From the Department of Medicine, Solna

NEUROIMMUNE MECHANISMS IN CHRONIC INFLAMMATION – TRANSLATIONAL STUDIES OF THE INFLAMMATORY REFLEX

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Neuroimmune mechanisms in chronic inflammation – Translational studies of the inflammatory reflex

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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The public defence will take place on Friday the 8th of June, 2018, at 9:00 am in the Rolf Luft lecture hall (L1:00, Karolinska University Hospital, Solna).

The true delight is in the finding out rather than in the knowing

- Isaac Asimov

ABSTRACT

A functional immune system is crucial for our survival from the pathogens and toxins we are constantly subjected to. For reasons only partially understood, in some individuals the immune response instead target self antigens, as suggested in rheumatoid arthritis (RA), or environmental non-pathogenic antigens, as suggested in allergy, leading to a failure of resolution and development of a state of chronic inflammation. Although current treatment strategies are largely effective at treating the peripheral inflammation, symptoms that can be attributed to the central nervous system (CNS) such as pain sensitization or fatigue often persist, causing considerable distress for the patient. A growing amount of evidence point towards that chronic inflammatory diseases are accompanied by central inflammation which could then be involved in driving CNS related symptoms.

In general, the immune response may result in damage not only to the pathogen but also to healthy tissues in the vicinity. Therefore it is essential that an inflammatory process is concluded as soon as the threat is cleared. Recently, the cholinergic anti-inflammatory pathway (CAP) was described promoting a fast vagus mediated control of systemic inflammation. The anti-inflammatory potential of CAP initiated clinical trials exploring the use of vagal stimulation as an alternative treatment strategy for immune suppression in human chronic inflammatory diseases such as rheumatoid arthritis. Even so, much remain to be understood regarding the mechanism of CAP and its anti-inflammatory extent.

In this thesis, work has been undertaken to explore the role of central nervous mechanisms in RA and seasonal allergy. Furthermore, CNS involvement in RA and other arthropaties was studied by mapping the cerebrospinal fluid proteome and its treatment associated changes. Additionally, CAP mechanisms were studied using animal models of endotoxaemia in a translational fashion. Whereas we could not detect an increased brain microglial activity in either RA or Allergy, we further confirm a state of autonomic dysregulation in RA with close associations to peripheral inflammation. We demonstrate for the first time that modern treatment strategies not only exert effects peripherally, but also lead to a central nervous reduction of inflammatory related proteins. We additionally identify several of these proteins as potentially important players to study further in the context of neuro immune responses. Furthermore, we provide evidence that the dependence of prostaglandins for a functional CAP is located to splenic events, demonstrating that prostaglandin E2 is important for acetylcholine production as well as immunosuppressive function in splenocytes. In addition, an activated CAP is shown to exert effects on additional immune cells and immune compartments than previously known.

Taken together, this thesis has contributed to further our understanding of CNS involvement in chronic inflammatory conditions, of effects exerted by commonly used as well as experimental treatment strategies and neural regulation of inflammation. In the future, the results here presented may hopefully benefit the patient by contributing to the development of improved treatment strategies and better understanding of disease pathology.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ett väl fungerande immunförsvar är viktigt för vår överlevnad och har utvecklats för att skydda oss mot infektioner och skadliga ämnen i vår omgivning som vi ibland kommer i kontakt med. Hos vissa individer fungerar dock inte immunförsvaret som det ska utan reagerar på kroppsegen vävnad, vilket sker vi ledgångsreumatism. Det kan även hända att immunförsvaret reagerar på ämnen i omgivningen som inte utgör ett hot mot oss, t.ex. gräspollen hos dem med hösnuva. Detta leder till ett ständigt aktivt immunförsvar och bidrar till kronisk inflammation i den utsatta vävnaden. Oftast hjälper befintliga behandlingar bra, men symptom som har sitt ursprung i hjärnan så som trötthet eller ändrad smärtkänslighet påverkas inte i lika stor grad av dagens anti-inflammatoriska behandlingar. Senare tids forskningsrön pekar mot att det inte bara pågår inflammation i den utsatta vävnaden utan att mekanismer i hjärnan också kan vara påverkade hos personer med olika kroniskt inflammatoriska sjukdommar. Detta skulle kunna vara en av orsakerna till att hjärnrelaterade symptom är så svårbehandlade.

Även hos helt friska personer är immunförsvaret ett tveeggat svärd och måste noga kontrolleras så att inflammatorisk aktivitet inte pågår längre än nödvändigt. Detta eftersom det under inflammation kan uppstå skada även på frisk vävnad. Kroppen är dock smart och har utvecklat en rad olika kontrollmekanismer. En av dessa kontrollmekanismer kallas kolinerg anti-inflammatorisk signallering (KAS) och utförs av vagusnerven som även sysslar med att kontrollera t.ex. våra hjärtslag eller matsmältning. En aktiverad KAS leder till att immunceller i mjälten hämmas i sin inflammatoriska aktivitet som annars bidrar till inflammation i hela kroppen. KAS fungerar därmed som en broms på immunförsvaret och kan på så vis dämpa inflammation. Forskare har nyligen kommit på att med hjälp av svaga elektriska impulser kan vagusnerven stimuleras och därmed aktivera KAS hos personer med kronisk inflammatoriska sjukdommar som inte svarar på befintliga behandlingar, för att på så vis minska deras inflammation. Trots att KAS är en relativt ny upptäckt och vi fortfarande inte vet allt om hur KAS fungerar eller hur bred effekt den har på immunförsvaret så har kliniska prövningar med elektrisk aktivering av KAS hos ledgångsreumatiker visat sig vara ett lovande koncept.

Mot bakgrund av detta ämnar arbetena beskrivna i den här avhandlingen att utröna om det finns en aktivering av immun-påverkande mekanismer i hjärnan hos individer med ledgångsreumatism eller allergi och om det finns ett samband mellan dessa mekanismer och hjärnrelaterade symptom hos dessa patienter. Att ytterligare studera hur hjärnan påverkas av inflammation hos reumatiker genom att kartlägga proteiner i ryggmärgsvätska och hur de förändras av behandling. Och till sist att utöka förståelsen för hur KAS fungerar med hjälp av inflammatoriska djurmodeller.

I denna avhandling finner vi indirekt ytterligare stöd för pågående inflammatorisk aktivitet i hjärnan hos reumatiker. Dessutom visar vi att det finns ett samband mellan minskad vagusnervaktivitet och ökad perifer inflammatorisk aktivitet vilket är i linje med tidigare forskningsresultat. För första gången påvisar vi även att modern antiinflammatorisk

behandling, vilken har större effekt på hjärnrelaterade symptom än tidigare behandlingar, inte bara påverkar perifer inflammation utan även har effekter centralt i hjärnan. Vi kan även peka ut ett antal proteiner som är särskilt intressanta att studera vidare för att ta reda på deras roll vid inflammatorisk aktivitet i hjärnan och hjärnrelaterade symptom. Vi har även lyckats slå fast att KAS påverkar fler immunceller än vad som tidigare visats och att immuncellerna i mjälten är beroende av prostaglandiner, små substanser producerade i kroppen vid inflammation som bland annat ger upphov till feber, för att KAS-bromsen ska fungera korrekt.

Sammantaget bidrar forskningsrönen från den här avhandlingen till en utökad förståelse för vilken roll inflammation i hjärnan spelar vid kroniskt inflammatoriska sjukdomar, hur moderna behandlingsstrategier påverkar hjärnan samt nervsytemets förmåga att kontrollera inflamation. I framtiden förväntas dessa forskningsrön att bidra till utvecklingen av nya och bättre anpassade behandlingsmetoder för personer med kroniskt inflammatoriska sjukdommar.

SCIENTIFIC PAPERS

I. Forsberg A*, Lampa J*, Estelius J, Cervenka S, Farde L, Halldin C, Lekander M, Olgart Höglund C, Kosek E. **Cerebral glia activity in patients with rheumatoid arthritis.** *Manuscript.*

II. Tamm S, Cervenka S, Forsberg A, Estelius J, Grunevald J, Gyllfors P, Karshikoff B, Kosek E, Lampa J, Lensmar C, Strand V, Åkerstedt T, Halldin C, Ingvar M, Olgart Höglund C, Lekander M. **Evidence of fatigue, disordered sleep and peripheral inflammation, but not increased brain TSPO expression, in seasonal allergy: A [C-11]PBR28 PET study.** *Brain Behavior and Immunity* 2018; **68**: 146-157.

III. Estelius J, Lengqvist J, Ossipova E, Idborg H, Le Maître E, Andersson LA M, Brundin L, Khademi M, Svenungsson E, Jakobsson PJ, Lampa J. **Mass spectrometry based analysis of cerebrospinal fluid from arthritis patients – Immune related candidate proteins affected by TNF-blocking treatment.** *Manuscript.*

IV. Estelius J, Le Maître E, Revathikumar P, Chemin K, Lampa J. **Vagus nerve stimulation decreases activation of select $CD4^+$ T cell populations and NK cells in LPS-treated mice.** *Manuscript.*

V. Revathikumar P, Estelius J, Karmakar U, Le Maître E, Korotkova M, Jakobsson PJ, Lampa J. **Microsomal prostaglandin E synthase-1 gene deletion impairs neuro-immune circuitry of the cholinergic anti-inflammatory pathway in endotoxaemic mouse spleen.** *Plos One* 2018, **13** (2):20, e0193210

* Contributed equally

ADDITIONAL SCIENTIFIC PAPERS

Le Maître E, Revathikumar P, Estelius J, Lampa J. **Increased recovery time and decreased LPS administration to study the vagus nerve stimulation mechanisms in limited inflammatory responses.** *Journal of visualized experiments* 2017, **121** (epub) DOI: 10.3791/54890.

CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChR	Acetylcholine receptor
ACPA	Anti-citrullinated antibodies
ACQ	Asthma control questionnaire
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
ANS	Autonomic nervous system
APC	Antigen presenting cell
AR	Adrenergic receptor
AS	Ankylosing spondylitis
AVP	Arginine vasopressine
B2M	Beta-2-microglobuline
BBB	Blood-brain barrier
BL	Baseline
CADM3	Cell adhesion molecule 3
CAP	Cholinergic anti-inflammatory pathway
CFB	Complement factor B
ChAT	Choline acetyl transferatse
CNS	Central nervous system
CNTN-1	Contactin-1
COX	Cyclooxygenase
CRH	Corticotrophin releasing hormone
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular patterns
DAS28	Disease activity score 28
DMARD	Disease modifying anti-rheumatic drug
EAE	Experimental autoimmune encephalomyelitis
ECG	Electrocardiography
ESR	Erythrocyte sedimentation rate

FGG	Fibrinogen gamma
FMO	Fluorescence minus one
HAQ	Health assessment questionnaire
HLA	Human leukocyte antigen
HPA-axis	Hypothalamic-Pituitary-Adrenal-axis
HRV	Heart rate variability
ICAM-1	Intercellular adhesion molecule 1
IF	Immunofluorescence
IFNγ	Interferon gamma
IFX	Infliximab
Ig	Immunoglobulin
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JAK	Janus kinase
JCA	Juvenile chronic arthritis
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAC-1	Macrophage antigen complex-1
MFI-20	Multidimensional fatigue inventory
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
mPGES-1	Microsomal prostaglandin E synthase-1
MS	Mass spectrometry
NA	Noradrenaline
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NSAID	Non-steroidal anti-inflammatory drug
NTS	Nucleus tractus solitaries
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBR	Peripheral benzodiazepine receptor

PET	Positron emission tomography
PGA	Patient global assessment
PGE_2	Prostaglandin E2
PNS	Parasympathetic nervous system
PRR	Pattern recognition receptor
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RMSSD	Root mean square of successive differences
SJC	Swollen joint count
SNS	Sympathetic nervous system
TJC	Tender joint count
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T cell
TSPO	Translocator protein
VAS	Visual analogue scale
VNS	Vagus nerve stimulation
WT	Wild type

1 INTRODUCTION

For a long period of time being diagnosed with a chronic inflammatory disease could mean a life in constant pain, disability and an early death. With the understanding of the concept of autoimmunity and development of more efficient treatment strategies a chronic inflammatory diagnosis in the 21st century no longer carries such burdens. However, in spite of an increasing amount of research being done in this field, there are still numerous aspects of autoimmunity and mechanisms of specific chronic inflammatory diseases that remain elusive. In addition, although current treatment strategies are vastly improved, they are sometimes expensive and may still not be able to efficiently tackle every symptom associated with chronic inflammatory diseases, especially symptoms related to the central nervous system. Consequently, hospitalization and care of patients with such diseases remain a considerable economic burden for society. It is therefore vital to continue research to improve patient quality of life as well as to reduce the burden of patient care on society.

1.1 A BRIEF LOOK AT THE IMMUNE SYSTEM

Throughout history, the constant arms race between host and invading pathogens has led to the evolution of the ever more sophisticated defence mechanisms that we today know as our immune system.

1.1.1 Innate and adaptive immunity

The immune system is generally divided into two parts, innate and adaptive immunity. The innate immunity is considered the main provider of a quick but unspecific first line of defence against invading pathogens. Adaptive immunity instead predominantly gives rise to a slow but highly specialised second line of defence. Adaptive immunity may also give rise to immunologic memory, thus providing a highly efficient counter-attack system to combat re-infection.

The innate immune system is comprised of several cell types e.g. neutrophils and macrophages, detecting threats via specific pattern recognition receptors (PRRs) such as toll-like receptor (TLR) family members[1,2]. These receptors indiscriminately recognize highly conserved structures on e.g. invading pathogens, so called pathogen-associated molecular patterns (PAMPs), e.g. lipopolysaccharides (LPS), or signs of tissue damage via damage-associated molecular patterns (DAMPs)[3]. Tissue resident macrophages are usually the first cells to detect threats via their PRRs which may then trigger a pro-inflammatory response program. Circulating neutrophils and monocytes provide a reservoir of immune cells that can quickly be recruited to sites of inflammation to aid in the assault on the pathogen by phagocytosis and the release of toxic mediators such as microbicidal agents, H_2O_2 or NO[4,5]. At the battlefield pathogens and resulting debris is efficiently cleared by macrophages as well as neutrophils[4,5]. The innate immune system does not only consist of cells, but is also accompanied by a protein based system - the complement system.

The complement system consists of an array of proteins with inherent proteolytic capacity, predominantly produced by the liver and then released into circulation [6]. The proteolytic activity of circulating complement proteins may be triggered through three distinct pathways via interactions mediated by antibodies (classical pathway), lectins (lectin pathway) or by self activation of C3 convertase (alternative pathway)[6]. Activation of the complement system via any of these pathways promotes a cleavage cascade of the complement proteins into their active components [6]. Some complement components (e.g. C1q and C4b) can attach to antigens on the surface of pathogens or damaged cells (opsonisation) to guide and assist clearance by neutrophils and macrophages [6]. The complement components C5b, C6, C7 and C9 may instead together form pore structures known as the membrane attack complex (MAC) on the cell membrane of infected cells or invading pathogens, thus subjecting them to free diffusion [6,7]. When enough MACs are accumulated on the target cell surface it can no longer cope with the stress of free diffusion and dies [6,7]

The adaptive immune system is primarily comprised of a group of highly specific immune cells where each cell may recognise a unique peptide sequence. The major players in the league of adaptive immunity are different subtypes of T cells and the antibody producing B cells. This wide selection of specificities in the adaptive immune cell pool allows immune responses to become tailored to different pathogens. It also provides the ability for formation of an immunological memory which will lead to a quicker adaptive immune response upon reinfection with the same pathogen[8].

