ACh not only inhibits the production of proinflammatory cytokines, it also modulates proliferation and differentiation of cells. Differentiation depends on the target cell's ACh-specific expression profile. Upon activation, T cells increase the expression of the α7nAChR, ChAT, ACh, and AChE. In addition, activated T cells also release SLURP-1 (Kawashima et al 2012). SLURP-1 stands for the secreted mammalian Ly6/uPAR (the urokinase type plasminogen activator receptor)-related peptide-1. The SLURP1 gene is located on the long arm (q) of chromosome 8 at position 24.3.

SLURP-1 belongs to the Ly-6/uPAR superfamily of proteins. The family members play diverse roles in signaling, cell proliferation, differentiation and adhesion. There are sixty members of the family, including Ly6/uPAR and CD59. The members may be GPI-anchored to the plasma membrane or secreted. SLURP-1 has no GPI anchor and is secreted. It was discovered in 1999 in patients with the Mal de Meleda (MDM) disease, exhibiting a loss of function mutation (Adermann et al 1999). MDM is a genetic disease resulting from autosomal recessive mutations in the SLURP-1 gene. MDM patients show transgressive palmo-planter keratosis with erythematous borders, perioral erythema, brachydactyly, and nail abnormalities (Tjiu et al 2011; Figure 12). The keratosis in the disease may progress to dorsal surfaces of the hands and feet. The mutations deregulate epidermal cell homeostasis and leads to their enhanced proliferation and differentiation. The prevalence of the disease is 1 case per 100,000 in the population. It is so named, as it was first found and described in patients living in the Adriatic Island of Meleda (Neumann 1898; Perez & Khachemoune 2016).

SLURP-1 acts as a positive allosteric ligand for α7nAChR in T cells, keratinocytes and DCs (Chimenti et al 2003, Fuji et al 2014). SLURP-1 potentiates the effects of ACh on T

lymphocytes and other cells such as keratinocytes, epithelial cells and DC. During T cell activation, ACh and SLURP-1 binding to the α7nAChR fine-tunes T cell activation. Mutations arising in the gene encoding SLURP-1 have been shown to impair T cell activation (Tjiu et al 2011). Recombinant SLURP-1 affects the cholinergic pathway in three ways; first, it attenuates peripheral blood mononuclear cell proliferation; secondly, it increases ChAT gene expression in MOLT-3 cells, and lastly, it increases the synthesis and release of ACh by T cells. All these effects are abolished by the α7nAChR antagonists.

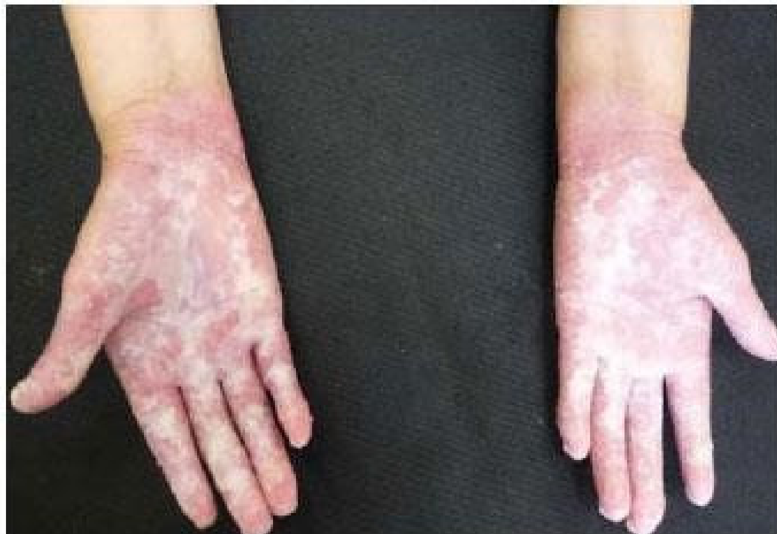


Figure 12. Features of palmo-plantar keratosis in a patient suffering from Mal de Meleda (MDM)

MDM patients show palmo-planter keratosis with erythematous borders, perioral erythema, brachydactyly and nail abnormalities. Similar lesions occur on the feet of the patients. (Permission to use granted by SciELO Brasil; originally published in Morais e Silva et al 2011).

SLURP-1 mRNA is expressed in the thymus and spleen (Moriwaki et al 2007). In addition, peripheral blood mononuclear leukocytes, DCs and macrophages have also been shown to express mRNAs encoding SLURP1. Barnes et al (2014) have shown that tonsillar CD205+ DCs also express SLURP-1 and are surrounded by CD4+ T cells, as well as other immune cells. The presence of SLURP-1 is essential for normal T cell activation. Other cell types that express SLURP-1 include keratinocytes, fibroblasts, neurons, and epithelial cells. Finally, SLURP-1 can be detected in saliva, tears, urine, and blood.

A SLURP-1 related molecule, SLURP-2, was discovered and found to be expressed in a tissue-specific manner. Unlike SLURP-1, SLURP-2 binds non-α7 (e.g. α3) nicotinic ACh receptors. It is expressed in keratinocytes, epithelial cells and immune cells (Moriwaki et al 2007). Mutation of the SLURP-2 gene in mice also causes plamo-plantar keratosis. SLURP-1 and -2 play a role in both keratinocyte turnover and wound healing in the skin and mucosae (Kong et al 2012). Interestingly, SLURP-2 is overexpressed in psoriasis (Tsuji et al 2003).

1.2 Human Immunodeficiency Virus (HIV)

1.2.1 The virus and the disease

The Human Immunodeficiency Virus type-1 (HIV-1) is a complex retrovirus, which is the causative agent of AIDS (Acquired Immune Deficiency Syndrome). HIV-1 caused an unprecedented global pandemic in the late 20th century. It is estimated that about 36.7 million people are infected by HIV-1 worldwide; with only 11 million people having access to anti-HIV medicines. In 2015, around 1.1 million individuals died from AIDS-associated illnesses, and about 2.1 million people became newly infected worldwide (World Health Organization 2016). These statistics show that the infection is still a global health problem.

The AIDS disease gained importance in the 1980s, when men in Los Angeles presented with multiple bacterial and fungal infections, as well as Kaposi's sarcoma. The disease was officially named "AIDS" in 1981 by the US Centre for Disease Control and Prevention. The retrovirus responsible for the disease was first isolated at the Pasteur Institute in France in 1983. At first, it was known by several names, including Lymphadenopathy Associated Virus (LAV) and Human T Lymphotropic Virus (HTLV)-III, before formally being renamed by the International Committee on Taxonomy of Viruses as the Human Immunodeficiency Virus (Sabin & Lundgren 2013). It is believed that HIV-1 originated from the Simian Immunodeficiency Virus (SIV) in chimpanzees due to increased contact between humans and non-human primates, as chimpanzees are illegally hunted in Africa for "bush meat". Some researchers have claimed that the oral Polio vaccine, which was grown in chimpanzee cells infected with SIV, may have resulted in the transmission to humans and the eventual development of HIV (Sabin & Lundgren 2013). Two types of HIV have been identified: HIV-1 and HIV-2. HIV-1 is more virulent and is responsible for the global pandemic. HIV-1 is composed of three subgroups: M, N and O; with M being the most prevalent. HIV-2 is not as virulent or as widespread as HIV-1 and the infection is confined to West Africa. HIV-2 is more similar to the Simian Immunodeficiency Virus (SIV).

1.2.2 Structure of an HIV-1 virion

HIV is a retrovirus of about 100 nm in diameter. The structure of a typical mature HIV virion is shown in Figure 13. The viral envelope is a lipid bilayer derived from the cell membrane during viral budding. The viral envelope proteins are studded in the envelope. Each envelope protein comprises a surface unit (SU) or glycoprotein (gp)-120, and a transmembrane part TM), gp-41. The SU is attached non-covalently to the TM. The gp120/41 complex is found as trimmers on the surface of the virion. Beneath the envelope lies the viral matrix comprising of the matrix (MA) protein, p17. The viral capsid comprises the capsid protein, p24. The viral nucleocapsid contains two copies of the single stranded viral RNA, viral reverse transcriptase (RT), integrase (IN), and protease (PR). In addition to the major structural proteins, group-specific antigen (gag), polymerase (pol), and envelope (env), the HIV-1 genome encodes two regulatory proteins, Regulator of expression of viral proteins (Rev) and Transactivator (Tat); and four accessory proteins, which include the Viral infectivity factor (Vif), Negative factor (Nef), Viral protein R (Vpr), and Viral protein U (Vpu). The proteins play a diverse role in ensuring the infection of non-dividing host cells, efficient replication, the budding of virions, and evasion from the host's antiviral immune factors (Li et al 2005). The non-immune cellular factors such as the apolipoprotein B mRNA editing enzyme/catalytic polypeptide-like (APOBEC)-3G, SAM- and HD domain-containing protein (SAMHD)-1, and tetherin, inhibit HIV replication by different mechanisms. The virus has developed strategies to overcome and to evade the host's antiviral activities (Malim & Bieniasz, 2012)

Mature HIV-1 Virion

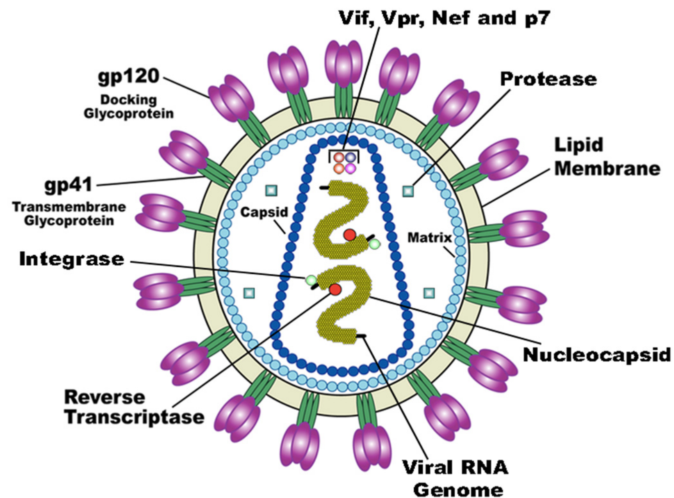


Figure 13. The Structure of a mature HIV-1 virion

The proteins making up the HIV-1 virion include the viral envelope studded with surface proteins, glycoprotein-120, and transmembrane gp-41; while the viral capsid houses the capsid protein p24, two copies of single stranded viral RNA, viral reverse transcriptase, integrase and protease. (Permission to use granted by National Institutes of Health; obtained from https://commons.wikimedia.org/wiki/File:HI-Virion-en.png).

1.2.3 HIV life cycle

CD4+ T cells are the main target of HIV infection. As the first step of infection, the viral envelope protein gp120 binds to the viral receptor, CD4 molecule, on target cells (Figure 14). This induces a conformational change in the receptor, exposing the fusogenic gp41 regions that bind the co-receptors, C-X-C chemokine receptor type 4 (CXCR4) for T cell (T) tropic or C-C chemokine receptor type 5 (CCR5) for macrophage (M) tropic HIV strains. After binding and penetration, the RNA genome is converted into DNA by the RT contained within the virus. The viral complementary DNA (cDNA) strand is then inserted by the viral integrase into the host genome. Upon cell activation, more viral proteins and viral RNA copies are made, which then assemble to form new virions. The newly formed virions hijack the multi-vesicular body-forming machinery for budding off the infected cells (Gomez & Hope 2005).