The unique specificities of the adaptive immune system are mediated by B- and T cell receptors as well as major histocompatibility complexes (MHCs) which in humans are referred to as human leukocyte antigen (HLA)[9]. Activation of adaptive immune cells occur when the B- or T cell receptor recognises their specific target peptide sequence as it is presented on one of the two MHCs[10-13]. MHC-I is expressed on the cell surfaces of all kinds of cells in the surrounding tissues while MHC-II is predominantly expressed on specialised antigen presenting cells (APCs)[12]. Upon activation B- and T cells start to proliferate and at the same time undergo adaptation, a process termed affinity maturation, to fine tune its respective pathogen recognition and subsequent effector response [10,14,15].

An efficient immune response where innate and adaptive immunity effortlessly work together is orchestrated via a host of inflammatory mediators, known as cytokines and chemokines, which are small proteins produced and released into the microenvironment by immune cells as well as cells in the inflamed or damaged tissue[16].

Although effective at keeping infection at bay, especially the inflammatory innate immune responses may often cause substantial damage also to healthy cells and tissues in the vicinity of an ongoing immune response[17]. It is therefore important that such inflammatory processes are ended as soon as the threat is neutralised to limit tissue damage and to prevent the development of chronic inflammatory states[18]. Resolution of inflammation is considered an active process involving a variety of mechanisms contributing to shifting a pro-inflammatory

microenvironment into an anti-inflammatory/healing microenvironment[18]. Such mechanisms include transformation of macrophages into anti-inflammatory/healing phenotypes, limiting neutrophil recruitment into inflammatory sites and their inflammatory activity, release of pro-resolving mediators e.g. resolvins and importantly immune suppression via neural pathways[18].

1.1.2 Cells of the immune system

The cells of the immune system are produced in the bone marrow from common haematopoietic stem cells through a set of developmental stages termed haematopoiesis.[19].

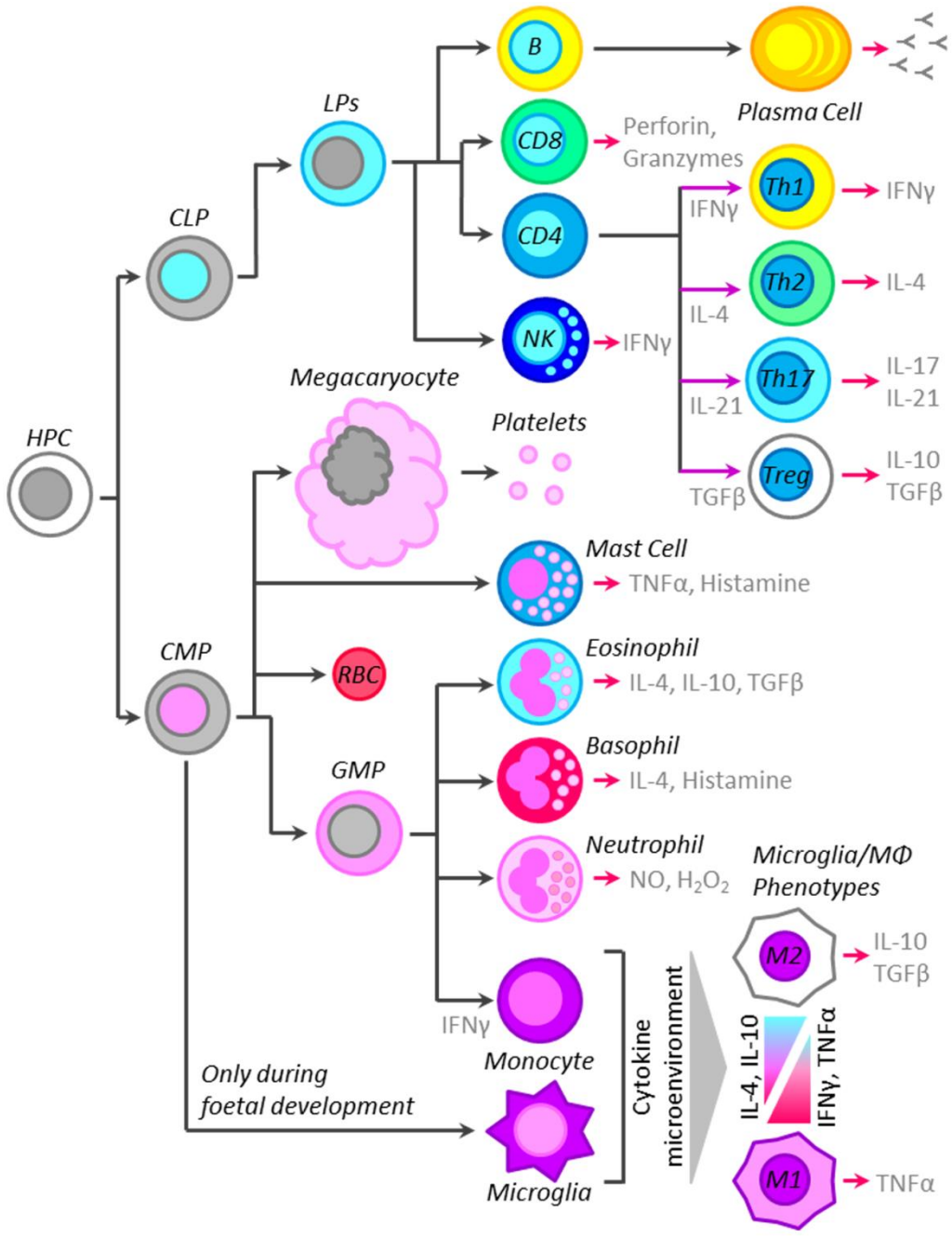


Figure 1 Schematic overview of the haematopoietic development and differentiation of immune cells of interest for this thesis. HPC: Haematopoietic stem cell, CLP: Common lymphoid progenitor, LPs: Lymphoid Progenitors, CMP: Common Myeloid Progenitor, GMP: Granulocyte Monocyte Progenitor, RBC: Red Blood Cell, MΦ: Macrophage. Adapted from [4,20-22].

As can be discerned from **figure 1** our immune system is an exceedingly complex network including many types of cells, each with its specialised function and responsibility, all highly interdependent on each other. All immune cells may be equally important since they are in one way or another involved in every aspect of immune responses in both health and disease. However, in the following section only the immune cells of particular relevance for the works in this thesis are briefly introduced.

1.1.2.1 Granulocytes

Many of the cell-types included in the innate immune system contain intracellular granules filled with various microbicidal agents ready to be released to the demise of any invading pathogen. They are therefore collectively called granulocytes and consist of neutrophils, eosinophils, basophils and mast cells.

Eosinophils, basophils and **mast cells** are normally involved in immune responses to parasites by releasing various parasitocidal proteins and enzymes such as proteoglycans and histamine stored in their intracellular granules[4,23]. However, they have also been shown to readily produce other immunoregulatory mediators such as prostaglandins and cytokines indicating that they also may play a role in directing the immune response[4,23,24]. Eosinophils and basophils are recruited to inflamed tissues from the blood while mast cells are tissue resident cells. Interestingly, mast cells are closely involved with the nervous system and have been shown to express receptors for neurotransmitters e.g. acetylcholine, and can be found in abundance in certain areas of the central nervous system (CNS) such as the thalamus[25]. They have additionally been suggested to play a part in pain transmission [26].

Eosinophils, basophils and mast cells also have the ability to react to immunoglobulin (Ig) E, the principal antibody involved in allergy and asthma[27]. Together with their inherent histamine production they are consequently highly involved in various aspects of allergy and asthma pathogenesis [4,23,24].

1.1.2.2 Monocytes and Macrophages

Monocytes are innate immune cells which circulate in the blood and upon infection migrate to sites of inflammation in response to interferon gamma (IFNγ). At the inflammatory site monocytes may differentiate and adopt characteristics of macrophages or dendritic cells depending on the local cytokine microenvironment [28]. Monocyte derived macrophages have been shown to be avid phagocytosers and producers of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα)[28]

Macrophages residing in tissues are under normal conditions predominantly contributing to the maintenance of homeostasis and continuously sample the tissue in search for threats[4] Monocyte derived- as well as tissue resident macrophages are highly plastic and upon activation can transform into a variety of different phenotypes with specialised functions depending on the current cytokine environment[4,20]. The phenotypes are ranging in a broad spectrum from the classically activated pro-inflammatory (M1) macrophages producing large

quantities of e.g. TNFα, promoting Th1 and Th17 responses, to the regulatory IL-10 producing macrophages and the suppressive alternatively activated anti-inflammatory (M2) macrophages [4,20]. Macrophages are one of the cell types that transcend the boundaries of innate/adaptive immunity with important functions in both systems [4]. Additionally, splenic macrophages have been shown to be essential players in neural regulation of inflammation[29].

1.1.2.3 Natural killer (NK) cells

NK cells are important players of innate immunity, efficiently killing infected, foreign or tumour cells. In addition, NK cells are also avid cytokine producers and are considered an important source of cytokines, particularly IFNγ, in the early immune response[30]. The activation of NK cells is determined via a delicately balanced interaction of activating and inhibitory surface receptors that are skewed towards activation by e.g. recognition of infected cells[31,32]. Intriguingly, a potential involvement of NK cells in contributing to disease pathology is discussed in several autoimmune diseases including different forms of arthritis [33-35] as well as depression[36].

1.1.2.4 CD4⁺ T cells

T cells are known to be produced in the bone marrow and to continue their development in the thymus, entering the blood as antigen inexperienced (naïve) cells[21]. Following activation naïve cells may differentiate into their effector function and upon clearance of the threat most effector cells are reported to die, leaving only a small number of cells (memory cells) to rapidly generate an already fine-tuned response upon re-encountering the same threat[37].

T cells are generally divided based on their surface expression of CD8 and CD4 molecules[38]. $CD8^+$ T cells display cytotoxic properties and are principally involved in immune responses directed toward intracellular pathogens[38]. $CD4^+$ T cells may instead upon activation give rise to a plethora of specialized effector cells predominantly involved in different aspects pathogen immune responses and immune regulation[39]. Like for macrophage phenotypes, regulation of $CD4^+$ effector T cell differentiation and subsequent function may be attributed to the nature of the activation stimulus and the cytokine environment[39].

Th1 cell differentiation is shown to be dependent on a cytokine environment containing IL-12 and IFNγ[21,40]. Once differentiated, Th1 cells predominantly take part in combating intracellular pathogens by production of large quantities of IFNγ[40]. Elevated levels of IFNγ are shown to contribute to the maintenance of the response of phagocytosing innate immune cells[20,40]. IFNγ expression is mediated via the lineage specific transcription factor T-bet[21].

Th2 cell differentiation has instead been shown to be dependent on IL-4 and IL-2 in the local environment[21,39]. Differentiated Th2 cells are predominantly involved in responses against parasites and allergens via the transcription factor GATA3 mediated IL-4 production[21,39].

Th17 cell differentiation is furthermore shown to depend on the presence of IL-21 as well as IL-6, IL-23 and TGFβ[21]. The main effector cytokine of Th17 cells is reported to be IL-17 with expression mediated via the Th17 principal transcription factor RORγt[21,41]. Th17 cells are

described to be particularly adept at helping to combat fungal or extracellular bacterial infections[21] but may also be closely linked with autoimmunity. For example, Th17 cells have been shown to be involved in bone destruction in an experimental model of rheumatoid arthritis (RA)[42].

Regulatory T cells (Tregs) are generally divided into natural Tregs developing in the thymus and induced Tregs developing in the periphery from ordinary naïve $CD4^+$ T cells[43]. Tregs are principally inhibitory and are thus described to be important in suppressing illicit pro-inflammatory responses to self[43]. This inhibitory function may be exerted on the immune system through several processes including production of potent anti-inflammatory cytokines such as IL-10 and TGFβ[43]. Because of their important immunosuppressive function, dysregulation of Tregs are often ascribed as one of the contributing factors to the development of chronic inflammation and autoimmunity[43].

1.1.2.5 B cells

Unlike T cells, B cells are known to undergo development in the bone marrow through a set of developmental stages and then migrate into lymphoid tissues[44]. In lymphoid tissues B cells mostly reside in special areas termed B cell follicles where they largely depend both upon having antigen transported to them and T cell help for activation[44]. The process of B cell transformation into actively antibody releasing plasma cells involves antibody class switching and affinity maturation and the characteristic formation of germinal centres[44]. Fully activated B cells may then respond by proliferation and production of antigen specific antibodies[44]. Like T cells, after resolution of inflammation a minority of adapted B cells are shown to remain to build up the memory pool[45]. Many autoimmune diseases, including arthritis, are characterised by autoantibodies[46]. However, the pathogenicity of the respective autoantibodies is not fully determined. Furthermore, B cell contribution in allergy via production of IgE antibodies is well established[27]. Consequently B cells are an important cell type to consider for many chronic inflammatory conditions.

1.2 A DISTURBANCE IN THE SYSTEM - CHRONIC INFLAMMATION AND AUTOIMMUNE DISEASE

To have such a delicately balanced immune system is a true evolutionary advantage to protect us from harm. However, the capacity for specific recognition and the ability to mount a perfectly tailored response to a wide variety of distinct pathogens and threats increase the risk of creating autoreactive immune cells[47]. It was long believed that it was impossible for the immune system to break tolerance and turn on the host[48]. As science progressed it soon became evident that even though rigorous control checkpoints are in place throughout both T- and B cell development, autoreactive cells may escape and in a susceptible host contribute to the development of autoimmunity and chronic inflammation[45,49]. Although the exact pathways leading to autoimmunity and chronic inflammation are incompletely understood and likely vary between diseases, several possible mechanisms for triggering cells initially considered non-autoreactive at the checkpoints have been suggested[50]. During e.g. bacterial infection concentration of self-antigens normally not seen in the extracellular environment

may become increased, often as a result of tissue damage[50]. In a process termed bystander activation, elevated levels of self-antigen coinciding with an environment of strong pro-inflammatory signals may lead to the activation of autoreactive immune cells[50] (**figure 2**). Immune cells may additionally come across pathogen derived peptides that share resemblance with self-peptides[51]. During such situations autoreactive immune cells that target host tissues may become activated by the cross reactive antigen and start responding to host tissue after clearance of the initial infection, a mechanism termed molecular mimicry[51] (**figure 2**). During infection with some pathogens post-translational changes of native proteins may be induced by the pathogen. For example, during periodontal infection with *p. gingivalis* citrullination (i.e. the enzymatic transformation of arginine into citrulline) has been shown to increase locally[52]. This process of exacerbated citrullination is discussed as one of the driving factors of RA development[53]. Such processes may lead to immune recognition of the same type of post-translational modification not only on the initial protein but also on other non-related proteins via a mechanism termed epitope spreading[54].