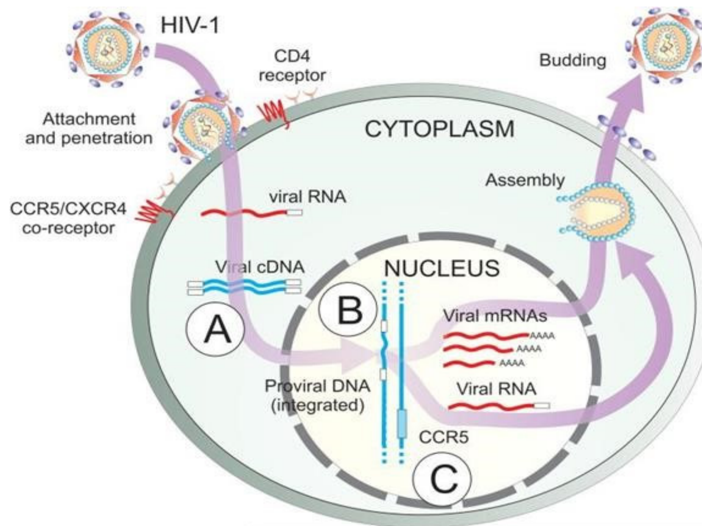


Figure 14. Cycle of HIV-1 replication

This figure illustrates the steps in the life cycle of HIV infection. The viral envelope protein gp120 binds to the CD4 receptor molecule and penetrates the target CD4+ cells. Viral RNA is then reverse transcribed into DNA by the RT contained within the virus, which is then integrated into the host genome. By hijacking the host's replication system, the virus is able to replicate uncontrollably and infect the host. (Permission to use granted from Taylor & Francis online, originally published in Saayman et al 2015).

1.2.4 Stages of HIV infection

HIV infection could be arbitrarily divided into three different clinical stages (Figure 15) and are described below:

i) Acute or primary infection: This stage usually lasts six months after the initial infection. Patients exhibit mild flu-like symptoms. However, during this time, massive replication of the virus occurs, followed by dissemination in the host. With the induction of HIV-specific CD8+ T lymphocytes (CTL), the viral load decreases (An & Winkler 2010).

ii) Chronic infection: This stage can last for several years, typically 4-7 years. Normally, infected individuals show no clinical signs. However, during this phase, there is massive replication of the virus accompanied by destruction and regeneration of the CD4+ T cells. The virus-specific immune response (antibodies and CTL) keeps the virus under control. The viral load in this early stage, called the viral load set-point, determines the prognosis of the infection.

iii) AIDS: This is the final stage in which host's immune response is no longer able to control viral replication. The host becomes immunodeficient; CD4+ T lymphocyte counts fall below 200 per µl of blood and the host becomes infected with opportunistic infections, develops neurocognitive defects, and AIDS-related cancer such as Kaposi's sarcoma. If infection is not treated, death occurs after a variable period of illness.

The median time to develop AIDS in untreated patients from initial infection is 8-10 years. However, some infected individuals do not develop AIDS. It is worth noting that a small fraction (5-10%) of HIV-infected individuals are able to control HIV replication for more than seven years without receiving anti-retroviral treatments. These individuals are called slow progressors. Some of them may control the infection and show no signs of disease for more than 15 years. They are called elite controllers. Several factors, including infection with mutant viruses, strong immune responses, and/or anti-viral host genetic factors may underlie their ability to control the infection (Blankson 2010).

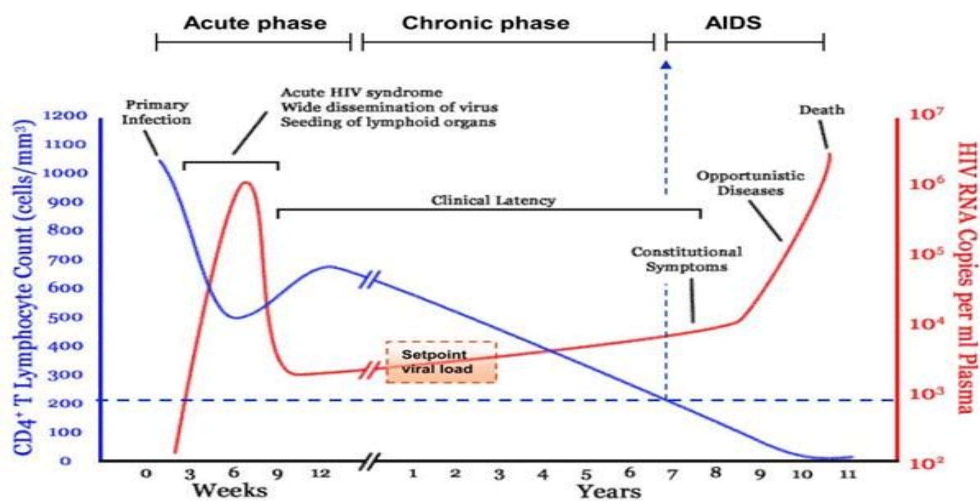


Figure 15. Clinical stages of HIV infection

The HIV infection is divided into three stages (primary/acute, chronic, and AIDS). The division is based upon the duration of the infection, CD4+ T cell counts, viral load in the blood and clinical symptoms. The chronic infection, when the infection is asymptomatic, is also called the period of clinical latency. (Permission to use granted by PubMed Central, originally published in An & Winkler 2010).

1.2.5 Immunopathogenesis of HIV infection

HIV induces a strong immune response in the host. Both HIV-specific antibodies and T cell (CD4+ and CD8+) responses can be readily demonstrated in infected individuals. However, the high mutation rate of the virus enables it to evade the host's immune responses. Furthermore, depletion of CD4+ T cells deprives the generation of virus-specific antibodies and CTLs. As a result, T cells become weak and exhausted. They express a variety of co-inhibitory molecules such as programmed cell death protein 1, (PD-1), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte-activation gene 3 (LAG-3) and CD160 (Mohan et al 2014).

HIV-infected individuals exhibit chronic and aberrant immune activation of all essential cellular components of the innate and adaptive immune systems including T cells, B cells, Natural Killer (NK) cells, plasmacytoid dendritic cells (pDC) and the complement system (Imran et al 2016). Increased expression of CD38, a marker of T cell activation, is considered a predictor for a bad prognosis in HIV-infected individuals. Furthermore, infected patients also show signs of chronic inflammation. The systemic inflammatory response results largely from a defect in the intestinal barrier. CD4+ T helper 17 (TH17) cells residing in the gut are major targets of the virus because they express relatively high levels of CCR5 on their surface. The massive depletion of these cells in the gut occurs during all stages of the infection (Brenchley et al 2004; Brenchley & Douek 2012). These cells secrete IL-17 and IL-22, which are important for maintaining the intestinal barrier function. The loss of these cells explains defective intestinal integrity in HIV-infected individuals. HIV viral proteins also contribute to disrupt mucosal tight junctions by inducing proinflammatory cytokines from intestinal epithelial cells. The defective intestinal barriers lead to the translocation of bacterial products and fragments into the blood. This microbial translocation causes immune activation and inflammatory responses in the infected individuals. Further, viral nucleic acids and proteins also activate the immune system and induce inflammation by activating a variety of Pattern Recognition Receptors (PRRs) of which Toll-like Receptors (TLRs), RIG-like helicases (RLH), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and inflammasomes are the most important. The virus has also been shown to kill infected CD4+ T cells via pyroptosis; a type of cell death accompanied by the production of proinflammatory cytokines such as IL-1β

and IL-18. The virus-infected individuals show increased levels of proinflammatory cytokines, chemokines and pro-coagulation mediators in the blood such as IL-6, IL-18, IL-7, chemokine (C-X-C motif) ligand (CXCL)-10/IP-10, D-dimer and sCD14 (Catalfamo et al 2012). Paradoxically, immune over-activation and chronic inflammation further weaken the immune system, and due to its high rate of replication and mutability, the virus is capable to evade the immune system. This results in immunodeficiency and patients become highly susceptible to opportunistic infections, cancers and neurocognitive defects. The infection, if untreated, invariably results in death.

1.2.6 Antiretroviral drugs

Tremendous efforts have been made in developing very effective antiretroviral drugs (De Clerc 2009, Cao et al 2015). The drugs belong to the following classes:

Nucleoside/Nucleotide RT Inhibitors (NRTI): This was the first class of drugs developed against HIV. They inhibit the viral RT, interacting with the catalytic site of the enzyme. This class includes zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), abacavir (ABC), and tenofivir (a nucleotide RTI). All are 2',3'dideoxynucleoside (ddN) analogues and compete with normal deoxynucleoside triphosphates inside cells. The drugs are incorporated during reverse transcription but terminate the reaction. AZT, in combination with 3TC, has been shown to effectively inhibit vertical transmission of the virus from infected mothers to their newborn babies (McIntyre et al 2009).

Non-Nucleoside RT Inhibitors (NNRTI): These drugs interact with an allosteric site located near the catalytic site of the RT. This category of drugs includes nevirapine, etravirine, delavirdine, and efavirenz.

Viral Protease Inhibitors: These drugs inhibit viral proteases and thus prevent the processing of precursor viral proteins into mature ones. This category of drugs includes ritonavir, indinavir, lopinavir, nelfinavir, saquinavir, amprenavir, atazanavir, and darunavir.

Integrase Inhibitors (INI): These drugs prevent the integration of the virus into the genome. This category of drugs includes Raltegravir and Elvitegravir.

Viral entry inhibitors: These inhibitors interfere with the entry of the virus into susceptible cells. Maraviroc, a CCR5 antagonist, is only effective against M-tropic viruses. An anti-CXCR4 (against T-tropic viruses), AMD3100, could not be used due to toxicity issues.

Fusion Inhibitors: The drug enfuvirtide is a polypeptide homologous to the heptad repeat region of gp41 and prevents the fusion of the viral lipid bilayer with that of susceptible cells. Consequently, the virus cannot enter and infect cells.

1.2.7 Anti-retroviral drugs prolong survival but do not cure HIV infection

The use of different anti-retroviral drugs in combination, often referred to as the highly aggressive anti-retroviral therapy (HAART) or simply as combination anti-retroviral therapy (cART), has saved the lives of millions of HIV-infected individuals. Today, if treated, patients no longer die from AIDS or from any AIDS-related illness. However, cART is not a cure. The treatment does not eliminate the virus and the virus can persist in a latent form in these individuals in immunologically and pharmacologically privileged sites called viral sanctuaries. Such sites include the gut, brain, testes, B cell follicles in lymph nodes, lungs and cornea. The residual viral loads at these sites constitute what are generally referred to as the viral reservoirs (Kimata et al 2016). These reservoirs are the main hurdle in curing HIV-infected individuals. The main cell type infected in these reservoirs is the long-lived memory CD4+ T cells. As a result, HIV-infected patients have to take cART to suppress viral replication and to reduce the level of proinflammatory mediators in the circulation, thereby partially restoring immune responses. However, the levels of the pro-inflammatory mediators rarely return to physiological levels and remain above normal values. Furthermore, the virus found in these reservoirs continues to undergo replication at very low undetectable levels. This low-level of viral replication and the continuous use of anti-retroviral drugs contribute to the chronic low levels of inflammation and immune activation found in these patients. Although HIV patients do not die from AIDS, they become more susceptible to AIDS-unrelated co-morbidities including enhanced aging, cardiovascular diseases, metabolic syndrome, liver and kidney diseases, osteopenia/osteoporosis, neurocognitive disease and cancers. Researchers are investigating strategies to deplete viral reservoirs. It has been found that an early initiation of anti-retroviral therapy after infection significantly reduces the size of the viral reservoirs. At the same time, a

variety of anti-inflammatory drugs (e.g. statins and aspirin) can be used to reduce the adverse consequences of chronic inflammation in HIV-infected patients (Bandera et al 2016).

1.2.8 Regulation of CAP in HIV infection

Little is known about how CAP is regulated in HIV-infected individuals. The HIV envelope protein, gp120, was shown to bear significant sequence homology with snake curare-mimetic neurotoxins. Gp120 has also been shown to bind the nAChR α7 subunit and to increase the expression of α7nAChR. However, the protein does not reduce production of pro-inflammatory cytokines from macrophages and microglia (Neri et al 1990, Ballester et al 2012, Delgado-Vélez et al 2015). In the neurons obtained from post-mortem brains of HIV-infected individuals with HIV-associated neurocognitive defects (HAND), the expression of α7 was upregulated but dup-α7 was downregulated. *In vitro*, gp120 mimicked these changes in neuron receptor expression (Ramos et al 2016). Gp120 was also shown to induce increased production of mucus from bronchial epithelial cells, which express CXCR4. The signalling pathway implicated in mucus secretion comprised activation of the CXCR4-α7-nAChR-γ aminobutyric acid (GABA) receptor-the epidermal growth factor receptor (EGFR) cascade (Gundavarapu et al 2013). *In vitro,* as well as *in vivo*, ACh mimetics, AcChE inhibitors (Galantamine and Pyridostigmine), and α7 agonists inhibit PMA and ionomycin-induced T cell activation, proliferation and production of proinflammatory cytokines (Valdés-Ferrer et al 2009; Pohanka et al 2011).

In summary, chronic inflammation accompanying HIV infection is the main cause of AIDS immunopathogenesis and later, CD4+ T cell depletion. It causes aberrant immune activation. In cART-treated individuals, low grade inflammation persists even after successful suppression of viral replication (Ahmad & Rinaldo 2017). Unfortunately, cART does not cure HIV infection. As a result, HIV-infected individuals must take antiretroviral drugs for life. Once cART is stopped, latent virus residing inside the reservoirs start replicating within days. Lifetime use of cART not only puts a lot of burden on healthcare resources, it is also toxic for patients. The drugs, especially PIs and NRTIs, exert toxic side-effects (Hester 2012). The

upregulation of body’s natural homeostatic anti-inflammatory pathways could constitute an alternative strategy to reduce inflammation and immune activation. In this regard, we need to learn about the function of the extra-neuronal cholinergic system in these patients. This new knowledge may help identifying new molecular targets to reduce inflammation and strengthen the immune response in HIV-infected patients. Based on our actual understanding of the extra-neuronal cholinergic system, we hypothesize that the extra-neuronal anti-inflammatory cholinergic pathway becomes hypo/non-functional in HIV-infected individuals. The main objective of this thesis was to understand how the extra-neuronal anti-inflammatory cholinergic pathway is affected during HIV infection and contributes to chronic inflammation and immune activation.

2 Hypothesis & Objectives

2.1 Hypothesis

We hypothesize that HIV infection dysregulates the extraneuronal cholinergic system, which promotes chronic inflammation and immune activation. New knowledge about this dysregulation may be exploited to attenuate inflammation and reduce immune activation in HIV-infected patients.

2.1.1 Rationale

It has been well documented that patients infected with HIV have chronic inflammation and show aberrant immune activation. Activated immune cells, especially CD4+ T cells, produce ACh and SLURP-1, which act in autocrine and paracrine manners to fine tune T cell activation and prevent over activation (Kawashima et al 2012, Fuji et al 2014). Furthermore, ACh and SLURP-1 may also attenuate inflammatory responses by signalling T cells and other immune cells. As HIV replication is more efficient in activated T cells, it is possible that a dysregulation of the cholinergic system may further promote HIV replication. This provides a rationale for investigating the regulation of the ENCS during HIV infection. Furthermore, we would like to investigate whether this system could be exploited to reduce HIV replication and benefit HIV-infected patients.

2.2 Main objective

The main objective of the study was to investigate how the ENCS is regulated in HIV-infected individuals and understand whether this system could be manipulated to reduce HIV replication and immune activation in HIV-infected individuals.

2.2.1 Specific aims

- To examine the production of ACh and soluble SLURP-1 in HIV-infected individuals
- To investigate the effects of different cholinergic components on HIV replication
- To investigate the expression of α7nAChR and β2 adrenergic receptors on peripheral blood monocytes and CD4+ T cells of HIV-infected individuals

3 Materials & Methods

3.1 Antibodies & reagents

The antibodies used in this study and their sources were: rat anti-human α7nAChR (catalog # 838401 from Biolegend (San Diego, CA, USA), rabbit anti-human nicotinic acetylcholine receptor α7 from Abcam (Toronto, ON, CA) (catalog # ab10096), rabbit polyclonal anti-human dup-α7nAChR (catalog #MBS719199) from Biosource (Westmount, QC, CA), mouse anti human beta-2 adrenergic receptor from Serotech (Burlington, ON, CA) (catalog # MCA2784), Brillant violet 421-conjugated mouse anti-human CD4 from Biolegend (catalog # 317434), PerC-Efluor 710-conjugated mouse anti-human CD3 from Ebioscience (San Diego, CA, USA) (Cat # 46-0037-42), PE-conjugated mouse anti-human CD25 from Ebioscience (catalog # 12-025941), mouse anti-human CD14 from Ebioscience (catalog # 17-0149-41), FITC-conjugated goat anti-mouse IgG from Biolegend (catalog # 405305) and FITC-conjugated goat anti-rabbit IgG from Ebioscience (catalog # 11-4839-81). Other reagents used included: recombinant human (rh) SLURP1 protein from Abnova (Walnut, CA, USA) (catalog # H00057152-P01), phytohaemagglutinin (PHA) and recombinant human IL-2 from Sigma-Aldrich (St-Louis, MI, USA), a selective α7nAChR agonist (Cat # 4477) and a selective α7nAChR antagonist (MG 624; catalog # 1356) from Tocris (Bristol, UK) and Hemicholinium-3, a competitive inhibitor of choline for synthesis of ACh, (catalog # H108; Sigma-Aldrich used at 100 μM concentration).

3.2 Isolation of peripheral blood mononuclear Cells (PBMC)

Peripheral blood samples from healthy volunteers were collected after obtaining their written informed consent. They were diluted with an equal volume of Phosphate Buffered Saline (PBS; pH 7.2) and PBMCs were isolated by centrifugation on Ficoll-Hypaque (Pharmacia, Montreal, QC, CA) density gradients as described previously (Ahmad et al 2002). PBMC were collected, washed with PBS and re-suspended in culture medium.

3.3 Cell culture

PBMCs were cultured in RPMI-10 (RPMI 1640 medium supplemented with 10% FBS) containing 100 U/mL penicillin and 100 µg/mL streptomycin from Invitrogen, 10 U/mL recombinant human IL-2 (Sigma-Aldrich), 10 µg/ml PHA (Sigma-Aldrich). The cell cultures were incubated at 37°C in 5% CO2 and 85% humid conditions. Three days of culture yielded PHA blasts. The cells were cultured in RPMI-10 and the medium was changed twice weekly.

3.4 Virus preparation

The T-tropic viral strain NL4.3 was prepared in our laboratory (Iannello et al 2010a). The proviral DNA for the viral strain was amplified from the plasmid pNL4.3 using a commercial kit (Qiagen, Germantown, MD, USA). To produce virus, 1.00 µg of plasmid DNA was transfected in HEK293T (an Adenovirus-transformed human embryonic kidney cell line expressing the SV-40 large T antigen; ATCC, CRL-11268) using polyethylenimine (catalog # 408712 from Sigma-Aldrich) and an in-house protocol (Iannello et al 2010a). The transfected cells were incubated in the culture medium (RPMI-10) at 37°C in humidified 5% CO_2 atmosphere. After 24 hours, culture supernatants were collected, clarified by centrifugation at 14,000g for 30 minutes. The supernatants were titrated for p24 contents using a commercial ELISA kit from ABL (Rockville, MD, USA). The supernatants containing 98.0 ng per ml of p24 were aliquoted and stored at -80°C until used. The supernatants from mock-transfected HEK293T cells were used as mock viral preparations.

3.5 *In vitro* infection of cells with HIV-1

In separate experiments, PHA blasts and MOLT-4 cells were infected in vitro with a T-tropic HIV-1 strain (NL4.3) as described in Iannello et al 2010a and Iannello et al 2010b. Briefly, $5x10^6$ cells were cultured for 2 h at 37°C with 100 µl of the viral preparation (containing 98 ng of p24 per ml) or with the same volume of the mock viral reparation (see below). The infected cells were washed with the culture medium to remove residual virus. To investigate the effects of different reagents on HIV replication, they were added to the cell cultures. The reagents and the concentrations used included HC-3 at 5 µM, α7nAChR agonist at

2 nM, α7nAChR antagonist at 50 μM, and SLURP-1 at 5 μg/ml concentration. The cells were cultured at 37°C for different lengths of time (indicated in individual experiments). Culture supernatants were collected and their p24 concentrations were determined.

3.6 Flow cytometry

The expression of β2 adrenergic receptor and α7nAChR in different subsets of PBMC was determined after incubating cells with the marker-specific primary antibodies on ice for 30 minutes. After washing and centrifuging, cells were incubated with the fluorochrome-conjugated secondary antibodies. The stained cells were then washed with PBS containing 0.5% BSA and stained with flurochrome-conjugated anti-CD3, anti-CD56, anti-CD4, and anti-CD25 for gating CD4+T cells or with anti-CD3 and anti-CD14 for gating monocytes (described above in section 3.1. Antibodies & Reagents). Stained cells were washed with PBS and re-suspended in 2% paraformaldehyde (PFA). The cells were then analyzed using the BD-LSR Fortessa (BD Bioscience, San Diego, CA). Data was acquired and analyzed using FACSDiva (BD Bioscience,) and FlowJo (Treestar, OR, USA) softwares, respectively.

3.7 Study participants

Plasma and serum samples were used to determine ACh and SLURP-1 contents in the blood. Samples were collected from a cross-section of HIV-infected individuals representing different stages of the infection, and from HIV seronegative healthy control subjects (Table 1). Samples were collected after obtaining the written informed consent from participants. For ACh measurement, we used blood samples from 8 individuals with primary HIV infection (PHI; defined as being within 6 months of date of infection), 16 patients with chronic HIV infection (CI; having the infection for more than 6 months and not treated with ART), 20 patients with chronic HIV infection receiving ART, 8 patients as long-term non progressors (LTNP; infected for more than 7 years without receiving ART and not having any AIDS-defining condition) and 16 HIV seronegative healthy controls. SLURP-1 was measured in the blood from 23 CI patients treated with ART, and 24 CI patients not receiving ART, 8 Primary

HIV infection (PHI) receiving ART and 9 PHI patients not receiving ART, 12 LTNPs and 34 healthy control donors.