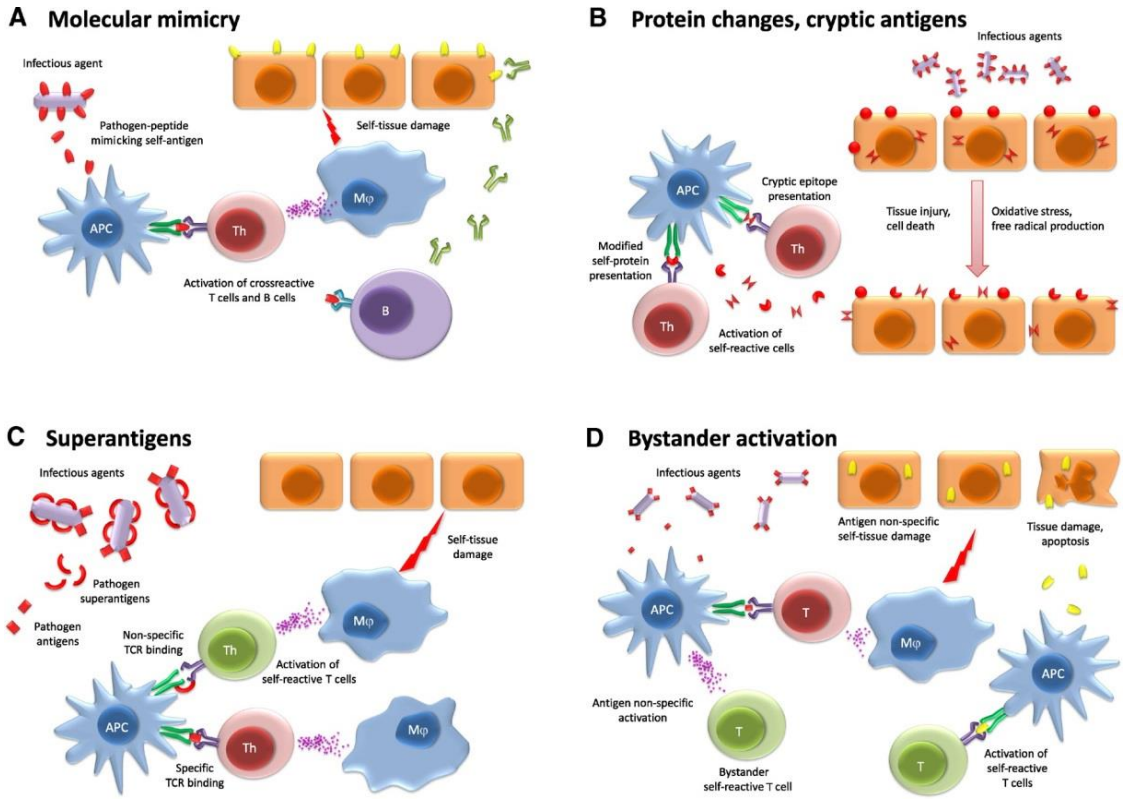


Figure 2 Examples of circumstances that may lead to activation of autoreactive immune cells during inflammatory events including **A)** Molecular mimicry and **D)** Bystander activation. Reprinted by permission from Journal of Leukocyte Biology [55]

Together, these mechanisms provide potential pathways where failure of resolving the immune response may lead to a state where the immune system is constantly triggered may lead to the development of various chronic inflammatory diseases such as allergy and arthritis. With chronic inflammatory conditions on the rise in the developed world further investigation into disease etiology is warranted.

1.2.1 Seasonal Allergy (Allergic Rhinitis)

Seasonal allergy is reportedly increasing, reaching a prevalence of around 30% in Sweden[56] and between 10-40% world-wide [57,58], however, regional variation has been shown to be high[59]. Seasonal allergy is commonly characterised by symptoms such as sneezing, itchy eyes and nose and rhino conjunctivitis likely caused by local mucosal inflammation initiated by the inhalation of allergens[58,60]. Risk factors for developing seasonal allergy include both environmental (e.g. western lifestyle, reduced childhood infections, exposure to airborne pollutants and altered gut microbiota) and genetic risk factors (e.g.polymorphisms in IL-4 and IgE receptor genes and the HLA-DR locus)[61].

Seasonal allergy is considered a Th2 driven disease mediated by defective IgE antibody production leading to mucosal inflammation and influx of eosinophils and basophils[58,60]. Allergens have been shown to drive differentiation of Th2 cells which via IL-4 excretion may promote IgE production from B cells[58,60]. IgE may then be captured by the numerous Fc receptors expressed on the surfaces of mast cells, eosinophils and basophils[58,60]. During exposure to allergen, i.e. to pollen during pollen season, the allergen can crosslink the surface-bound IgE on mast cells, eosinophils and basophils and may thus induce histamine release and an inflammatory response[58,60]. Developing allergy is considered a two-step process with a hypersensitivity reaction during the first encounter with the antigen and allergic response during the subsequent allergen encounters[58,60]. Treatment for seasonal allergy was long consisting only of avoiding allergen exposure and pharmacotherapy targeting histamine release (i.e. anti-histamines) or corticosteroids targeting local inflammation[62]. Recent advances in immunotherapy provide an attractive treatment strategy whereby the immune system is slowly desensitised to the allergen leaving a majority of subjects free of symptoms[58,63].

1.2.2 Rheumatoid arthritis (RA)

Rheumatoid arthritis is a chronic inflammatory disease characterised by joint swelling and subsequent joint destruction due to synovial inflammation[64]. RA mainly affects the smaller joints in the hands although involvement of the large joints such as knees and shoulders are also frequent. RA is one of the more common autoimmune diseases with an overall incidence of 41/100,000 in Sweden and a world-wide prevalence of around 1%[64-66]. Autoantibodies are considered a frequent feature in RA, where the RA-specific anti citrullinated protein antibodies (ACPAs) are associated with more severe disease[67,68].

In RA, major risk factors include both genetic and environmental contributors. For example, a genetic risk factor attribution of about 50-60% is inferred from twin studies[65]. Among the strongest genetic risk factors associated with RA development are specific RA associated HLA alleles (e.g. HLA.DRB1 and PTPN22), affecting both disease susceptibility and disease severity[65,69]. Furthermore, both viral (e.g. Epstein-Barr[70]) and bacterial (e.g. *P. gingivalis*[71]) infections as well as exposure to small particular substances (e.g. textile dust[72] or cigarette smoking[53]) have been reported as environmental factors contributing to the risk of developing RA[65]. Interestingly, risk factors are not fully shared between $ACPA^+$ and $ACPA^-$

disease, as illustrated by the risk of cigarette smoking which is only an important risk factor for the development of ACPA$^+$ RA[53].

The underlying mechanisms driving the disease are still not completely understood, but are gradually becoming clearer and growing evidence indicate that both innate (e.g. NK cells[73]) and adaptive (T- and B cells[74,75]) immune cells contribute to disease pathogenesis. Involvement of many pro-inflammatory cytokines such as TNFα produced by innate immune cells are additionally considered important[69,76]. It is further understood that the triggering event most likely takes place in the mucosal tissues and probably happen several years before disease onset, as a rise in autoantibody titres is detectable long before diagnosis[77,78].

Treatment strategies for RA include both pharmacological and biological approaches directed to immune suppression with the goal of reaching inflammatory remission and symptom relief. The first-line approach is the use of methotrexate, a synthetic disease modifying anti-rheumatic drug (DMARD). If methotrexate is found to be inefficient other DMARDs and biologic treatments are tried. Biologic treatments were introduced in the form of TNF blocking antibodies in the 90's and to date five such biologic drugs (infliximab, etanercept, adalimumab, golimumab and certolizumab pegol) are available on the market[79]. These biologics have been shown to have similar efficacy in RA[79]. Additional biologic treatments include abatacept inhibiting T cell co-stimulation, tocilizumab inhibiting the IL-6 receptor, rituximab depleting B cells and anakinra blocking the IL-1β receptor.[80,81] Furthermore, the targeted synthetic DMARDS baracitinib and tofacitinib which are inhibiting janus kinase (JAK), one of the key proteins in the signalling pathway of pro-inflammatory cytokine production, have been recently added to the pool of available anti-rheumatic drugs[82].

In addition, concomitant therapies such as corticosteroids which can be administered orally or locally via intra-articular injection[83], pain relieving non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen as well as physical exercise are pivotal in the optimal management of RA. Interestingly, a higher degree of physical activity before RA diagnosis is likely beneficial for the outcome of the disease[84].

With all these treatments at hand severe joint destruction and deformation traditionally associated with longstanding RA has become a rare phenomenon. Despite the availability of these treatments some symptoms such as pain and fatigue remain elusive and difficult to treat despite being frequently reported in RA patients, although, TNF-blockade is able to ameliorate these symptoms to some extent[85,86].

1.2.3 Other arthropaties

Several autoimmune diseases affecting joints exist beside rheumatoid arthritis. In this section the arthropaties important for the work in this thesis are briefly introduced.

1.2.3.1 Psoriatic arthritis (PsA)

PsA patients show psoriatic skin involvement in addition to joint inflammation, however, PsA can present with a wide variety of symptoms that together with the lack of specific biomarkers makes diagnosis challenging[87]. Incidence rates of PsA of around 6/100,000 have been reported[88]. There seem to be a strong genetic component of PsA, since family history generates an increased risk for development of PsA, and some HLA alleles are indicated[87]. Also environmental risk factors such as viral infection are discussed, although the specific nature of genetic and environmental factors remains to be discovered[87].

PsA is associated with a substantial effect on the patient's quality of life, predominantly attributed by the patients' own assessment to chronic pain and fatigue[89]. As many as 50% of PsA patients have been reported to suffer from fatigue[90]. Together with chronic pain being a common feature in PsA, the need to further understand PsA pathophysiology is illustrated[87].

1.2.3.2 Ankylosing spondylitis (AS)

In ankylosing spondylitis it is mainly the sacroiliac joints in the hip bone and vertebrae of the lower back that are subjected to inflammation which subsequently can lead to bone formation along the spine as the disease progesses[91]. Prevalence of AS varies substantially depending on country, with a prevalence reported between 0.1 and 1.4%[92]. Although, AS is generally reported more frequently in males than females a Canadian study report a recent increase in females diagnosed with AS[93].

AS have for a long time been known to have a genetic predisposition connected to the HLA-B27 loci with an excess of 90% of patients being reported as HLA-B27 positive[91]. The underlying mechanisms driving AS are poorly understood. One of the possible mechanisms discussed is molecular mimicry between *Chlamydia*[94] and enterobacterial[95] peptides and the self-peptide cytokeratin[96]. Another possibility put forward is related to the deposition of the HLA-I associated molecule beta-2-microglobulin (B2M) in synovial tissues[97]. In this model increased release of B2M from AS associated HLA-B27 sybtypes in complex with peptide is suggested, leading to increased B2M levels which may accumulate in synovia there contributing to an inflammatory response[97]. Treatment of AS include NSAIDs as a fist-line strategy and when these drugs are insufficient, TNF-blockers have provided efficient treatment effects in a majority of patients[98].

AS is commonly associated with physical as well as centrally associated symptoms such as fatigue, extensive pain and depression[99]. In a Scottish cohort moderate pain was reported in 70% of patients and severe pain in 15%[99]. In this cohort, fatigue was identified as one of five potentially modifiable factors contributing significantly to patient poor quality of life[99]. A study by Brophy and colleagues report that fatigue in AS is primarily associated to pain and that both pain and fatigue levels are reduced after initiating anti-TNF therapy[100].

1.2.3.3 Juvenile chronic arthritis (JCA)

While RA presents predominantly in middle aged women, Juvenile chronic arthritis is a heterogeneous group of diseases affecting joints in children under the age of 16 and has a reported prevalence of 33/100,000[101]. Since there is such large heterogeneity in JCA genetic risk factors are complex with many genes involved, but a strong association is reported to the HLA genes HLA-DR/DQ and HLA-DP[88]. Furthermore, seasonal variation in disease development suggests additional environmental involvement[88].

Fatigue as well as persistent pain are symptoms frequently reported also in JCA patients and are reported to be important contributors to a lower quality of life[102]. Interestingly, a correlation has been shown between measures of pain and fatigue in JCA[102]. In a study by Bromberg et.al. where JCA patients completed a one month symptom diary, it is revealed that none of the patients were completely free of pain during the study period with up to 86% of participants reporting at least one entry of high pain[103]. Interestingly, there were no reported difference in either pain or fatigue levels between patient groups receiving biologic treatments compared to conventional DMARD treatment, highlighting the difficulty of conventional treatments to sufficiently deal with these symptoms[103].

It is important to note that although disease pathology/etiology may differ between the arthropaties here presented, important features such mechanisms linked to fatigue and pain and a substantial involvement of TNFα have been described in all of them. On the basis of TNF involvement in the pathogenesis of these diseases, use of TNF-blockade generally show good efficacy on disease progression, and CNS related symptoms such as fatigue has also been proven to be relieved to some extent[104,105]. Together this indicates a possible role of TNF driven peripheral and/or central inflammation in part driving CNS related pathology in these diseases.

1.3 A BRIEF LOOK AT THE NERVOUS SYSTEM

The nervous system is vital for our body to function properly. It is generally divided into the peripheral nervous system (PNS) including the peripheral nerves and the central nervous system (CNS) including the brain and the spinal cord. The peripheral nerves are either afferent, i.e. leading information from the periphery to the CNS or efferent, i.e. communicating information from the CNS to the periphery. The CNS is organised into different structures and brain centres each highly interconnected and each specialised at processing information and directing response actions regarding respective organs or functions [106].

The PNS can in turn be additionally divided into two sections. The somatic nervous system handling voluntary movements via innervation of skeletal muscles and the autonomic nervous system (ANS) handling and directing involuntary bodily functions[106].

1.3.1 The autonomic nervous system (ANS)

The peripheral nerves of the ANS are described to innervate most of our internal organs including the smooth muscle cells of the heart as well as secretory glands[106,107]. The main function of the ANS is considered to be maintenance of bodily homeostasis. Typically homeostasis of any given organ or process is maintained by a reflex arch. The universal reflex arch generally consists of visceral afferent neurons together with efferent preganglionic neurons synapsing on post ganglionic neurons that are innervating the target organ or tissue[106,107]. The afferent neurons are able to sense the state of the organ and this information is then relayed to the corresponding control centres in the CNS. In the CNS the incoming information is processed and information about the appropriate response to take to restore homeostasis is communicated to the organ[107]. The ANS can be further divided into the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS)[107]. The two systems are each considered responsible for a variety of autonomic functions relating either to a body at rest or a body at an alerted state often termed the "rest and digest" and "fight or flight" responses. The PNS mainly governs rest and digest responses such as reduction of heart rate and release of digestive enzymes while the SNS mainly governs the fight or flight responses such as elevated heart rhythm and adrenaline release.

Neurotransmitters released at nerve terminals and synapses ensure quick and accurate signal transduction between neurons. Acetylcholine (ACh) is the principal neurotransmitter of the preganglionic neural communication in both PNS and SNS, however, SNS post ganglionic signal transduction is mainly mediated via noradrenaline (NA) while PNS post ganglionic signalling continues to be principally mediated by ACh[107,108]. The effect of NA and ACh release in the target tissues can then be further fine-tuned by an array of respective receptors. For NA two subtypes of receptors have been described, the α- and β adrenergic receptors, each family consisting of further subdivisions based on receptor location, function and intracellular signalling pathway [107]. Two families of receptors are described also for ACh, the nicotinic and muscarinic receptors. The muscarinic receptors are mainly found in target tissues where they are involved in intracellular signalling processes mediating the target response[107]. The nicotinic receptors are instead predominantly found on post ganglionic neurons, there important for mediating signal transduction via regulation of intracellular ion levels (Na^+ and Ca^{2+})[107]. To maintain a tight control of the effector tissue, the neurotransmitters are quickly cleared after release, either by degradation (ACh) or by reuptake (NA) [107].

The organs controlled by the ANS are for the major part innervated by neurons of both PNS and SNS origin as illustrated in **figure 3**. These two systems, often providing opposite responses, are working together in a finely balanced fashion to regulate and maintain homeostasis [108].