Tableau I. Demographic and clinical parameters of the study participants

Category (number)	Age Range	CD4+ counts Range	CD8+ counts Range	VL (log10) Range
PHI ART- (12)	22-44	380-1037 (402)	520-2510 (980)	3.50-4.275 (4.222)
PHI ART+ (13)	28-57	240-900 (367.5)	360-1080 (519)	1.60-3.41 (2.10)
CI ART- (43)	22-56	201-1037 (430)	480-2180 (760)	4.376- 5.684 (5.189)
CI ART+ (46)	22-56	400- 780 (710)	360-1080 (1065)	1.602-2.822 (1.65)
LTNP (12)	-	-	-	ND
Healthy (50)	-	-	-	-

PHI: Primary HIV infection, CI: Chronic HIV infection, CD4+ and CD8+ counts represent counts of the respective T cells per mm3, VL represents copies of HIV RNA per ml in Log10, ND: Non-detectable. A dash (-) means unknown.

3.8 Measuring ACh and SLURP-1 concentrations

The levels of ACh in the blood samples were determined using a commercial kit, Choline/ Acetylcholine Assay Kit (BioSource). The kit measures choline in the samples before and after releasing it from the ACh. The kit can detect ACh in the samples in the range of 31.2 nM/L to 1000 nM/L SLURP-1 in the samples was quantified using a commercial ELISA kit from CUSABIO (College Park, MD, USA) with a minimum detection limit of 31 pg/ml.

3.10 Statistical analysis

Group means were compared using Student's t-test for comparison between two groups and by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests for comparing means between more than two groups. Analyses were performed using SPSS (IBM, Chicago, IL, USA). Differences were deemed significant at $p \leq 0.05$.

4 Results

4.1 Levels of ACh in HIV-infected individuals

In a cross sectional study, we measured levels of ACh in the blood of HIV-infected individuals and HIV-seronegative healthy controls. Patients were separated according to their stages of the infection. Results are shown in Figure 16. While no significant differences were found in ACh concentrations between HIV-infected individuals and healthy controls ($p>0.05$), LTNP had significantly higher concentration of ACh compared with healthy controls and HIV-infected individuals ($p<0.01$).

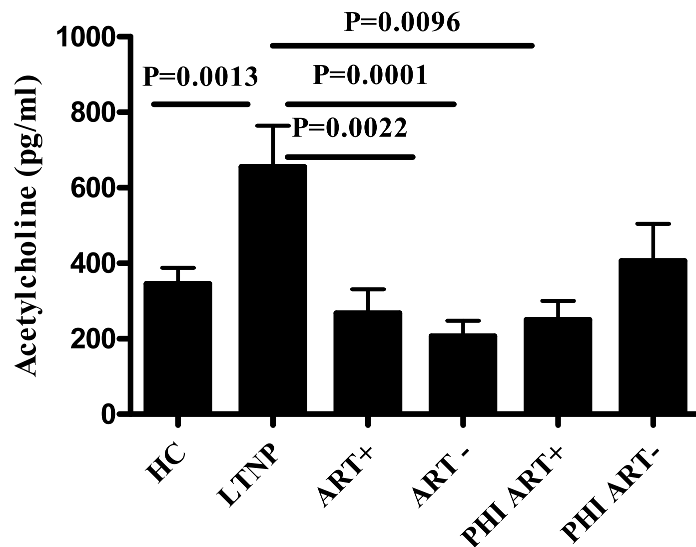


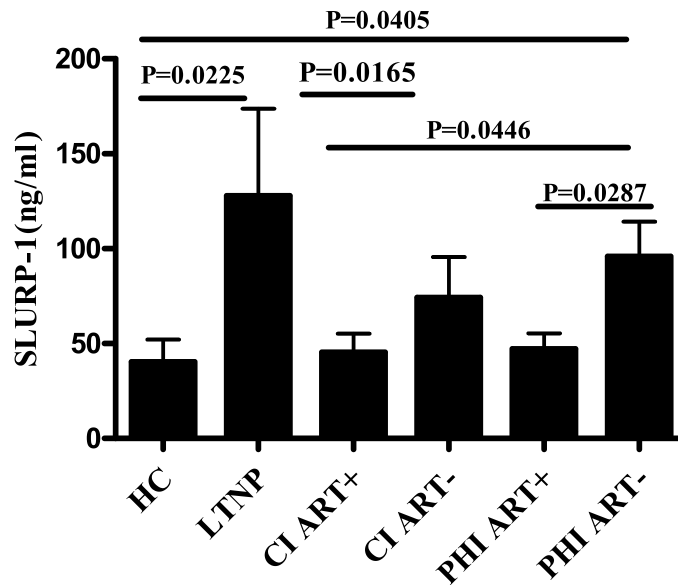
Figure 16. Concentration of ACh in the circulation of HIV-infected individuals

ACh concentrations were measured using a commercial choline/acetylcholine Assay Kit. The number of donors in each category included: healthy controls (HC; 34), long-term non-progressors (LTNP; 12), chronically infected anti-retroviral therapy+ (ART+; 23), chronically infected anti-retroviral therapy- (ART-; 25), primary infection without ART (PHI ART-; 9) and primary HIV infection with ART (PHI ART+; 9). The figure depicts mean ± SE. Data were analyzed using one way ANOVA and means were compared with Tukey's post-hoc tests.

4.2 Levels of SLURP-1 in HIV-infected individuals

As shown in Figure 17A, SLURP-1 levels did not differ significantly ($p>0.05$) between healthy controls and HIV patients (Figure 17A). However, these levels were significantly higher in LTNP as compared with those in healthy controls and in virus-infected individuals as well ($p<0.01$). Interestingly, SLURP-1 levels tended to increase in ART-naïve PHI and CI individuals as compared with their ART+ counterpart donors. Similarly, no difference was found between ART+ and ART- chronically infected patients ($p>0.05$). Furthermore, no difference in SLURP-1 contents was found between PHI ART+ and PHI ART- patients (Figure 17B).

A



B

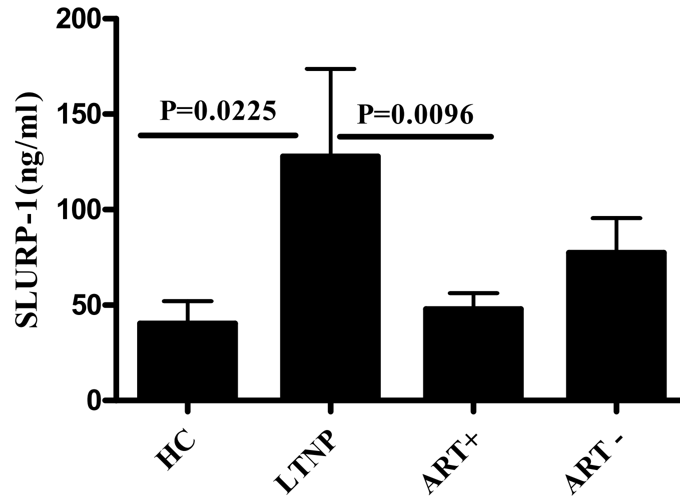


Figure 17. Comparison of SLURP-1 concentrations between ART+ and ART- HIV-infected individuals

SLURP-1 concentrations were measured by ELISA. (A) The Panel shows SLURP-1 concentrations (mean ± SE) in the blood of healthy controls (HC; n= 16), long-term non-progressors (LTNP; n= 8), chronically infected anti-retroviral therapy+ (CIART+; n= 20), chronically infected anti-retroviral therapy- (CHIART-; n= 16), primary infection without ART (PHI ART-; n= 9) and primary HIV infection with ART (PHI ART+; n=8). One way ANOVA and Tukey's post-hoc tests were used for data analysis. (B) Panel B compares concentrations (mean ± SE) of SLURP-1 between ART+ (24) and ART- (20) patients irrespective of stage of the infection. No significant difference ($p>0.05$) was found between these two groups. Data were analyzed using one way ANOVA and Tukey's post-hoc tests.

4.3 Effects of α7nAChR agonist and antagonist on HIV replication

We determined the effects of an agonist and an antagonist of the α7nAChR on HIV replication in human PHA blasts. The agonist or antagonists were added to the cell cultures immediately after *in vitro* infection of the cells. The culture supernatants were obtained on day +3 post-infection and quantified for p24 content. As shown in Figure 18, the addition of the receptor agonist significantly decreased ($p<0.01$) p24 contents in the culture supernatants. In contrast, the antagonist significantly increased ($p<0.05$) p24 contents in the culture supernatants.

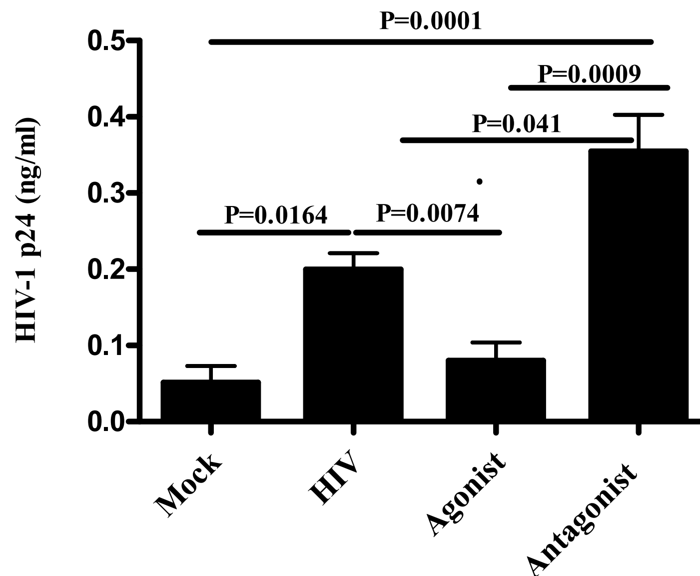


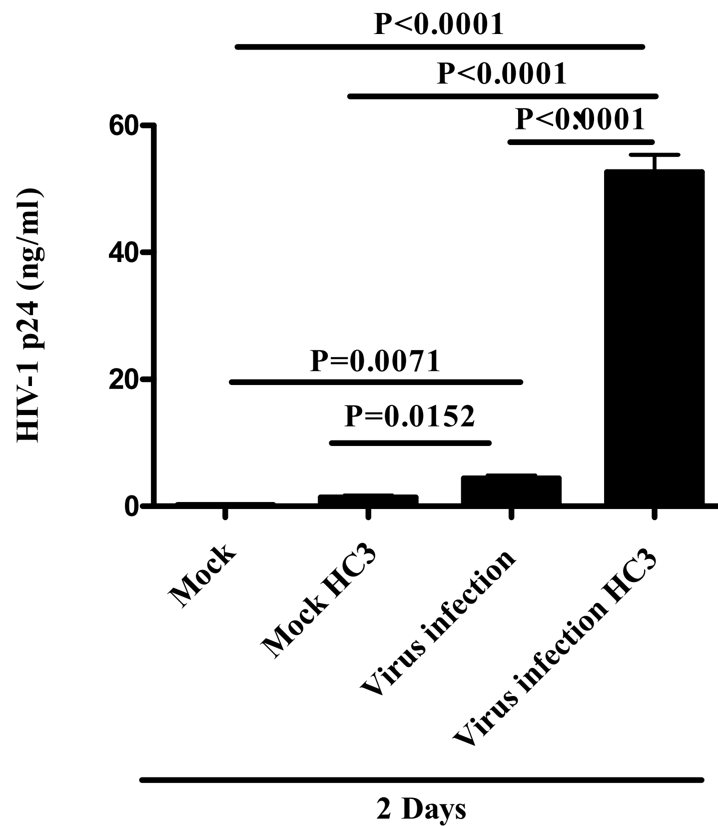
Figure 18. Effect of the α7nAChR agonist and antagonist on HIV replication