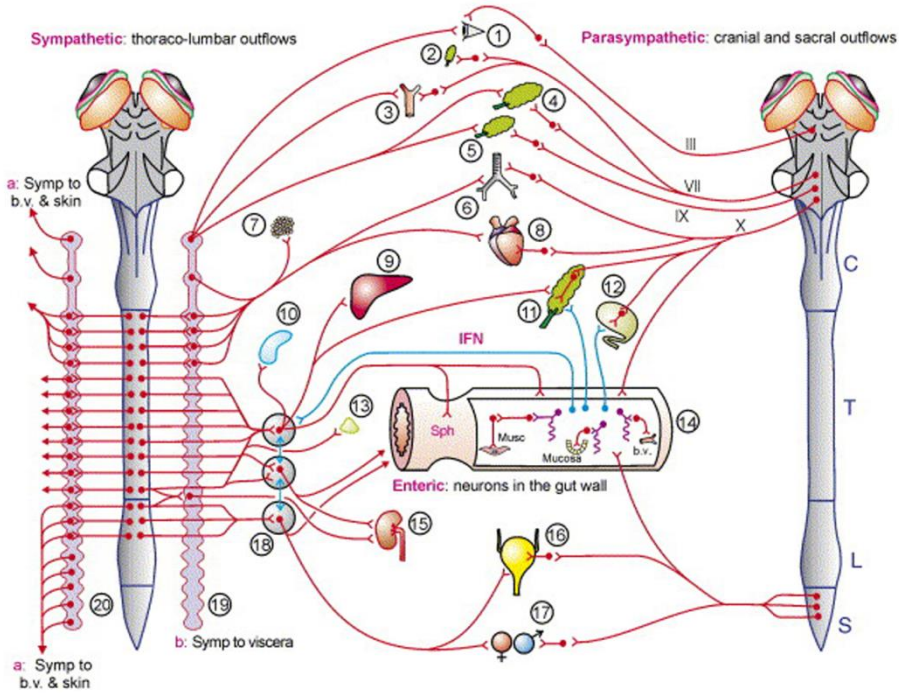


Figure 3 Overview of the organisation of the sympathetic and parasympathetic efferent arms of the autonomic nervous system with characteristic dual innervation of multiple internal organ systems. Parasympathetic vagus nerve (X) innervation is including the heart (8) and intestine (14). Sympathetic innervation is including the heart (8), intestine (14) and the spleen (10). Reprinted by permission from Autonomic Neuroscience[106].

1.3.2 Protecting the CNS

The neurons in the CNS are largely taking care of the processes keeping us alive. For optimal signal transduction and functioning a highly specialised microenvironment is required. It is thus essential for the body to protect not only the neurons but also the CNS microenvironment to ensure that any of our vital functions are not compromised.

1.3.2.1 The blood brain barrier (BBB)

The blood brain barrier (BBB) initially evolved to provide and maintain a highly controlled ionic microenvironment for optimal neural communication within the CNS, and is now integral for maintaining CNS micro environmental homeostasis. The BBB is comprised of the endothelial cells, and the specialised tight junctions between them, forming the capillaries supplying the CNS with oxygen and nutrients. The tight junctions provide a barrier for larger water-soluble blood borne molecules such as cytokines as well as cells and pathogens, effectively blocking their entry to the CNS. The endothelial cells are in turn encapsulated by a layer of extracellular matrix proteins forming the basement membrane which is produced in part by the endothelial cells themselves and in part by astrocytes. The BBB capillary is then hugged by the different cell types of the CNS, primarily pericytes and astrocytes which are involved in regulating BBB function and permeability, but also including microglia and neurons. [109]

To not deprive the CNS of essential nutrients and ions required for optimal performance, there are a variety of BBB transport systems in place for ions and macromolecules needed by

the CNS. Lipid soluble molecules may however diffuse freely into the CNS across the endothelial cells[109]. Transport across the BBB of lipid mediators with poorer diffusion qualities e.g. being acidic, may be facilitated via active efflux carriers while larger water soluble macromolecules can be transported via solute carriers as well as receptor mediated or adsorptive mediated vesicular transport systems[109,110]. For example, the presence of saturable transportations systems across the BBB for cytokines such as TNFα as well as interferons has been reported[111]. However, some substances do not have to cross the BBB to have an effect on the CNS. For example, binding of some cytokines or PAMPs such as IL-1β or LPS to receptors on the blood facing side of the endothelial cell has been shown to induce endothelial cell production of small inflammatory mediators such as NO or prostaglandins which easily diffuse through the BBB[112,113]. It has also been shown that the BBB endothelial cells can be induced by LPS on the blood facing side to produce and release pro-inflammatory cytokines at the CNS facing side[114]. Additionally, pathways for cellular entry across the BBB have been described including modified permeability at tight junctions (paracellular pathway) or directly through the endothelial cells, a process called diapedesis[109,110]. The permeability of the tight junctions of the BBB may quickly be controlled either by factors circulating in the blood or factors being locally produced in the CNS to modify entry via the paracellular pathway[109,110]. There are also specialised areas of the CNS where the permeability of the BBB is found to be increased through fenestration allowing larger molecules such as cytokines, or as demonstrated by Carrithers et.al lymphocytes, to pass through[115,116]. Such areas are found in the circumventricular organs and the choroid plexus[115]. BBB structure and transport systems are summarised in **figure 4**.

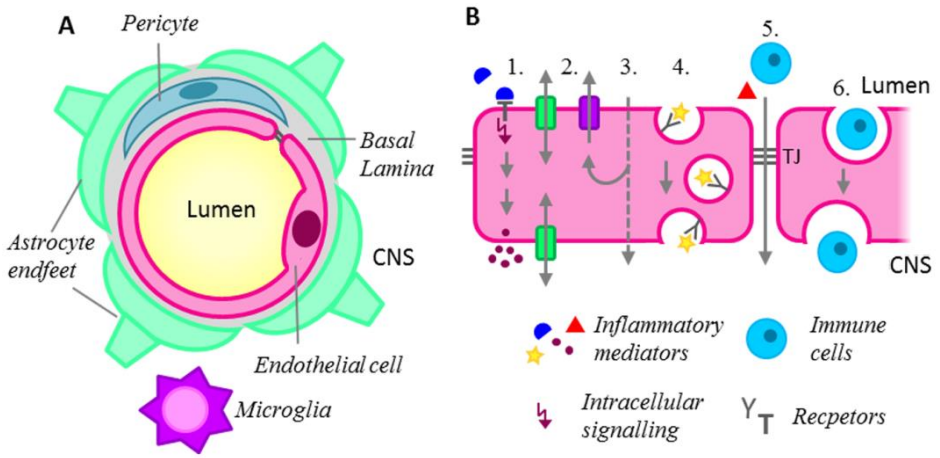


Figure 4 Schematic overviewshowing blood-brain barrier structure and transport systems. **A)** BBB structure. **B)** Pathways across the BBB including: 1. Receptor mediated induction of polar endothelial cell inflammatory mediator production, 2. Transport via efflux or solute carriers, 3. Diffusion, 4. Receptor mediated transcytosis 5. Paracellular pathway, 6. Diapedesis. Adapted from [109]

There are a number of pathologic conditions where the integrity of the BBB is reported to be affected ranging from mild disruption (e.g. during episodes of epilepsy) to complete BBB breakdown (e.g. amylotrophic lateral sclerosis)[109,110]. Although not leading to BBB integrity loss, some conditions may affect the transport systems instead. One such condition is Alzheimer's disease where the amyloid-β protein transport system is shown to be up

regulated[110]. Interesting to note is that certain PAMPs such as LPS and pro-inflammatory cytokines such as TNFα and IL-6 have been shown to be able to alter BBB permeability[109,117,118]. Consequently, alterations in BBB functionality may contribute to CNS inflammation and is something that should be considered for further investigation in chronic inflammatory conditions.

1.3.2.2 Immunoreactive cells in the CNS

The CNS was initially described to be an immune privileged site, where the immune system was denied entry due to the highly impermeable nature of the BBB. However, the CNS is not left unprotected and the presence of microglia cells, a CNS resident cell type with immune functions was described during the 1930's by Pio del Rio-Hortega[119].

Microglia belong to the group of cells known as glial cells, whose main function is to maintain homeostasis in the CNS and act as supporter cells for neurons ensuring and maintaining appropriate neuronal function[110]. Microglia closely shares ancestry with macrophages and have been shown to arise from a common myeloid progenitor residing in the yolk sack during embryonic development[120]. During normal conditions microglia are predominantly present in a "resting state" where they are easily recognisable with long thin processes protruding from a small cellular body[121]. In this resting state microglia are primarily involved in controlling neuronal proliferation, differentiation, novel synaptic formation and modification of existing synapses[122]. In accordance with its myeloid origin microglia are also constantly surveying the environment for threats[121]. If a threat is encountered, e.g. through recognition via DAMPs, microglia may quickly become activated and changes phenotype to an amoeboid shape indistinguishable from macrophages [121] Activated microglia reportedly start phagocytosing pathogens and debris, excrete toxic substances such as nitric oxide and pro-inflammatory cytokines such as TNFα, IL-1β and IL-12 but also IFNγ to recruit more immune cells from the circulation and coordinate phagocytosis[123]. Like macrophages the effector function of microglia has been shown to vary depending on the stimuli and it is likely that a similar spectrum of effector phenotypes exist[121]. After an insult is cleared microglia and blood derived monocytes/macrophages are also found to be involved in tissue regeneration[123].

Due to their quick action and prompt inflammatory response microglia are highly involved in CNS pathology and are thus commonly used as markers for ongoing inflammation in the CNS[124]. Interestingly, data from *in vitro* as well as animal studies identify the translocator protein (TSPO) as upregulated primarily on microglia subjected to pro-inflammatory activation, and to a lower extent on astrocytes and macrophages, indicating TSPO as a useful marker of microglia activation[125,126]. This led to development of radioactive tracers directed against TSPO for the use of *in vivo* positron emission tomography (PET) imaging of central glia activation[127]. TSPO upregulation and glial activation has since been shown in a variety of acute and chronic inflammatory settings including peripherally induced endotoxaemia in non-human primates[128] and humans[129], human alzheimer's disease [130], stroke in animal models[131] and humans[132] as well as experimental arthritis[133]. Although, microglial activation is gaining interest in relation to inflammatory diseases, further research is needed to fully

elucidate the role of microglial activation in inflammatory conditions and relations to CNS related symptoms and pathologies.

Astrocytes are another type of glial cells with the ability to respond to insults[134]. In healthy CNS two phenotypes of astrocytes have been described, protoplasmic and fibrous, distinguished by morphology and location[135]. With their processes they reportedly form close connections to the CNS vasculature and make contact with neighbouring astrocytes and neurons[135]. Their main function at homeostasis is to supply the neurons with all the nutrients and components they need as well as regulating blood flow and synaptic transmission[135]. Astrocytes are shown to readily respond to injury or pathogenic derived threats, and like microglia responses are ranging from pro-inflammatory to anti-inflammatory depending on strength and type of stimuli[134]. The inflammatory functions of astrocytes are not fully understood but include: 1) formation of a tight barrier creating containment of the affected area, thus protecting neighbouring healthy CNS tissues, and 2) modulation of the inflammatory response exerted by microglia and incoming immune cells via production of pro- or anti-inflammatory mediators such as TNFα and IL-6 or TGFβ[134].

In contrast to microglia, astrocytes (together with polydendrocytes, oligodendrocytes and neurons) originate from a neural progenitor[22].

It has become evident that the immune system and the CNS is intricately linked and **immune cells** are to some extent allowed to cross the BBB to perform immunosurveillance of the CNS, however, in a healthy state this migration is considered to be tightly controlled and kept at a low frequency[136]. Immune cells are also regularly found crossing the part of the BBB protecting the cerebrospinal fluid (CSF) and in CSF of healthy individuals a cell count of approximately 3000 cells/ml can be expected including cells of both innate and adaptive immunity[136,137].

1.4 NEUROIMMUNE REGULATION

As described, the CNS is constantly subjected to immune surveillance and any subsequent immune responses are normally tightly controlled by the cells in the CNS. However, the CNS has been shown to not only have the ability control the immune system at home ground, but also to have the ability to regulate peripheral immune responses. This central control of inflammation is mainly directed via two pathways, the hormone mediated Hypothalamic-Pituitary-Adrenal (HPA) axis and the neuronally mediated inflammatory reflex. Like big brother watching, having additional central control systems in place may thus provide a safeguard against over activation of the immune response and a prolonged state of inflammation.

1.4.1 The HPA-Axis and the role of prostaglandins

The HPA axis is a feedback regulated neuroendocrine system influencing homeostasis of for example digestive and cardiovascular systems via controlled release of glucocorticoids such as cortisol from the adrenal glands[138]. The glucocorticoids induced by induction of the HPA-

axis may also exert important immunosuppressive functions[138]. Initiation of the HPA-axis involves signalling by neurons innervating the paraventricular nucleus of the hypothalamus resulting in the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) into the circulation[138]. CRH is in turn described to act in concert with AVP on the pituitary gland triggering the release of adrenocorticotrophic hormone (ACTH), involved in the release of glucocorticoids such as cortisol from the adrenal glands[138]. The immunosuppressive properties of glucocorticoids are well established and exert its effect via widely expressed glucocorticoid receptors on the immune cells[138]. The immunosuppressive effects on immune cells include inhibition of pro-inflammatory cytokine release which may instead favour generation of anti-inflammatory immune cell phenotypes and thus promotes resolution[138].

It has become established that the HPA-axis may effectively be triggered by inflammatory mediators such as cytokines and prostaglandins[139]. However, inflammatory induction of the HPA-axis may also be provided via direct input from immunosensory afferent vagal neurons [140] as illustrated in **figure 5**. Interestingly, it has been demonstrated that there may be dysfunction of the HPA-axis in chronic inflammatory conditions including RA where the amount of glucocorticoids released is not enough to counteract the ongoing inflammatory response[141].

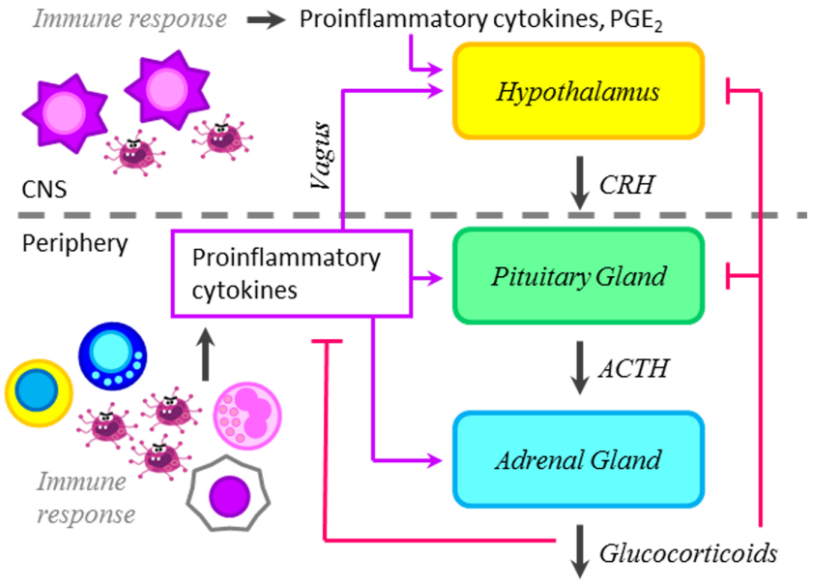


Figure 5 Schematic overview of the HPA-axis and relation to inflammatory events. Engagement of the HPA-axis leads to release of glucocorticoids from the adrenal gland. Glucocorticoids feeds into the negative feedback loop inhibiting the HPA-axis response (pink lines). HPA axis can be initiated by peripheral production of cytokines in part sensed via the vagus nerve as well as via central production of pro-inflammatory cytokines and prostaglandins (purple arrows). Glucocorticoids additionally promote a shift toward anti-inflammatory cytokine production which negatively regulates the HPA axis response (pink lines) Adapted from [142]

1.4.1.1 Prostaglandins

Prostaglandins are a group of small lipid molecules produced from arachidonic acid by a number of cell types and are shown to be involved in many different processes including

release of neurotransmitters[143]. They are predominantly constitutively expressed in the CNS and tissues, where synthesis is mainly mediated via the constitutively expressed enzyme cyclooxygenase-1 (COX-1)[143,144]. Importantly, prostaglandin production (particularly prostaglandin E_2 (PGE_2)) may readily be induced by inflammatory stimuli such as LPS or pro-inflammatory cytokines in a range of cells including macrophages and endothelial cells[143,144]. In such cases PGE_2 production is mediated by the inducible COX-2 and subsequent processing by the microsomal prostaglandin E synthase-1 (mPGES-1)[144,145]. There are four receptors capable of binding PGE_2 described, namely EP1-4, each with a variety of subtypes that may affect the response outcome[146].