The receptor agonist and antagonist were added to PHA blasts infected with NL4.3. Three days post-infection, culture supernatants were collected, centrifuged and quantified for their p24 contents. The Figure shows mean ± SE of p24 contents in mock infected, HIV infected, HIV infected + agonist and HIV infected + antagonist from three independent experiments. Data were analyzed by one way ANOVA and Tukey's post-hoc tests

4.4 Effect of ACh depletion on HIV replication

We determined the effects of ACh depletion on HIV replication in human PHA blasts. After infecting PHA blasts with HIV-1, we incubated cells with HC-3, which acts as a competitive inhibitor of ACh production (Guyenet et al 1973). The culture supernatants were collected on day +2, +3 and +4 post-infection and quantified for p24 contents. The reduction/depletion of ACh in PHA blasts by HC-3 increased virus production (10 fold higher) compared with non-treated cells (Figure 19A) on day +2 post-infection. The increase in virus production decreased progressively on day +3 and +4 compared with day +2. The increase in virus production remains significant compared with HC-3 untreated cells (p≤0.05); (Figure 19B).

A



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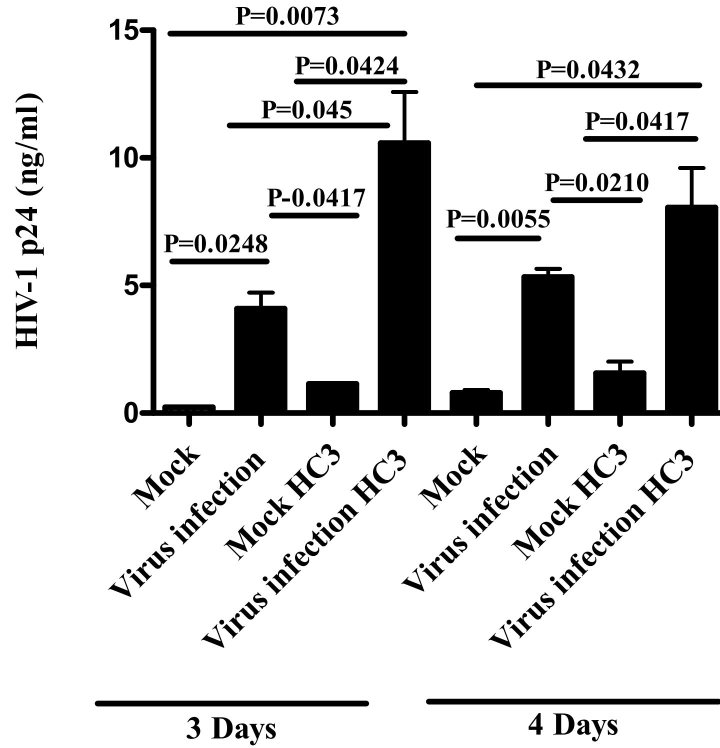


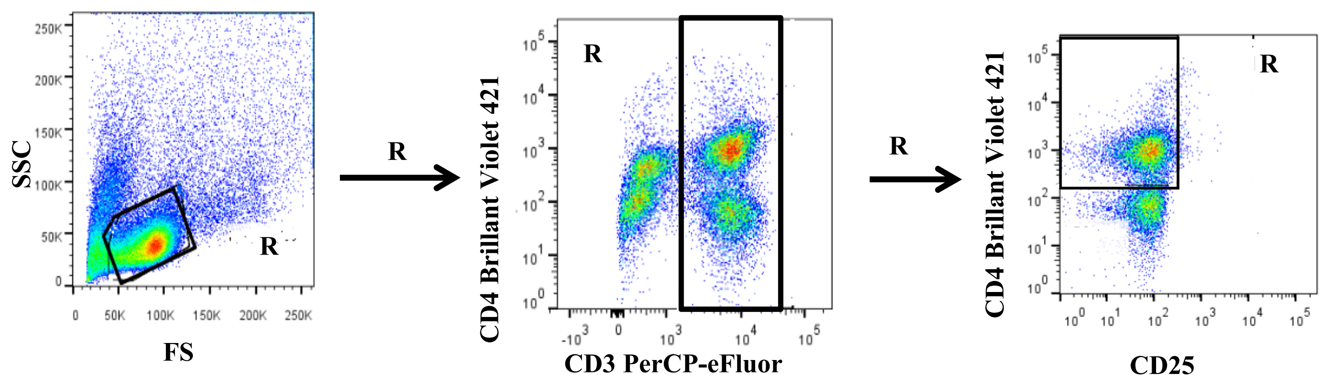
Figure 19. Effect of reduction/inhibition of ACh synthesis on HIV replication in PHA blasts

Addition of HC-3 to HIV-infected PHA blasts obtained from a healthy donor increased production of HIV (measured by their p24 contents) on day +2 post-infection (A) and on day +3 and +4 post-infection (B). Data were analyzed using ANOVA and Tukey's post-hoc tests. The results (mean ± SE) generated from three independent experiments are shown in each panel.

4.5 Expression of α7nAChR on CD4+ T cells

The expression of α7nAChR was evaluated using a combination of five monoclonal antibodies: PerCP-eFluor 710-conjugated anti-CD3, APC-conjugated anti-CD56, Brilliant violet 420-conjugated anti-CD4, rabbit anti- human α7nAChR and FITC-conjugated goat anti-rabbit IgG. CD4+ T cells were gated as shown in Figure 20A. Specificity of the antibodies for the receptor is shown in Figure 20B. Expression of α7nAChR on CD4+ T cells from healthy and HIV-infected individuals is shown in Figure 20C. Mean Fluorescence Intensity (MFI) of the expression of α7nAChR on CD3+CD4+ CD25- T cells of 5 HIV-infected and 5 HIV healthy controls is shown in Figure 20D. The expression of the receptor was significantly decreased on CD4+ T cells of HIV-infected individuals compared with healthy controls ($p<0.01$). In a separate experiment, we determined the expression of α7nAChR on CD3+CD4+ T cells of 6 individuals with chronic HIV infection (3 ART- and 3 ART+) and 3 healthy control subjects. As shown in Figure 20E, the expression of α7nAChR was significantly decreased on CD4+ T cells in HIV-infected individuals in both ART- and ART+ patients ($p<0.01$). Furthermore, expression of the receptor was significantly higher on these cells in ART+ patients compared to ART-naïve ones ($p<0.01$).

A



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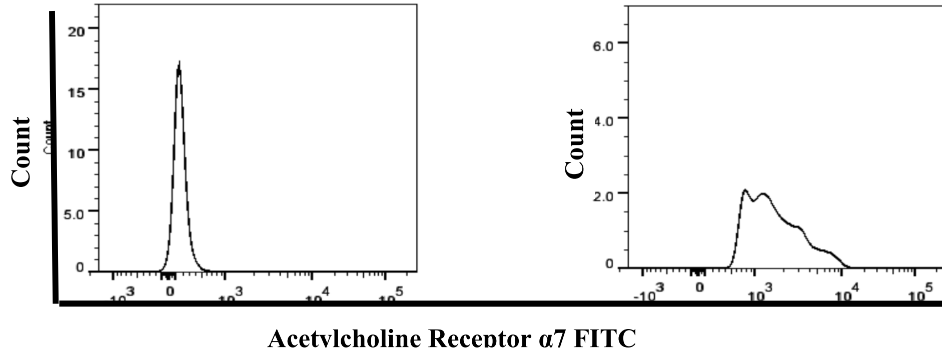
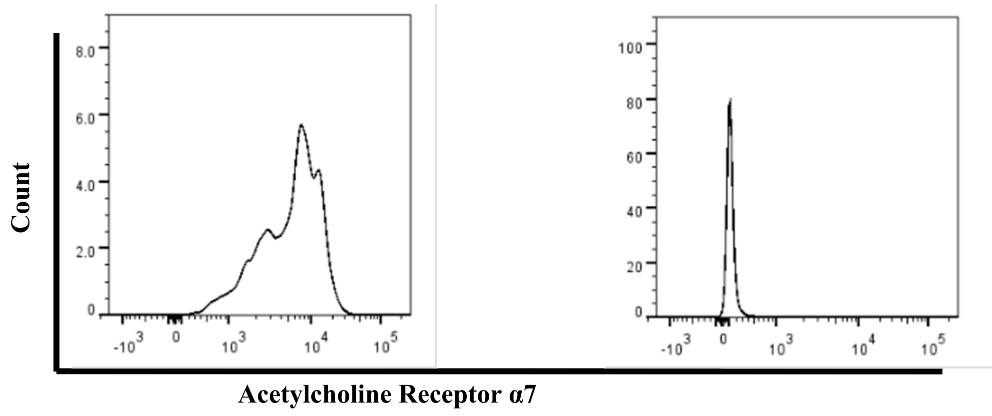


C



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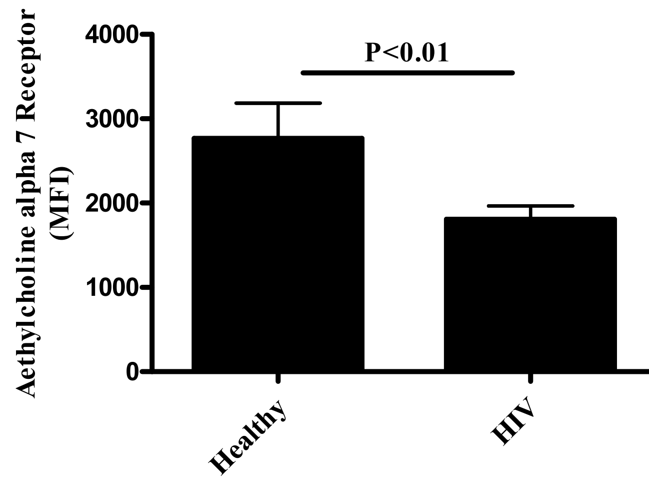