Inflammatory induced PGE_2 may reach the CNS, either entering via circumventricular organs or through induced production by BBB endothelial cells[147]. In the CNS PGE_2 has been shown to be involved in induction of fever and pain responses as well as CRH release via EP3 receptor interaction on neurons in the hypothalamus[139,148,149]. Prostaglandins are also shown to be important for the induction of "sickness-syndrome", i.e. inflammatory mediated behavioural changes such as reduced food intake and reduced social encounters illustrating the ability of the immune system to induce behavioural changes in the host[150].

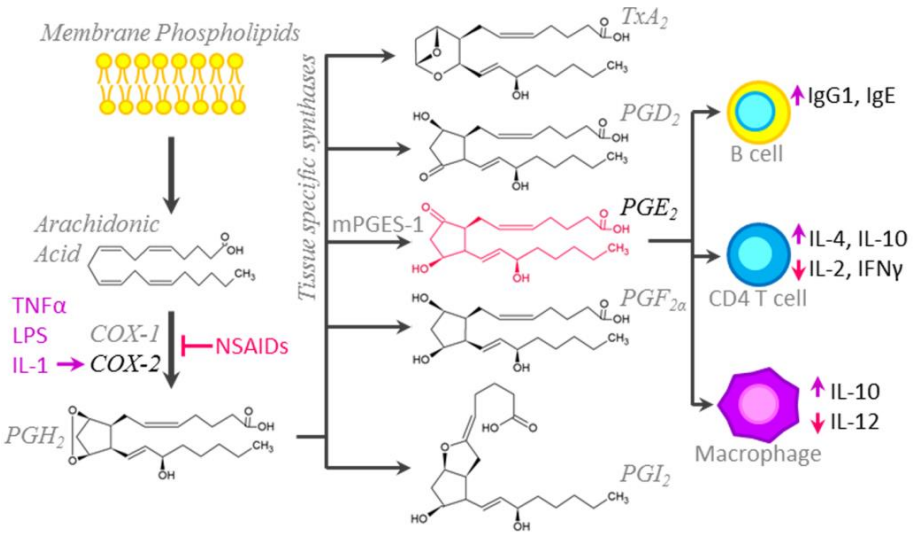


Figure 6 Schematic overview of prostaglandin synthesis and reported PGE_2 effect on immune cells. Adapted from [143,151]

Prostaglandins may also exert direct effects on the immune cells themselves such as $CD4^+$ T cells, B cells and APCs which by individual effects are seemingly driving the immune system toward a Th2 or Th17 response[143,152]. However, PGE_2 mediated immunomodulation is exceedingly complex with sometimes opposing effects on immune cells reported. Although immunomodulatory effects of PGE_2 is intensely studied much remain to be elucidated regarding mechanisms of actions[152].

In different disease pathologies, prostaglandins may have a pronounced pro-inflammatory role, as exemplified by Rheumatoid arthritis. In RA mPGES-1 is reported to be upregulated in the cells of the synovial membrane (i.e. fibroblasts and macrophages) in active disease and PGE_2 production is readily induced by pro-inflammatory cytokines in these cells[144].

Exacerbated prostaglandin production in the synovium is thus thought to be one of the contributing factors leading to disease pathology in RA.

Blocking prostaglandin production via specific COX2 or general COX1/2 inhibitors (NSAIDs) are commonly used to treat febrile, inflammatory or painful conditions[146]. COX inhibitors often provide efficient symptom relief in chronic inflammatory conditions such as arthritis[153]. However, chronic use of NSAIDs is associated with elevated risk for severe side effects such as gastrointestinal ulcers (though this side effect is reduced in modern selective COX2 inhibitors), predisposing to thrombus formation and cardiovascular events[154,155]. Together with emerging evidence that prostaglandins also may have beneficial anti-inflammatory functions, the use of COX inhibitors may have to be re-evaluated when used as an anti-inflammatory treatment option[146].

1.4.2 Immunomodulation by the SNS

To date no major parasympathetic innervation has been found reaching the primary (bone marrow and thymus) and secondary (spleen, lymph nodes, mucosa associated lymphoid tissue) immune organs.[156] Instead these sites are shown to be richly innervated by NA releasing sympathetic neurons [156]. Several studies have reported sympathetic nerve endings in close proximity to many types of immune cells including lymphocytes and macrophages[156]. Interestingly, in the spleen sympathetic innervation (via the splenic nerve) is highly localised with nerve terminals focusing in the reactive T- and B cell zones[156]. NA released by the splenic nerve may act on either α- or β NA receptors on the local immune cells. However, it is generally the β_2AR that is expressed on immune cells and the effect of physiological NA concentrations on splenic immune cells has been shown to be predominantly anti-inflammatory[156]. This effect includes suppression of cellular cytotoxic activity and by shifting cytokine production from the Th1 driving TNFα and IL-1 towards Th2 driving IL-4 and IL-10[156].

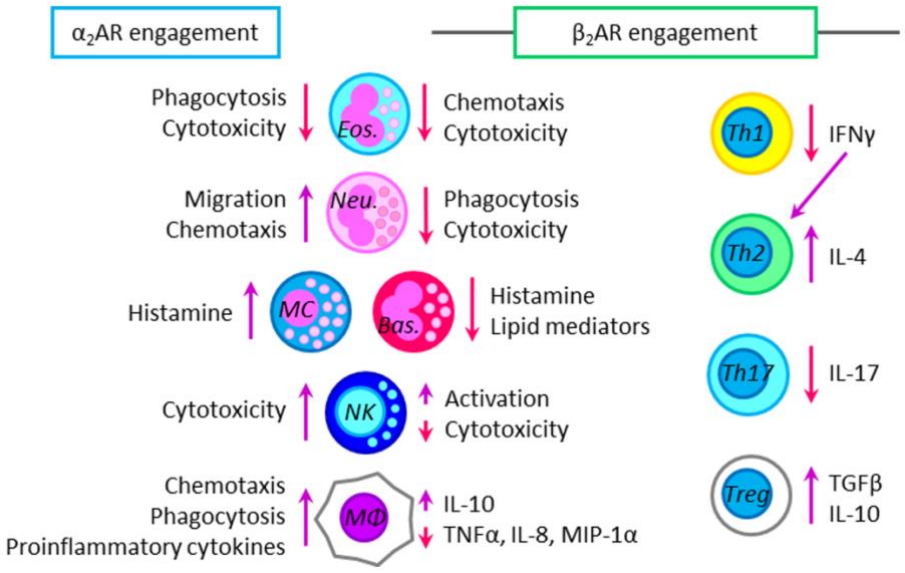


Figure 7 Effects of NA engagement of α_2AR or β_2AR on immune cells. Adapted from [157].

LPS has been shown to induce activity in the splanchnic as well as splenic nerves generating an elevation of circulating levels of NA[158,159]. NA released by the splenic nerve has furthermore been shown to induce anti-inflammatory effects in activated splenic macrophages expressing the β_2AR[160].

1.4.3 Immunomodulation by the PNS - The vagus nerve and the inflammatory reflex

As any function mediated by the ANS is generally regulated by both SNS and PNS input logic would dictate the presence also of parasympathetic immunomodulation.

The largest nerve of the PNS is the Xth cranial nerve, also known as the vagus nerve, accounting for roughly three quarters of the parasympathetic neurons (most being afferent fibres) and is innervating most internal organs[107,161]. As the vagus is part of the "rest and digest" responses its main function entails inhibition of stress induced actions (e.g. to dampen inflammation) and induction of resting responses (e.g. decrease heart rate). In contrast to the SNS, post ganglionic neurons in the PNS are generally located within the target tissue and PNS mediated effects thus tend to be localised to the specific target tissue[107].

Afferent vagal neurons have been demonstrated to terminate in the nucleus tractus solitaries (NTS), a small nucleus in the brain stem associated with reflex control[108]. The central projections of the vagus nerve were largely elucidated in the 70's and 80's by a series of animal experiments[108]. From the NTS neurons project to many brain centres, including the hypothalamus and locus coeruleus, in an intricate network as depicted by **figure 8**. Efferent vagal outflow is primarily generated from the nucleus ambiguous and the dorsal motor nucleus of the vagus, both located in the caudal medulla of the brain stem[108,161]. Importantly, while no direct parasympathetic innervation to immune organs to date has been demonstrated, the vagus has been shown to innervate sympathetic ganglia as well as the adrenal gland[162]. This supports more indirect routes of efferent vagal immunomodulation e.g. mediated via controlling sympathetic innervation of immune organs or by modulating HPA-axis output[162].

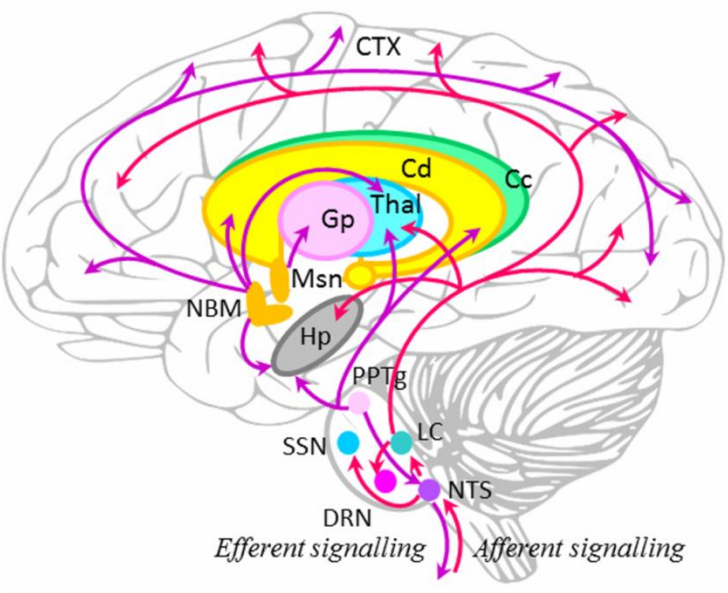


Figure 8 Central projections of the vagus nerve in the human CNS. The afferent vagus nerve projects to several control centres deep in the brain including the Locus Coeruleus (LC), Thalamus (Thal) and Hippocampus (Hp). The efferent projections driving vagal outflow originates from structures such as nucleus basalis of Meynert (NBM) and Pedunculopontine tegmental nucleus (PPTg). CTX: Cortex, Cc: Corpus callosum, Cd: Caudate nucleus, Gp: Globus Pallidus, Msn: Medial septal nuclei, SSN: Superior salivatory nucleus, DRN: dorsal raphe nucleus, NTS: Nucleus tractus solitarius. Modified from [163].

Neurons have been shown to sense and become activated by inflammatory mediators such as IL-1β or LPS leading to induction of sickness syndrome responses [113,164,165]. Investigations of a neuronally mediated immune-to-brain axis of communication was initiated based on a growing amount of evidence showing that such neuronal functions was blockable by vagotomy[164,165].

Together with the finding that an inflammatory response could be abrogated by electrical stimulation of the vagus nerve (VNS) a reflex based model of vagal immunomodulation was ultimately put forward by Tracey and co-workers under the name of the inflammatory reflex[166-168]. In this model afferent vagal neurons sense peripheral inflammation and relay this information to the NTS where efferent vagal neurons are engaged. Efferent vagal activity in turn leads to an attenuation of the inflammatory response by suppression of pro-inflammatory cytokine production by macrophages in the spleen[166,167].

1.4.3.1 Cholinergic anti-inflammatory pathway (CAP)

By further investigations into the mechanism of the inflammatory reflex it was determined that its anti-inflammatory effect was mediated via ACh, since TNFα production was effectively reduced in endotoxaemic wild type (WT) mice but not in mice deficient in nicotinic α7 acetylcholine receptor (α7AChR)[169,170]. The efferent arm of the inflammatory reflex was thus named the cholinergic anti-inflammatory pathway (CAP). Further in vitro investigation led to the conclusion that the nicotinic receptors mediating this response was present on macrophages since nicotinic agonists could block TNFα release in WT but not α7AChR deficient macrophages[169]. Furthermore, important studies demonstrated a

dependency of the spleen for a functional CAP[171,172]. Splenectomy was shown to not only revoke induced anti-inflammatory effects of CAP, but to also be the main contributor to circulating levels of cytokines produced during endotoxaemia[171,172]. Importantly, further studies also showed the significance of the splenic nerve in the CAP, because by cutting it the anti-inflammatory properties of CAP were abolished thus demonstrating a connection between the PNS and SNS in regulation of peripheral inflammatory responses[173]. The splenic nerve is part of the SNS and subsequently releases NA. Macrophages are known to express receptors for both ACh (α7AChR) and for NA (β_2AR) and both receptors have been shown to be able to suppress pro-inflammatory cytokine production[29,168,169,174]. Further scrutiny of the splenic events leading to attenuation of cytokine production was able to identify the origin of splenic ACh to a subset of $CD4^+$ memory T cells testing positive for choline acetyl transferase (ChAT), the enzyme responsible for ACh production[175]. Rosas-Ballina and colleagues showed that anti-inflammatory CAP responses were un-effective in mice lacking these T cells, but that it can be partially restored by adoptive transfer[175]. Based on these findings, a model of CAP was presented where efferent vagal signalling initiates NA release in the spleen via the splenic nerve. NA engages with α7AChR on a subset of splenic memory T cells, which respond by upregulation of ChAT expression and ACh production. ACh in turn act on splenic macrophages inhibiting their production of pro-inflammatory cytokines[175]. However, the exact mechanism whereby efferent vagal signalling leads to splenic NA release is yet to be fully understood and alternative mechanisms have been suggested[159].