E

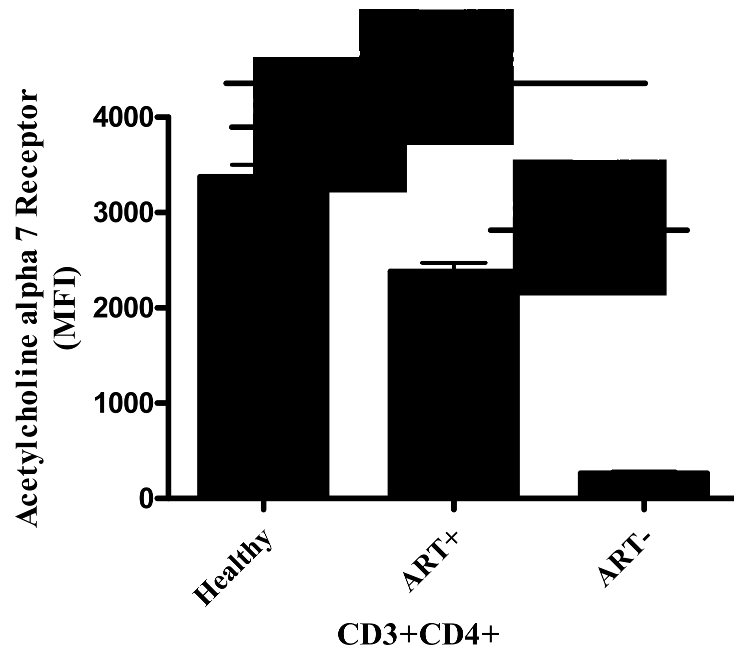


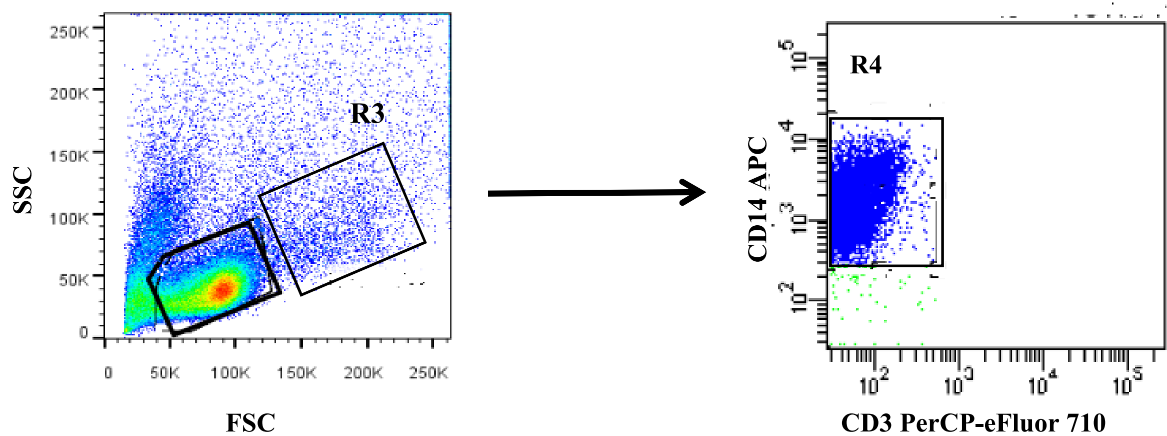
Figure 20. Expression of α7nAChR on CD4+ T cells in HIV-infected individuals

(A) Gating strategy used for CD4+ CD25- T cells. Lymphocytes (R1) were gated using FSC and SSC. Then CD3+ cells were gated (R2). Finally, CD3+CD4+ CD25- cells were gated (R3) and used for determining expression of α7nAChR. (B) For the specificity of the antibody for α7nAChR, cells were incubated with control antibodies (left panel) or receptor-specific (rabbit anti-human α7nAChR; right panel), washed and stained with FITC-conjugated goat anti-rabbit IgG. (C) The typical histograms for the expression of α7nAChR on CD4+ T cells from a healthy individual (left panel) and from an HIV-infected individual (right panel). (D) The Figure shows mean ± SD of the MFI of the expression of α7nAChR on CD4+ T cells from 5 HIV-infected and 5 healthy controls. The mean values were compared using a T test. The expression of α7nAChR on CD4+ T cells is significantly reduced in HIV-infected individuals ($p<0.01$). (E) Expression of α7nAChR on CD4+ T cells in healthy controls and HIV-infected individuals treated or not with ART (three individuals in each group). The Figure shows MFI (mean ± SE) for each group. The data were analyzed using ANOVA and Tukey's post comparisons.

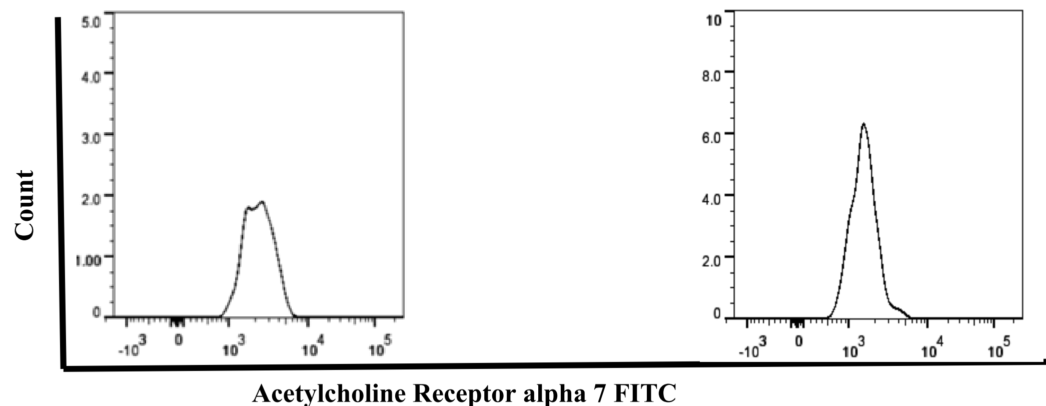
4.6 Expression of α7nAChR on monocytes

The expression of α7nAChR was determined by flow cytometry on CD3-CD14+ peripheral blood monocytes of HIV-infected individuals and healthy controls. Monocytes were gated as shown in Figure 21A. The expression of α7nAChR on monocytes of HIV-infected and healthy control individuals is shown in Figure 21B. MFI data for the expression of α7nAChR on monocytes of 5 healthy and 6 HIV-infected individuals were analyzed using T-test and are shown in Figure 21C. No difference was observed ($p>0.05$). This may be due, at least in part, to high variability of the expression of α7nAChR on monocytes obtained from HIV-infected patients.

A



B



C

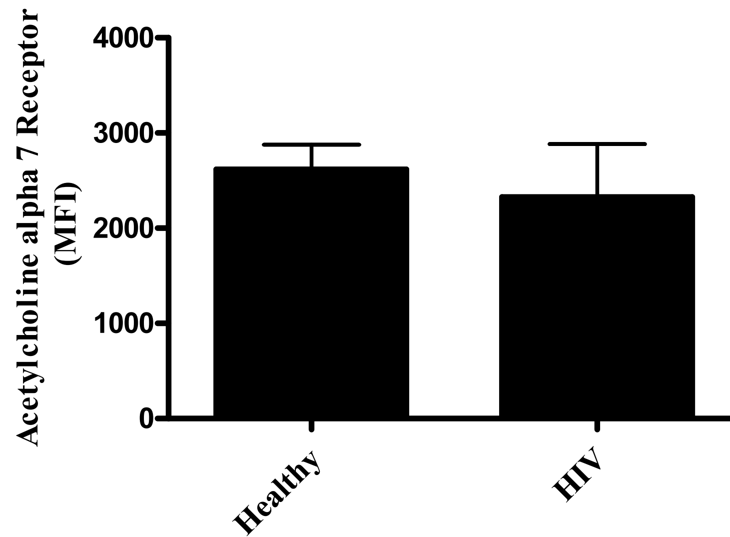


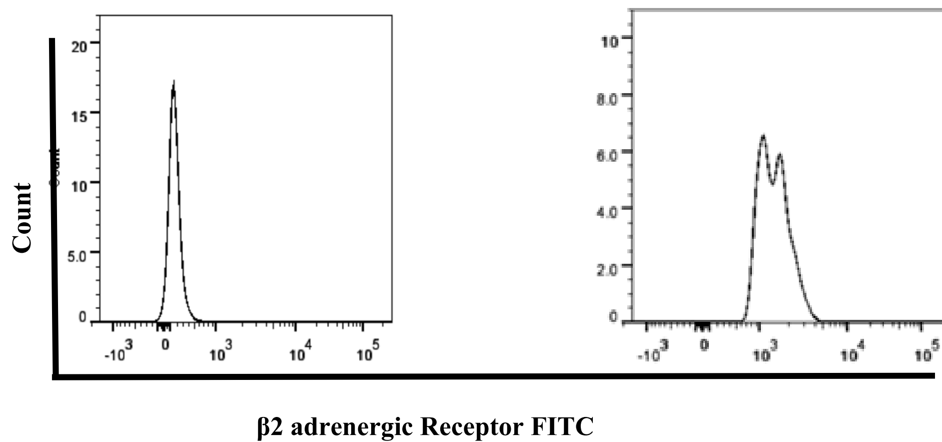
Figure 21. Expression of α7nAChR on CD3-CD14+ monocytes in HIV-infected individuals

(A) Strategy for gating CD3-CD14+ monocytes. Monocytes (R3) were gated using FSC and SSC. Then CD3-CD14+ monocytes were gated (R4) and used for determining expression of α7nAChR. Specificity of the antibodies for the receptor was shown above in Figure 20B. (B) Typical histograms for the expression of α7nAChR on monocytes from a healthy control (left panel/C) and an HIV-infected individual (right panel/P). (C) The panel depicts mean ± SD of MFI (in arbitrary units) of expression of α7nAChR on CD3-CD14+ monocytes from 5 healthy control and 6 ART- HIV-infected individuals. Means for the two groups were compared using T test. The difference in the mean values between the two groups was not significant ($p>0.05$).

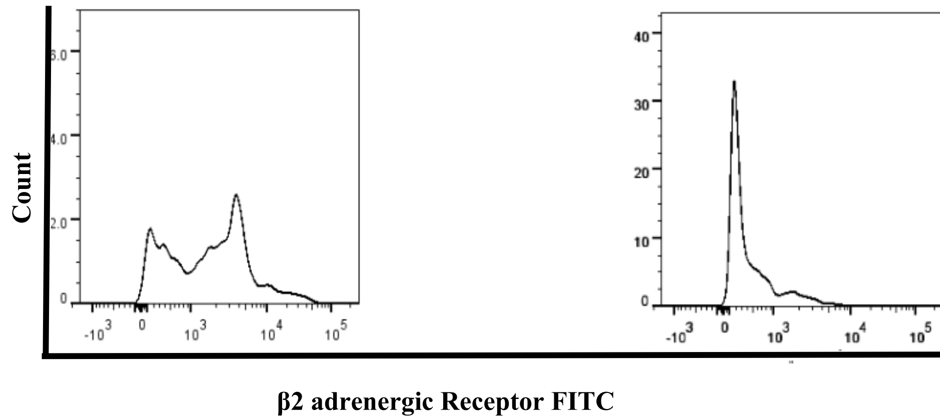
4.7 Expression of β2 adrenergic receptor on peripheral blood CD4+ T Cells

The expression of β2 adrenergic receptors was determined by flow cytometry using receptor-specific (mouse anti-human β2 adrenergic receptor) and cell type specific monoclonal antibodies (Brillant violet 421-conjugated mouse anti-human CD4, PerC-Efluor 710-conjugated mouse anti-human CD3 and PE-conjugated mouse anti-human CD25) as described in Materials & Methods section. Strategy for CD4 gating was similar to experiments as described earlier in Figure 20A. Specificity of antibodies is shown in Figure 22A. The expression of β2 adrenergic receptor on CD4+ T cells of healthy (control) and HIV-infected individuals is shown in Figure 22B in left and right panels respectively. The expression of the receptor on these cells was determined in 5 HIV-infected ART- and 5 healthy controls. The results are shown in Figure 22C. The expression of β2 adrenergic receptor tended to decrease on CD3+CD4+CD25- T cells of HIV-infected individuals although the decrease was not significant ($p>0.05$).