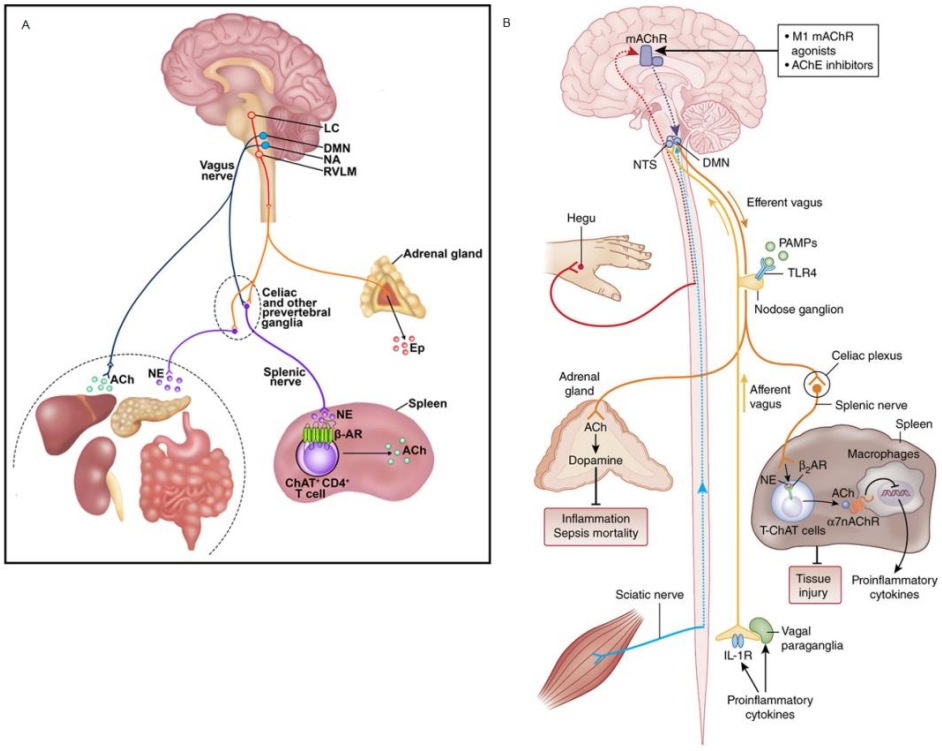


Figure 9 Autonomic control of inflammation. **A)** Systemic and local inflammation is controlled via autonomic innervation of the spleen and other organs including the intestine. Reprinted by permission from Immunity[176]. **B)** Schematic overview of anti-inflammatory effects mediated via the vagus nerve where efferent signalling involving the spleen represents the CAP. Reprinted by permission from nature neuroscience[177].

The molecular events of CAP in splenocytes are comparably well established. In macrophages, ACh interaction via the α7AChR has been shown to affect several internal signalling pathways, each affecting the pro-inflammatory response to LPS. For example, treatment with ACh or the ACh agonist nicotine was shown to promote the ability of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibito (IκB) to block nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) mediated transcription of pro-inflammatory genes leading to reduced TNFα release in monocytes, and to limitation of leukocyte recruitment ability in endothelial cells[178,179]. Furthermore, engagement of the α7AChR is known to affect the Jak2-STAT3 signalling pathway and in macrophages has been shown to induce STAT3 activation leading to inhibition of NFκB mediated production of pro-inflammatory cytokines[180,181]. In addition to limiting production of pro-inflammatory cytokines, engagement of α7AChR has been shown to inhibit expression of several surface proteins involved in driving the inflammatory response in immune cells[182].These proteins including CD14, TLR-4, intercellular adhesion molecule-1 (ICAM-1), B7.1 and CD40, which are all LPS inducible[182]. It is thus indicated that the anti-inflammatory effect extend beyond reducing splenic pro-inflammatory cytokine production, highlighting the need for extensive studies on CAP effects in a broader context.

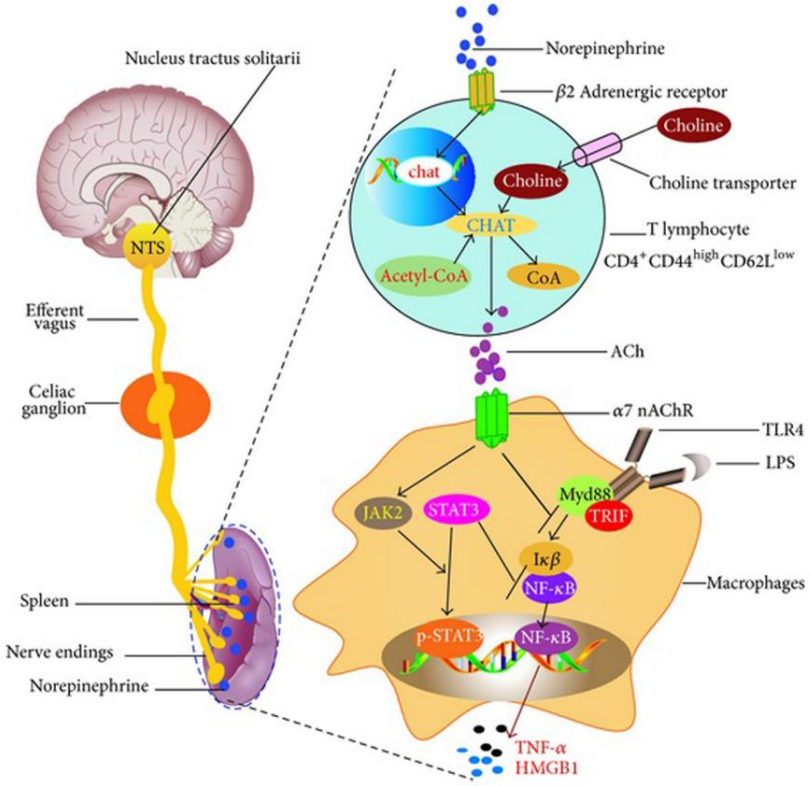


Figure 10 Schematic overview of intracellular signalling events leading from neuronal NA release to the inhibition of pro-inflammatory cytokines in macrophages. NA is released from splenic neurons following efferent vagal nerve activity. NA binds to β_2AR on ChAT$^+$ T cells which leads to upregulation of ChAT and subsequent increase in ACh production. Elevated levels of ACh in turn engages α7nAChR on macrophages initiating intracellular signalling events leading to the promotion of factors which inhibit NFκB mediated pro-inflammatory cytokine production. Reprinted by permission from BioMed research international[183].

While many aspects of neuroimmune regulation via the inflammatory reflex remain to be studied, altogether, the CAP makes a promising pathway to target in the quest for new anti-inflammatory treatment strategies. By better understanding the mechanism behind the inflammatory reflex and the CAP we may also increase our understanding of mechanisms leading to disease pathogenesis in chronic inflammatory conditions.

1.5 CNS RELATED SYMPTOMS

Apart from the disease specific symptoms, patients with chronic inflammatory disorders such as arthritis and allergy are often reported to suffer from more diffuse symptoms that can be attributed to the CNS such as altered pain perception, autonomic dysfunction, severe fatigue, and cognitive or mood alterations. These symptoms are often hard to treat and commonly the regular disease controlling treatment has little or no effect on these types of symptoms, causing a substantial reduction in quality of life for these patients.

1.5.1.1 Pain

In rheumatic disease, inflammation of the affected joint gives rise to pain and stiffness. Frequently the presence of pain persist in the patient despite achieving good inflammatory control by medication[86]. Generally, pain is a physiological response to harmful stimuli that evolved to protect us from harm. Inflammation is one of the stimuli shown able to trigger pain via receptors recognising pro-inflammatory mediators (e.g. cytokines or PAMPs) on nociceptive neurons[184]. An additional physiologic response of inflammatory pain is a sensitisation of nociceptive neurons for a period of time after the inflammatory stimulus has disappeared to ensure that the injured area is protected from further damage [184]. This process of sensitisation is complex and shown to involve many mechanisms and players resulting in an altered pain perception controlled both locally and centrally[185]. Ordinarily, such adaptive sensitisation returns to normal after a period of time, however in some conditions this state of pain sensitisation may become chronic[184]. It has been shown that RA patients display altered pain perception, reacting to normally non-painful stimuli in an area unaffected by inflammation, typical of central sensitisation[186]. Together with evidence of central sensitisation in animal models of arthritis [187] and the reported increased incidence of fibromyalgia during the course of RA[188] a substantial involvement of central sensitisation in arthritis pain pathology is suggested. However, the exact nature of events leading to pain sensitisation is still incompletely understood.

1.5.1.2 Fatigue

Fatigue is a common symptom in chronic inflammatory diseases, including arthritis and seasonal allergy, and is reported by patients as one of the key factors negatively affecting their quality of life[189-192]. The prevalence of fatigue is high, with reports of more than 40% of arthritis patients being affected and remains one of the most important symptoms in allergic rhinitis[189,192]. The mechanism(s) behind fatigue is to date poorly understood, but emerging evidence suggests complex interactions between a number of different factors. Studies in Sjögren's syndrome suggest a genetic component as well as involvement of the IL-1β

system[193,194]. Moreover, several studies in arthritis show relationship between fatigue and parameters such as functional health status, mental status and level of pain[85,190]. In these studies, there was no clear relation between fatigue and systemic inflammation, however, the role of inflammation in fatigue is controversial and other investigations have proven that immune-suppressive treatments are effective to ameliorate fatigue. For example, biologic agents targeting TNFα have been shown to alleviate fatigue to some extent [85,195] and there have been additional reports of substantial decrease in fatigue following treatment with IL-1β receptor blockade[196]. Furthermore, it was previously shown that elevated IL-1β levels in CSF of RA patients correlated positively with fatigue[197]. Together this indicates that although the pathogenesis of fatigue is multifactorial, certain inflammatory mediators may contribute in driving this symptom. Additionally, work by Hifinger and colleagues and Feldhusen et.al. shows that both country of residence and season of the year affects fatigue severity, demonstrating increased fatigue rate scores during wintertime and in wealthier countries respectively[198,199]. Moreover, a recent metabolomic study performed by Surowiec and colleagues instead show a strong association between fatigue and patterns related to oxidative stress[200] all together shedding light on the complexity of the pathogenesis of fatigue.

In allergic rhinitis fatigue is generally attributed to treatment side effects and the symptom of a blocked nose which is shown to severely affect sleep quality [201]. However, when dissecting the exact nature of fatigue using multidimensional questionnaires Marshall and colleagues show that only mental fatigue parameters are affected in allergy, suggesting CNS involvement[202]. Further studies are thus needed to explore the connection between fatigue and CNS in allergy disorders.

1.5.1.3 Altered autonomic activity

Dysfunction of the autonomic nervous system is recognized as a trait for several chronic inflammatory diseases including RA[203]. It is well established that RA patients have an increased risk of early mortality due to cardiovascular events such as myocardial infarction or stroke which has a reported worldwide prevalence of almost 10% in RA pateints[204]. The increased risk of cardiovascular events is considered to be an effect of a dysregulation in the autonomic cardiovascular reflexes and heart rate variability (HRV)[205]. HRV is a measure of autonomic balance between the PNS and the SNS which can be calculated from an ordinary electrocardiography (ECG) recording[206]. Several studies confirm that not only RA patients and patients with other arthropaties, but also allergy patients as well as patients suffering from other chronic inflammatory diseases such as SLE or multiple sclerosis display autonomic dysfunction, although subtle differences between the diseases are reported[205,207-210]. For example, RA patients are shown to have elevated basal heart rate and reduced vagal (parasympathetic) tone[205,211]. This autonomic imbalance may not only contribute to the increased risk of cardiovascular events, but may also contribute to the development of the chronic inflammatory state via impaired neuro-immunoregulatory functions[212]. Together this indicates that altered autonomic function may be connected to the inflammatory status rather than with any particular inflammatory disease. This has recently been reported in

general population where decreased parasympathetic activity associates with measures of systemic inflammation[213]. In contrast, patients suffering from allergic rhinitis is reported to have a dysfunction in the sympathetic regulation[210]. However, it is yet to be discovered what this means for the inflammatory status in allergy patients.

1.5.2 The role of inflammatory mediators

There is a growing amount of evidence pointing toward an ongoing inflammatory response in the CNS in both arthritis and allergy which may contribute to the development of CNS related symptoms and associated pathologies.

In allergic rhinitis CNS related symptoms are well documented but poorly understood and have been sparsely studied[202]. However, emerging evidence from animal studies point towards CNS involvement at multiple levels of allergic rhinitis. For example, in the brains of allergic mice IgE and IgG levels has been shown to be elevated[214]. Additionally, microarray assessment has shown altered expression patterns of inflammation related genes in the CNS of allergic mice[215].

In mouse models of arthritis upregulation of pro-inflammatory cytokines in the spinal cord, including TNFα, IL-6 and IL-1β, is reported by several studies[197,216]. These cytokines together with PGE_2 are furthermore implicated in the process of pain sensitisation[184]. Additionally, activation of glial cells in the spinal cord has been linked to pain sensitisation via cytokine (e.g. TNFα) dependant mechanisms in murine experimental arthritis [217,218]. Interestingly, experimental arthritis can be ameliorated by blocking central TNFα or IL-1β production indicating a strong relationship between centrally produced inflammatory mediators and disease pathology[219,220]. Furthermore, central production of cytokines and PGE_2 are discussed in the pathogenesis of fatigue[221].

Also in a human setting involvement of inflammatory mediators in the CNS are indicated. For example, previous work in our group has demonstrated elevated levels of IL-1β in CSF of RA patients that also were shown to correlate with measures of fatigue in these patients[197]. We have also demonstrated that elevated CSF IL-1β levels is associated inversely with heart rate variability parameters describing autonomic function in RA patients[222]. Similarly, associations between HRV parameters and the pro-inflammatory mediators HMGB-1 and IL-6 has also been reported in RA patients by other investigators[212,223]. Intriguingly, functional magnetic resonance imaging studies on arthritis patients receiving TNF-blocking treatment furthermore reveal a normalisation of neuronal activation patterns in response to painful stimuli [224]. Thus further strengthening the theory that (inflammatory) agents targeted either directly or indirectly by anti-TNF therapy is contributing to altered pain perception

Together with studies investigating anti-inflammatory effects of central muscarinic ACh receptor engagement in a CAP context a potentially strong connection between central and peripheral inflammation and CNS related symptoms is implicated[225] .

1.5.3 Vagus nerve stimulation (VNS) as a treatment strategy

Since a suppressed or dysfunctional CAP is linked to chronic inflammatory conditions, treatment strategies aimed at stimulating or restoring this pathway should be able to alleviate disease in a significant number of patients. In light of this, electrical stimulation of the vagus nerve is gaining influence as a promising treatment strategy for reducing inflammation in chronic as well as acute inflammatory conditions. VNS by external or implantable devices has already been used in humans for several years for treatment of severe depression and drug-resistant epilepsy and is considered safe with only few side effects[226,227].

VNS has been shown to have a wide range of beneficial effects in various animal models of disease. For instance, in a model of colitis central activation of the cholinergic system was shown to decrease colitis related inflammation via pathways dependent on intact vagus and splenic nerve signalling as well as cellular events in the spleen[228]. By implantation of a neurostimulatory device, Levine and colleagues furthermore demonstrated that VNS is able to ameliorate disease severity in experimental arthritis in rats, in part via reduced systemic levels of pro-inflammatory cytokines[229]. Despite these studies being very recent, already implantable devices for VNS mediated activation of CAP are being tested in pilot clinical trials. In a study following a small group of epilepsy patients having VNS devices implanted, it was shown that release of the pro-inflammatory cytokines TNFα as well as IL-6 and IL-1β by LPS stimulation of peripheral blood cells was attenuated by CAP activation thus confirming the anti-inflammatory abilities of CAP in a human setting[230]. In the same study it was demonstrated that VNS by implantable devices in RA patients was able to effectively reduce TNFα production from peripheral blood cells as well as reducing disease activity scores[230]. A pilot trial of VNS for the treatment of patients suffering from Crohn's disease, a chronic inflammatory autoimmune disease affecting the gut, has also showed good results with reduced measures of disease activity[231]. Interestingly, VNS in the Crohn's patients was also shown to push HRV measures toward expected values in the healthy population[231]. Together this demonstrates the diversity of the beneficial potential of CAP activation in different chronic inflammatory conditions and the potential of CAP activation to be useful in many more diseases. However, it is also important to remember that VNS is a relatively new technique and we don't yet know the consequences (beneficial or harmful) of long term modulation of the immune system.