A



B



C

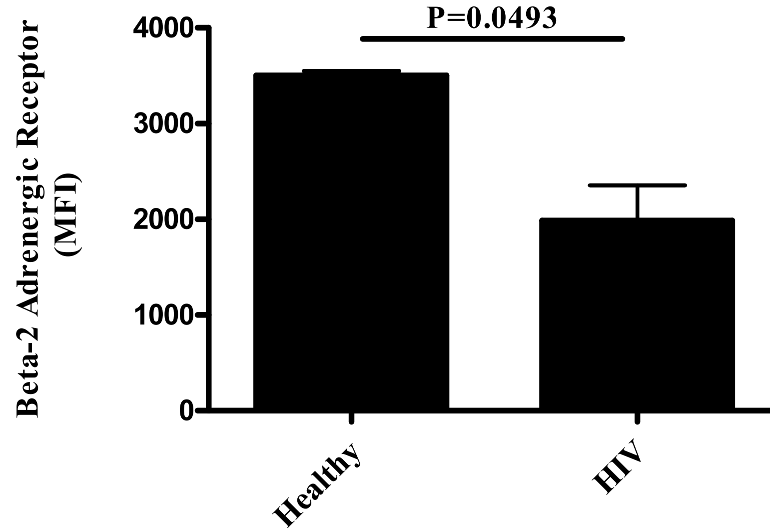


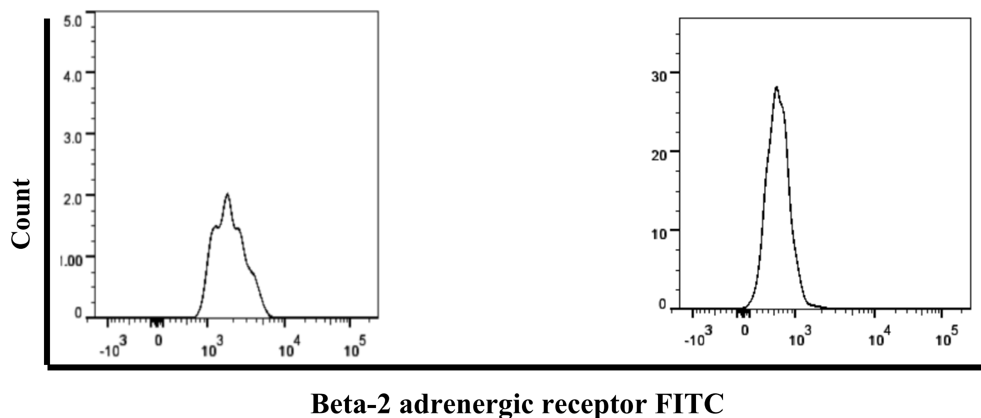
Figure 22. Expression of β2 adrenergic receptor on CD4+ T cells

(A) Gating strategy for CD4+ T cells was the same as shown in Figure 20A. For specificity of the antibodies, the cells were incubated with control antibodies (rabbit IgG; right panel) or with rabbit anti-human β2 adrenergic receptor (right panel), washed and stained with FITC-conjugated goat-anti-rabbit IgG. (B) The Figure shows typical histograms of the receptor expression on CD4+ T cells of a typical healthy control (left panel) and a typical HIV-infected individual (right panel). (C) Expression of β2 adrenergic receptor on CD4+ T cells of HIV-infected individuals. The Figure shows mean ± SD of MFI in arbitrary units from 5 healthy control and 5 ART- HIV-infected individuals. Means between the two groups were compared using T test. The differences in mean values were statistically non-significant ($p>0.05$).

4.8 Expression of β2 adrenergic receptor on CD3-CD14+ monocytes

The expression of β2 adrenergic receptor on CD3- CD14+ monocytes of peripheral blood of HIV-infected and healthy control individuals was determined by flow cytometry using receptor- and cell type-specific monoclonal antibodies. Strategy for gating monocytes was the same as described above in Figure 21A. Specificity of the anti- β2 adrenergic receptor was shown in Figure 22B. The expression of β2 adrenergic receptor on CD3-CD14+ monocytes from healthy (control) and HIV-infected individuals is shown in Figure 23A in left and right panels respectively. The expression of the receptor on monocytes was determined in 5 HIV-infected and 5 healthy control individuals. The results show a significant decrease in expression of the receptor on monocytes of HIV-infected individuals compared to those from healthy control individuals ($p<0.01$) (Figure 23B).

A



B

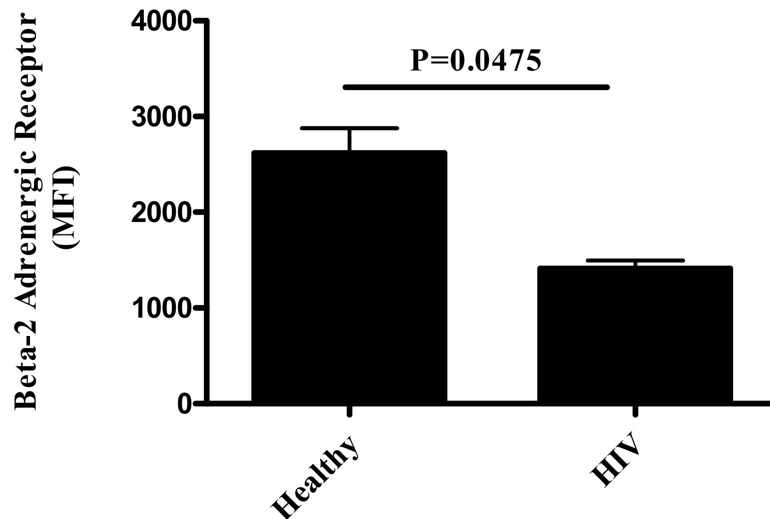


Figure 23. Expression of β2 adrenergic receptor on CD3-CD14+ monocytes in HIV-infected individuals

The gating strategy for the monocytes was essentially the same as shown above in Figure 21A. Specificity of the anti-receptor antibodies is shown above in Figure 22B. (A) Histograms showing the expression of β2 adrenergic receptor on monocytes from a healthy control (left panel) and from an HIV-infected individual (right panel). (B) The expression of β2 adrenergic receptor on CD3-CD14+ monocytes of HIV-infected individuals. The Figure depicts mean ± SD of MFI in arbitrary units from 5 healthy controls and 5 ART- HIV-infected individuals. Means between the two groups were compared using T test. Expression of the receptor decreased significantly in monocytes from HIV-infected donors as compared to those from healthy control donors ($p<0.01$).

5 Discussion

In this study, we investigated how components of the extra-neuronal cholinergic system are regulated in HIV-infected individuals. Our results show that ACh levels in the blood do not differ significantly ($p>0.05$) between HIV-infected individuals and HIV-seronegative healthy controls (Figure 16). In addition, we showed that ACh levels did not differ between primary and chronic infections, as well as between ART+ and ART- individuals. However, ACh levels tended to be higher in ART- PHI patients compared with ART+ PHI patients. Surprisingly, significantly higher levels of this neurotransmitter were observed in the blood of LTNP compared with those in HIV-infected and healthy control subjects ($p \leq 0.01$). Given the anti-inflammatory effects of ACh, it is tempting to speculate that the increased levels of ACh in the blood may contribute to diminish and/or delay occurrence of HIV-induced pathology in LTNP. Chronic inflammation is considered a major contributing factor for the progression of HIV (Nasi et al 2017). Although ACh is produced in the central and peripheral nervous tissues, it is rapidly degraded by AChE and does not contribute to ACh levels observed in blood (Olofsson et al 2012). Both immune and non-immune (e.g. intestinal epithelial) cells produce ACh upon activation and contribute to increase ACh concentrations in the circulation (Fujii et al 2012, Zdanowski et al 2015). Furthermore, production of ACh degrading enzymes (acetylcholinesterse and butyrylcholinesterase) decreases during an inflammatory responses (Das 2007, Olofsson et al 2012). This may represent a compensatory mechanism to diminish ACh degradation in order to maintain its concentrations in the blood and attenuate inflammation. Our results have some limitations. First, we used archived blood samples from HIV-infected and healthy control individuals. The freezing and thawing of blood samples may have caused some degradation of ACh and this may have affected our results. Future studies should be carried out with serum/plasma samples in which AChE inhibitors (e.g., physostigmine) have been added immediately after collection. Second, our sample size is relatively small. Therefore, these studies should be repeated using larger sample sizes.

The concentrations of SLURP-1 in the circulation of HIV-infected individuals mirrored those of ACh. SLURP-1 acts as an allosteric ligand for α7nAChR (Chimenti et al 2003, Fuji et al 2014). It is produced by activated T lymphocytes and epithelial cells. Upon activation, T

cells produce both ACh and SLURP-1, which bind sequentially to α7nAChR. SLURP-1 potentiates the effects of ACh and fine-tunes T cell activation. Dominant recessive mutations in the SLURP-1 gene result in Mal de Meleda, a genetic disease accompanied by palmo-plantar keratosis and aberrant T cell activation (Neumann 1898; Perez & Khachemoune 2016). Interestingly, SLURP-1 is very stable in the body and its concentrations can be readily detected in plasma, serum, urine and in other body fluids (Neumann 1898). Compared with ACh, SLURP-1 levels in serum samples from HIV-infected individuals show a clear trend of increase in primary and chronic HIV infections. Furthermore, SLURP-1 levels also tended to decrease in infected individuals receiving ART (Figure 17). This increase in SLURP-1 concentrations may result from increased activation of immune and non-immune cells in HIV-infected individuals. Similar to ACh levels, increased concentrations of SLURP-1 in the circulation of LTNP may play a role in their ability to control the progression of the disease.

The expression of α7nAChR is essential for the anti-inflammatory effects of ACh (Fujii et al 2007a, Fuji et al 2014, Kalkman & Feuerbach 2016). We used a specific agonist of α7nAChR to investigate the effects on HIV replication. While the agonist suppressed the antagonist increased HIV replication in human PHA blasts (Figure 18). Based on these observations, we postulate that an agonist of α7nAChR could potentially be used to reduce HIV replication and inflammation in HIV-infected individuals. The use of an agonist would be preferable to other ligands such as nicotine, which could also exert effects through other nAChRs and has been linked to cancer and other metabolic effects (Sanner et al 2015). A potential complication of the use of α7nAChR agonists could be their unintended effects on the central and peripheral nervous systems. However, potential side effects of the agonists on the central nervous system can be reduced by using agonists that do not cross the blood-brain barrier. Interestingly, the receptor agonists are currently being used to improve memory, cognition, and anxiety in humans in several brain diseases (Thomsen et al 2010).

We investigated the expression of α7nAChR on monocytes and CD4+ T cells in HIV-infected patients and in healthy individuals. Expression of α7nAChR was significantly reduced ($p<0.01$) on CD4+ T cells in ART- HIV-infected individuals compared with healthy controls. Furthermore, expression of α7nAChR was significantly higher in ART+ HIV-infected individuals compared to untreated (ART-) HIV-infected individuals ($p<0.01$). Nonetheless,

expression of α7nAChR was still higher in healthy controls compared with ART+ patients. Taken together, these results suggest that expression of α7nAChR is reduced on CD4+ T cells in HIV-infected individuals and that ART tends to increase its expression, yet it remains lower compared with healthy controls. Lower expression of α7nAChR may have implications for viral replication as well as for the inflammatory response to the infection. Given that α7nAChR stimulation decreases HIV replication in human PHA blasts, lower expression of α7nAChR may reduce the inhibitory effects of ACh on HIV replication as well as on the production of proinflammatory cytokines by immune cells. It is well known that α7nAChR is important for the induction of anti-inflammatory effects mediated by ACh (Wang et al 2003). Previous *in vitro* studies have demonstrated that gp120 can increase the expression of α7nAChR on monocyte-derived macrophages and that higher expression of the receptor was found on monocytes, macrophages, and T cells isolated from HIV-infected individuals (Bracci et al 1992, DelgadoVélez et al 2015). However, in these studies, expression levels of the receptor were measured using α-Bungarotoxin (α-BuTx), which can bind ACh receptors other than α7nAChR. In fact, α-BuTx was recently shown to bind and antagonize the γ aminobutyric acid (GABA) type-A receptors (Hannan et al 2015). These findings suggest that the reported increase in the expression of α7nAChR on immune cells in HIV-infected individuals should be re-assessed using receptor-specific monoclonal antibodies. Furthermore, the reported increase in expression of α7nAChR is not supported by functional studies. Despite increased expression of the receptor in gp-120 treated macrophages, ACh did not inhibit LPS-induced expression of proinflammatory cytokines (Delgado-Vélez et al 2015). Alternatively, immune cells treated with gp120 may have increased expression of some other α-BuTx-binding receptors that do not respond to ACh. A relative lack of response to ACh in immune cells from HIV-infected individuals strongly suggests that these cells may have down-regulated α7nAChR expression which supports our findings. Our results also provide an explanation for previous observations (Borges et al 2012) that noted a decrease in parasympathetic activity of HIV-infected individuals both at rest and after exercise. Studies on the expression of α7nAChR on human leukocytes have been complicated by recent discovery of a partially duplicated CHANA-7 gene (that encodes the α7 polypeptide, which pentamerizes to form an7nAChR). The duplicated gene, called CHRFAM-7A, is located on chromosome 15 about 1.6 megabase upstream of CHRNA7 (Costantini et al 2015a). The polypeptide encoded by CHRFAM7A gene has been

named as duplicated (dup)-α7. The dup- α7 polypeptide does not bind or respond to ACh, but acts as a negative regulator of α7nAChR expression. The ratio of expression between α7 and dup-α7 polypeptides is much lower on immune cells compared with the ratio found on nervous tissue cells. The α7nAChR receptor is known to be a homopentamer of α7 polypeptides. It has been suggested that α7 and dup-α7 polypeptide subunits may combine in different ratios to form heterogeneous novel nicotinic receptors for ACh on immune cells (Costantini et al 2015b; Figure 11). Since dup-α7 does not bind and respond to ACh, α7nAChRs (that contains one or more dup-α7 polypeptides) expressed on immune cells may have decreased responsiveness to ACh depending upon the number of dup-α7 subunits incorporated in them. Thus, the expression and accumulation of dup-α7 units into α7nAChR render these receptors (and the cells that express such receptors) less responsive to ACh (Figure 11). There are indications that the two genes (CHRNA-7 and CHRFAM-7A) may be regulated differentially in different human diseases. For example, an increase in expression of CHRFAM7A gene relative to the CHRNA7 gene occurs in the colon of IBD patients (Baird et al 2016). The α7nAChR expressed in the colon in these patients respond less to the anti-inflammatory effects of endogenous ACh and hence contribute to the persistence of chronic inflammation.

No study has so far examined the relative expressions of CHRNA-7 and CHRFAM-7A on immune cells in HIV-infected individuals. A practical difficulty in this regard is a lack of antibodies specific to dup-α7. In fact, it is feared that most (if not all) α7-specific antibodies might cross-react with dup-α7. Thus, the studies conducted on the expression of α7nAChR in human diseases will require confirmation by using antibodies that do not cross-react with dup-α7 polypeptide. A study in this regard has examined the relative expression of the CHRNA-7 and CHRFAM-7A genes in the post-mortem brains of HIV-infected individuals suffering from HIV-associated neurocognitive defects (HAND) by quantifying their transcripts (Ramos et al 2016). The results show that the expression of α7 is upregulated but that of dup-α7 is downregulated in these brains. *In vitro*, gp120 mimicked the receptor expression changes in neurons (Ramos et al 2016). It is noteworthy that α7 expression is normally higher than dup-α7 in brains, while the opposite is true for immune cells (Costantini et al 2015a, Costantini et al 2015b). Further studies are needed in order to determine whether similar changes occur in the

expression of these two genes in immune cells of HIV-infected individuals, and whether gp120 affects gene expression in immune cells similar to those reported in neurons.

Our results on the expression of α7nAChR receptor on immune cells of HIV-infected and healthy individuals have two caveats. First, these studies were conducted on a limited number of individuals (six HIV-infected and three healthy individuals) and hence need to be validated in a larger population. Second, we have used polyclonal antibodies for detecting α7nAChR expression. These antibodies are likely to cross-react with the dup-α7 polypeptide. Thus, future studies should be conducted using receptor-specific monoclonal antibodies that do not cross-react with different α7 polypeptides.

The expression the α7nAChR on peripheral blood monocytes did not differ significantly between HIV-infected and healthy controls ($p>0.05$) (Figure 21). This is interesting, as monocytes respond to ACh and reduce LPS-induced production of pro-inflammatory cytokines (Fujii et al 2007a, Rosas-Ballina et al 2011). We had anticipated that monocytes from HIV-infected individuals might show a significant decrease in the expression of this receptor and may be less responsive to the anti-inflammatory effects mediated by endogenous ACh. Nevertheless, these results are subject to the same caveats as described above for CD4+ T cells (i.e., the small number of patients studied and cross reactivity of used antibodies).

Additionally, we investigated the expression of β2 adrenergic receptor on immune cells in HIV-infected individuals. In mice, CD4+ T cells have been shown to be important in the function of the cholinergic anti-inflammatory pathway (CAP) that acts as a homeostatic reflex in response to increased concentrations of proinflammatory cytokines in the blood. CD4+ T lymphocytes respond to NOR released from sympathetic nerves that innervate the spleen and produce ACh (Rosas-Ballina et al 2011). Our results show that the expression of ACh tends to decrease in HIV-infected patients compared with healthy individuals but the difference between these two groups was not statistically significant ($p>0.05$). However, the expression of β2 adrenergic receptor was significantly decreased on monocytes of HIV-infected individuals. The implications of these findings are not completely understood. We speculate that these immune cells from HIV-infected individuals may respond less to NOR and could act as negative feedback players to increase the sympathetic tone observed in these individuals (Borges et al 2012).

We were interested in determining whether ACh concentrations affect HIV replication. For this purpose, we used HC-3, a compound that competes with choline for transport into cells. Given that choline is required for synthesis of ACh, the cells cultured in the presence of HC3 have a reduced ability to produce this ACh (Guyenet et al 1973). When HIV-infected PHA blasts were cultured in the presence of HC-3, they produced significantly higher amounts of HIV according to the viral p24 detected in their culture supernatants (Figure 19). Interestingly, the increase was the highest when determined 24 hours after treatment with HC-3. The increase in the viral replication gradually decreased over the next two days. These results suggest that decreasing the production of ACh by PHA blasts increases HIV replication. Although, we did not determine the concentrations of ACh in the culture supernatants, we investigated the effects of an agonist and an antagonist of α7nAChR on the viral replication. It is worth noting that this is the main nicotinic receptor on CD4+ T cells in humans and that this receptor is indispensable for the anti-inflammatory effects of ACh in humans and mice (Rosas-Ballina et al 2011). In order to determine the effects of α7nAChR activation on HIV replication, we used the receptor-specific agonists and antagonists and not ACh or nicotine, as these latter two compounds exert their effects on T cell by using α7nAChR as well as other receptors. Our results show that the receptor agonist decreases and the receptor antagonist increases HIV replication in human PHA blasts. Thus, α7 receptor on CD4+ T cells could represent a novel molecular target for diminishing viral replication in HIV-infected individuals. The agonists, due to their anti-inflammatory effects, could also reduce inflammation that accompanies HIV infections (Nasi et al 2017). Potential side effects of the agonists on the brain could be reduced by using an agonists that do not cross the blood-brain barrier.

Interestingly, α7nAChR agonists have been used to treat cognitive defects, dementia, and psychological disorders in human diseases like Alzheimer's, Schizophrenia, and Down's syndrome (Thomsen et al 2010). The expression of the receptor decreases in different areas of the brain in these patients, which might have contributed to the disease pathogenesis. Decreased expression of the receptor also occurs in lungs and brains of smokers. It is not yet clear what would be the global effects of the changes in the expression of the α7nAChR on immune cells in humans. However, it is noteworthy that an increase in the expression of dup-α7 (a negative regulator of α7) has been reported in the gut of IBD patients (Baird et al 2016). This may make

immune cells from these patients less responsive to effects of endogenous ACh and help in the persistence of intestinal inflammation. Taken together it is possible that lower α7nAChR expression may predispose humans to chronic proinflammatory diseases.

6 Conclusion

Extra-neuronal cholinergic system (ENCS) plays an important role in immune homeostasis in the body. It also exerts potent anti-inflammatory effects in the body. Results from the present study show that HIV causes significant perturbations in different components of the ENCS. Main disturbances noted included decreased expression of α7nAChR and β2 adrenergic receptor on CD4+ T cells in HIV-infected individuals. These disturbances could reduce the production of ACh from these cells and could make them less responsive to anti-inflammatory effects mediated by endogenous ACh. We found that reducing the production of ACh in human PHA blasts increased HIV replication in these cells. Most importantly, we successfully diminished HIV replication by increasing α7nAChR signaling with an agonist. In contrast, HIV replication was increased when α7nAChR signaling was reduced with an antagonists. These results have important clinical implications for HIV infections. They suggest that α7nAChR is an important receptor that could be targeted for controlling HIV replication. Current HIV therapies do not cure HIV infection and it is well known that latent HIV tends to hide in safe reservoirs inside patients. However, it is unclear if the use of an antagonist of α7nAChR could help to reveal these reservoirs by forcing latent viruses to replicate and become susceptible to HIV drugs. In addition, chronic low-grade inflammation and drug toxicity can predispose HIV-infected patients to a variety of non-AIDS-related co-morbidities. Agonists of α7nAChR may reduce HIV replication and reduce chronic inflammation in these patients. Interestingly, several receptor agonists have been developed and they are currently used to improve memory function, dementia, anxiety and other psychological diseases in humans. While some agonist and antagonist molecules can cross the blood-brain barrier, others have an action limited to the periphery. The finding that the modulation of α7nAChR signaling can impact HIV replication provides a novel perspective on HIV therapy that should be further studied in future pre-clinical studies.

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