In summary, there is great interplay and interdependence between the nervous and immune system, a delicate balance that can become dysregulated leading to chronic inflammatory conditions connected with different comorbidities and CNS related symptoms. The CNS is thought to be a central player in contributing to this neuro-immune dysregulation and already neuro-immunomodulation is targeted as a successful treatment strategy in chronic inflammatory diseases. However, much research remains to be done to completely understand the inner workings of neuro-immune regulation in health and its contribution to disease pathologies during dysregulation. Continued research in this field is therefore of outmost importance to be able to efficiently and safely use current treatment strategies as well as developing new ones for the benefit of the patients.

2 THE WORKS – AN OVERVIEW OF THE CURRENT STUDIES

2.1 OBJECTIVE

The work presented in this thesis use both human and animal settings to study different aspects of neuroimmune mechanisms in a translational manner.

2.1.1 General objective of the thesis

The overall aim of this thesis is to increase our understanding of neuroimmune communication and connections to CNS related symptoms in chronic inflammatory conditions.

2.1.2 Specific study objectives

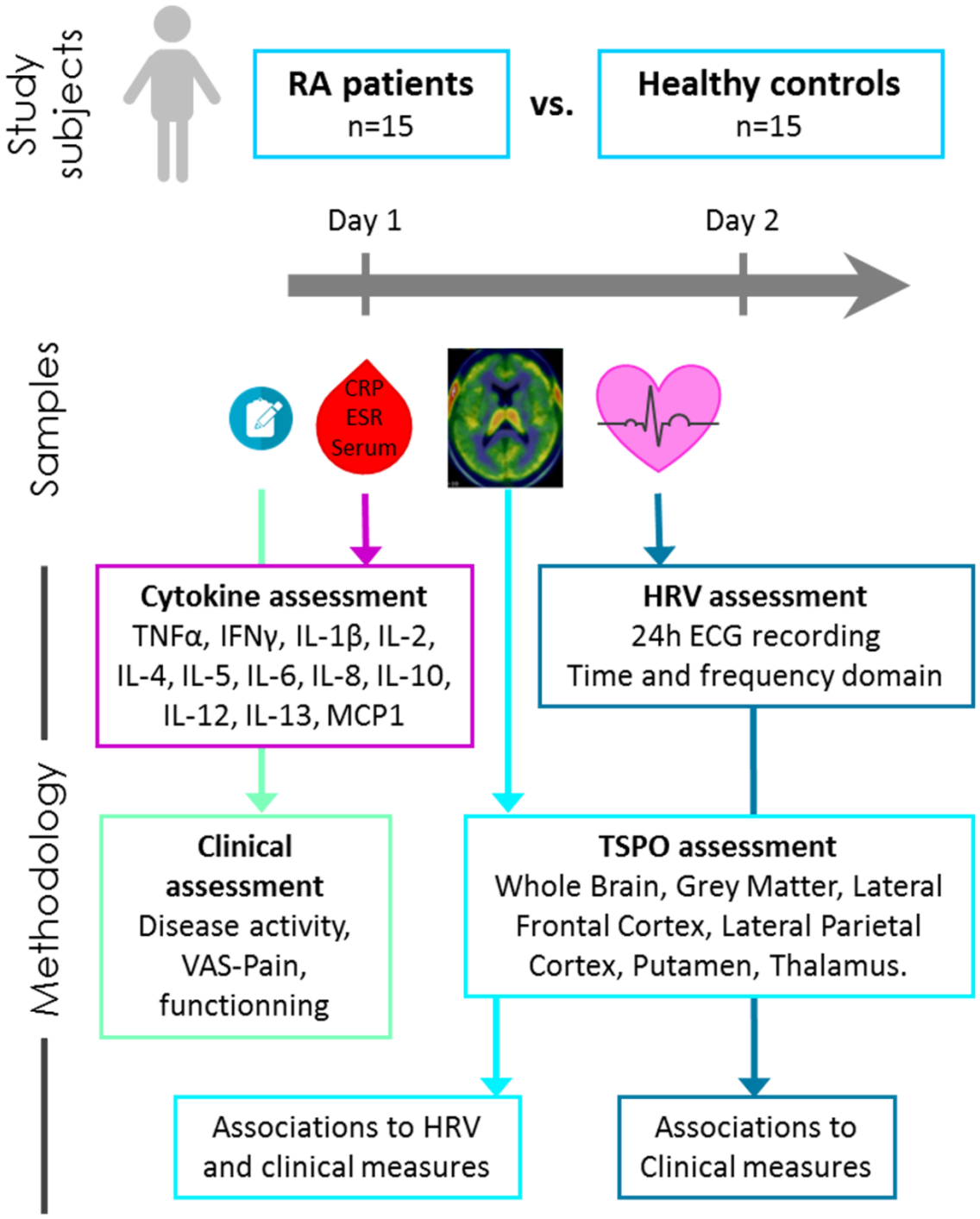
1. To explore patterns of glial activation in the CNS of patients with chronic inflammatory conditions (RA and severe seasonal allergy) by means of positron emission tomography (PET) and associations to CNS related symptoms, autonomic activity and peripheral inflammation. **(Study I and II)**
2. To evaluate the effects of anti-TNF treatment on the CSF proteome in arthritis patients and subsequent associations to disease activity, CNS related symptoms and peripheral inflammation. **(Study III)**
3. To investigate the immunomodulatory effects of VNS on early immune responses by means of flow cytometry in a murine model of acute endotoxaemia. **(Study IV)**
4. To decipher the mPGES-1 dependent prostaglandin involvement in the neuromodulatory ability of the CAP via VNS by *in vivo/ex vivo* and *in vitro* studies of murine splenocytes as well as human peripheral blood mononuclear cells (PBMCs) subjected to endotoxaemia. **(Study V)**

By achieving these goals, small steps forward are taken on the ultimate journey leading towards new and/or improved treatment strategies for the benefit of patients suffering from chronic inflammatory diseases.

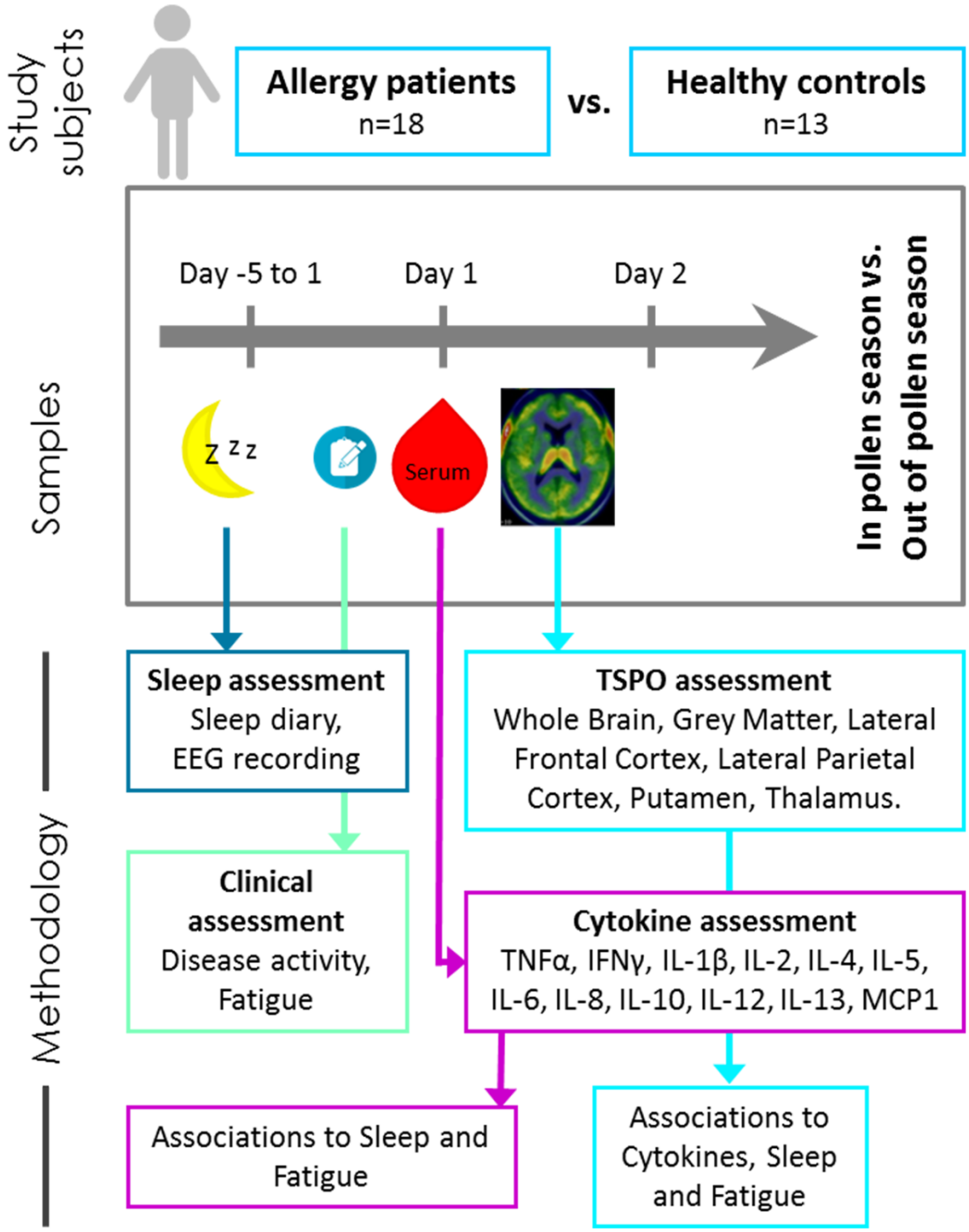
2.2 METHODOLOGICAL OVERVIEW

This section is meant to provide an overview of the experimental setup used in each study. More detailed descriptions of the specific methods used can be found in the respective method section of each paper or manuscript.

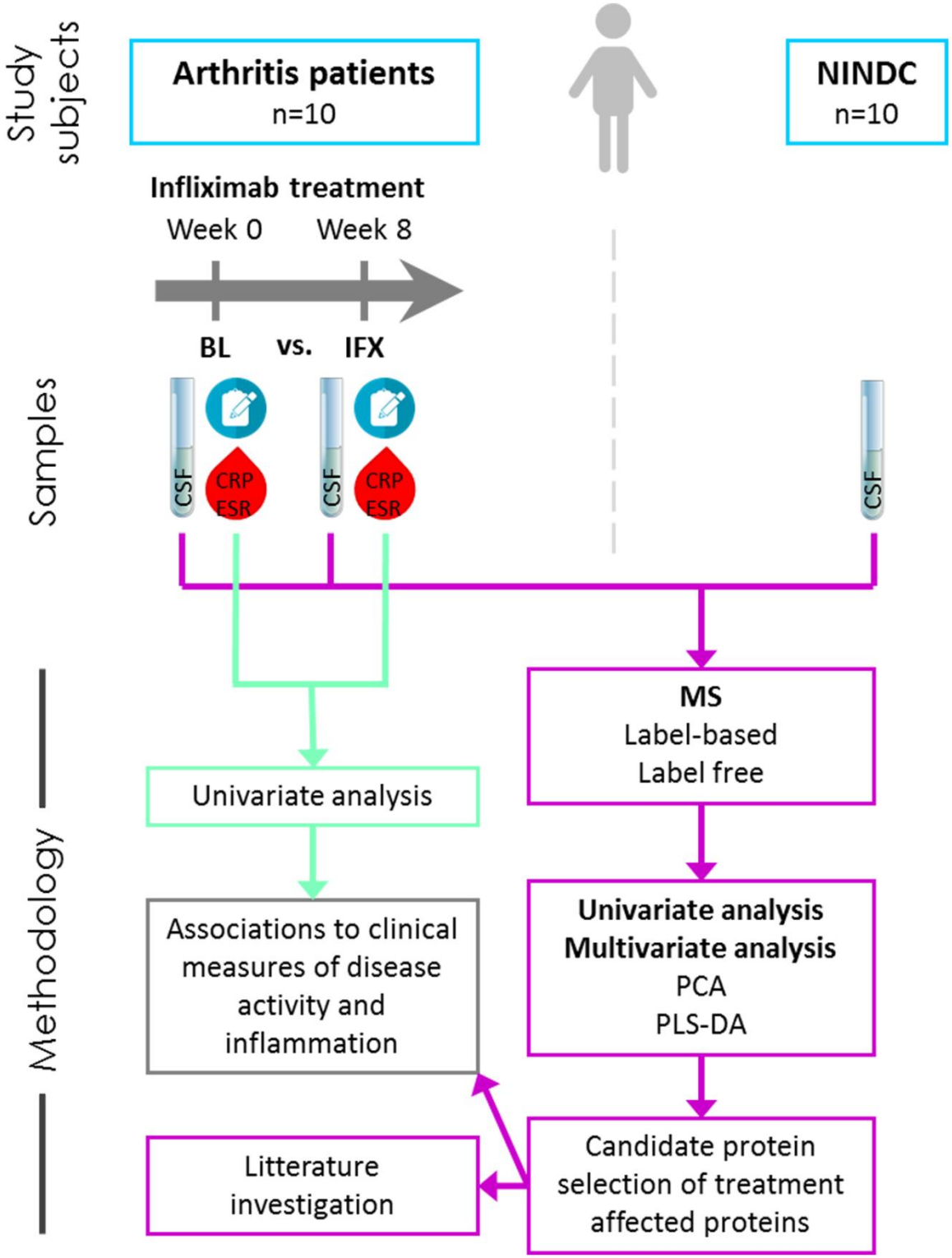
2.2.1.1 Study I



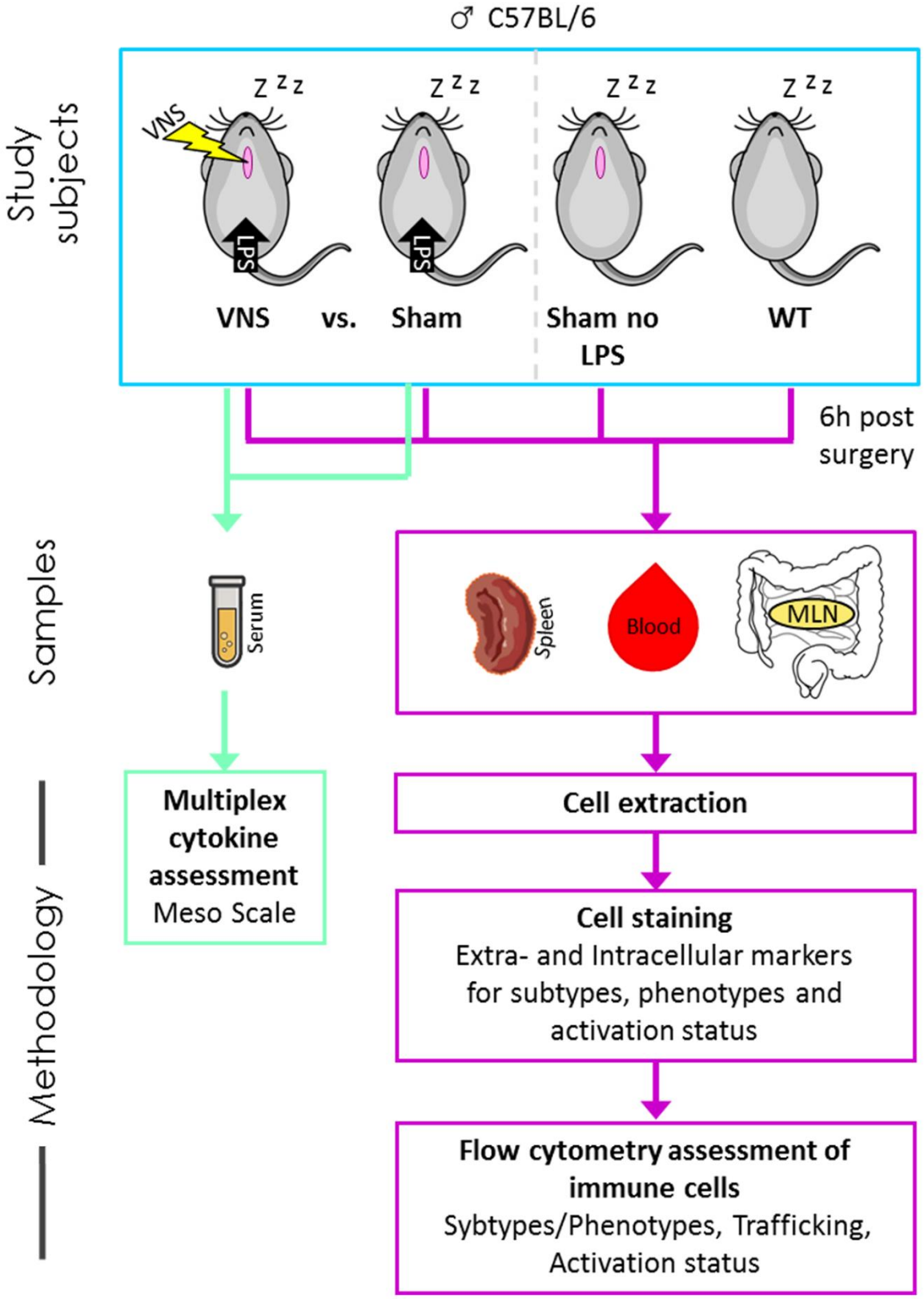
2.2.1.2 Study II



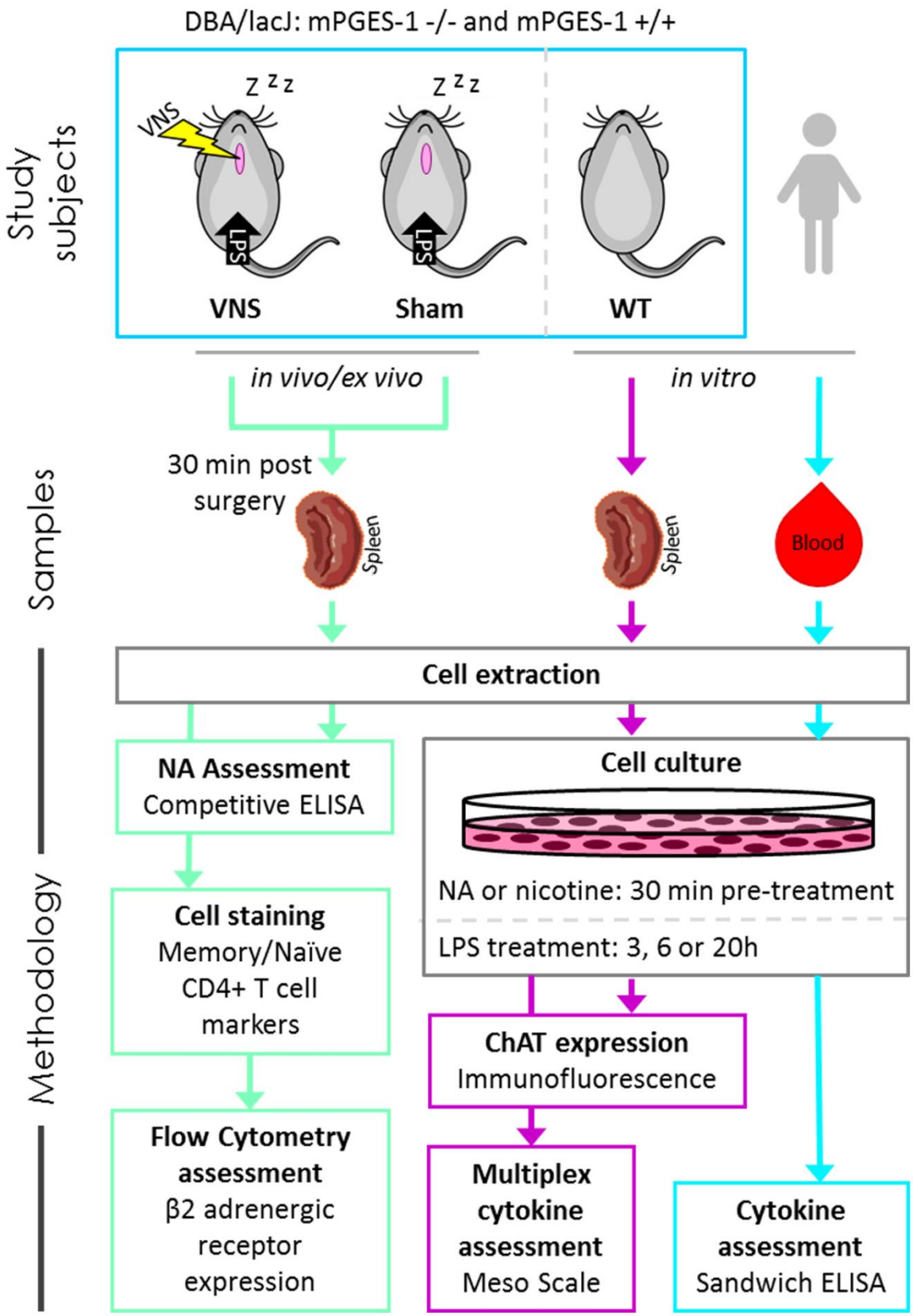
2.2.1.3 Study III



2.2.1.4 Study IV



2.2.1.5 Study V


DBA/lacJ: mPGES-1 -/- and mPGES-1 +/+
Study subjects
VNS
LPS
VNS
Sham
WT
in vivo/ex vivo
in vitro
Samples
30 min post surgery
Spleen
Spleen
Blood
Cell extraction
Methodology
NA Assessment
Competitive ELISA
Cell culture
NA or nicotine: 30 min pre-treatment
LPS treatment: 3, 6 or 20h
Cell staining
Memory/Naïve CD4+ T cell markers
ChAT expression
Immunofluorescence
Flow Cytometry assessment
β2 adrenergic receptor expression
Multiplex cytokine assessment
Meso Scale
Cytokine assessment
Sandwich ELISA

2.3 METHODOLOGICAL CONSIDERATIONS

A detailed description of the methods used will not account for the considerations relating to each method. In this section such considerations are discussed shedding light on current methodological advantages, issues and limitations.

2.3.1 Human subjects

In **study I, II and III** samples from human subjects were used. These studies were approved by the local ethics committee at Karolinska university hospital and subjects provided informed written consent. A summary of demography and characteristics of subjects included in these three studies are presented in **table 1**.

Table 1 Demography and baseline characteristics of study subjects included in study I, II and III

	Study I		Study II		Study III	
	RA	Control	Allergy	Control	Arthritis	Control
Demographics						
N	15	15	18	13	10	10
Sex (% female)	13 (86.7)	13 (86.7)	8 (44.4)	5 (38.5)	10 (100.0)	10 (100.0)
Age (median ± SD)	53 ±11.5	52 ±11.5	34 ±9.9	34 ±10.8	42 ±14.8	14.6
Diagnoses (n)	ACPA+ RA (15)	Healthy (15)	Allergy (18)	Healthy (13)	RF+ RA (3) RF- RA (2) JCA (2) PsA (2) AS (1)	Psychosis (3) Vertigo (2) Migraine (1) Tension Headhache (1) Paresthesia (1) Paraparesis (1) Trigeminal Neuralgia (1)
TSPO genotype						
High affinity binders N (%)	9 (60.0)	9 (60.0)	7 (38.9)	8 (61.5)	N/A	N/A
Mixed affinity binders N (%)	6 (40.0)	6 (40.0)	9 (50.0)	5 (38.5)	N/A	N/A
Low affinity binders N (%)	0 (0)	0 (0)	2 (1.1)	0 (0)	N/A	N/A
Disease characteristics						
DAS28 score (median ± SD)	3.2 ±1.4	N/A	N/A	N/A	5.1 ±1.6	N/A
SJC (median ± SD)	1 ±3	N/A	N/A	N/A	4 ±5	N/A
TJC (median ± SD)	5 ±3	N/A	N/A	N/A	6 ±6	N/A
ESR ≥ 20 (mm) N (%)	6 (42.9)	0 (0.0)	N/A	N/A	6 (60)	N/A
CRP ≥ 3 (mg/L) N (%)	6 (40.0)	2 (28.5)	N/A	N/A	10 (100)	N/A
Treatment						
DMARD (MTX) N (%)	11 (73.3)	N/A	N/A	N/A	10 (100.0)	N/A
Biologics N (%)	12 (80.0)	N/A	N/A	N/A	0 (0)	N/A
TNF-blocker N (%)	9 (60.0)	N/A	N/A	N/A	0 (0)	N/A
B cell blocker N (%)	1 (6.7)	N/A	N/A	N/A	0 (0)	N/A
T cell blocker N (%)	2 (13.3)	N/A	N/A	N/A	0 (0)	N/A
NSAID N (%)	7 (46.7)	N/A	N/A	N/A	8 (80.0)	N/A
Daily	1 (6.7)*	N/A	N/A	N/A	7 (87.5)	N/A
If needed	6 (40.0)*	N/A	N/A	N/A	1 (12.5)	N/A
Corticosteroids N (%)	0 (0.0)	N/A	N/A	N/A	4 (40.0)	N/A

AS, Ankylosing spondylitis; CRP, C-reactive protein; DAS28, Disease activity score 28; DMARD, disease modifying anti-rheumatic drug; ESR, Erythrocyte sedimentation rate; JCA, Juvenile chronic arthritis; MTX, Metotrexate; NSAID, Non steroidal anti-inflammatory drug; N/A, Not assessed; PsA, Prosiatic arthritis; RA, Rheumatoid arthritis; RF, Rheumatoid factor; SJC, swollen joint count; TJC, tender joint count; TNF, Tumour necrosis factor; TSPO, Translocator protein. * Patients were instructed not to take any NSAIDs two days prior to the study visit.

2.3.2 Evaluation of patient clinical characteristics

2.3.2.1 Disease activity

In RA, disease activity is measured using a standardized index, the disease activity score 28 (DAS28). DAS28 is calculated using the 28 tender joint count (TJC) and the 28 swollen joint count (SJC) results as well as ESR and patient global assessment (PGA)[232]. The cut-off points for disease activity characterization are classified as low (DAS28 ≤ 3.2), moderate (3.2 < DAS28 ≤ 5.1) or high (DAS28 > 5.1) with a cut-off point of DAS28 < 2.6 considered as clinical remission. In this thesis, DAS28 was used for assessment of disease activity in **study I and III**. For allergic subjects in **Study II** asthmatic symptoms was assessed using the validated asthma control questionnaire (ACQ)[233] and allergy symptoms were monitored by daily scoring of eye redness, itchy eyes, itchy nose, loss of smell, nasal congestion, runny eyes, runny nose, sneezing and swollen eyes during 7 days prior to the study visits.

2.3.2.2 Health assessment questionnaire (HAQ)

The HAQ is a validated questionnaire that in its full form covers the five dimensions of patient reported outcomes disability, pain, medication effects, cost of care and mortality[234]. In **study III** the short form HAQ only assessing disability was used.

2.3.2.3 Pain

There are several pain assessment methods described in the literature, including multi item and multidimensional tools[235,236]. In **study I and III** pain was assessed on a visual analogue scale (VAS). The pain VAS has been validated in RA[237] and comprises an accurate and easy to use tool in daily rheumatologic practice as well as research.

2.3.2.4 Fatigue

A number of methods of evaluation have been used to investigate fatigue in chronic inflammatory diseases. In **study I** we have used the VAS assessment validated in RA[238] and in **study II** we have used the validated multidimensional fatigue inventory 20 item general (MFI-20) [239] to evaluate fatigue. VAS fatigue provides a simple, but useful evaluation of fatigue. This single item scale has earlier been validated and described as more sensitive than longer scales[240]. On the other hand, the MFI-20 covers five dimensions of fatigue, namely general fatigue, physical fatigue, mental fatigue, reduced motivation and reduced activity providing an opportunity to assess different aspects of fatigue.

2.3.3 Positron emission tomography (PET)

Direct studies of the human CNS have historically been limited to post-mortem samples thus greatly restricting the types of research questions that can be asked and answered. With the emergence of PET in the second half of the 20th century, researchers were provided with a window into the brain, enabling investigation of its cerebral processes *in vivo* in a non-invasive manner. In this work PET is used in **study I and II** to assess translocator protein (TSPO) binding in the brains of RA patients, allergic subjects and healthy controls.

Selecting an appropriate radiotracer is essential for adequate PET analysis. In **study I and II** the radioactive tracer [11C]PBR28 was used which has been shown to be upregulated on activated microglia in response to peripheral inflammation in both human and animal settings[125,128,129]. Importantly, [11C]PBR28 has been shown to reflect activation of peripheral macrophages in a model of experimental arthritis[133] indicating functional use in human disease. In this tracer the radioactive carbon isotope ^{11}C is incorporated into a protein that will bind to the peripheral benzodiazepine receptor (PBR), now known as TSPO, upon injection into the study participants. It has however been shown that there exist a point mutation in TSPO at the binding site of the PBR28 tracer, giving rise to three groups with different binding affinity for [11C]PBR28[241]. Mixed affinity binders will generate a lower radiation signal than high affinity binders, while non-binders will produce no detectable signal at all[241]. To compensate for binding affinity differences TSPO genotype was assessed in **study I and II** participants, and patients and controls were matched according to genotype. Furthermore, to protect study participants from being subjected to unnecessary risks non-binders were excluded from participation.

During a PET scan participants are subjected to ionising radiation which always generates a risk for mutagenesis, however, radiation levels in **study I and II** are low (equivalent of a year's worth of background radiation) and well within the safety recommendations of the Swedish radiation safety authority. The half-life of the [11C]PBR28 traces is approximately 20 min compared to other commonly used tracers utilising e.g. ^{18}F which has a half-life of 110 min[242]. On one hand this quick radiation decay of the [11C]PBR28 tracer is beneficial for the participant as that means the tracer will become harmless in the body a few hours after its injection. On the other hand it can cause trouble for the researchers as injection of the tracer must be timed precisely with its production, which takes place on site.

The PET images are the result of radiation produced by the radioactive tracer as its emitted positrons decays in the target tissue in a burst of gamma radiation[242]. The radiation is captured by an array of detectors in the tomograph and by computer modelling is rendered into an analysable image. As with any imaging technique resolution is always an important issue to consider. In general PET is considered to have relatively low resolution limiting studies of individual small brain structures. As we were interested to study particular nuclei of the brainstem linked to the vagus nerve, which are small, we were unable to study them directly. To overcome that limitation, larger areas of interest known to be activated (cerebellum, insula, orbitofrontal cortex, temporal pole and thalamus) or deactivated (amygdala and hippocampus) by the vagus nerve were instead identified and combined together respectively and analysed as larger regions of interest.

In **study I and II** radiation in blood samples was continuously measured at set time intervals throughout the 90 min duration of each PET scan. This was done since the brain is supplied with large quantities of blood containing circulating levels of the tracer in an intricate network of blood vessels, to ensure that PET image analysis is not obscured by blood borne tracer interference.

2.3.4 Heart rate variability (HRV)

Heart rate is controlled by influences of both sympathetic and parasympathetic (vagal) nature. Therefore, by mathematical analysis of the distance between consecutive heart beats on an ECG recording, information can be inferred about HRV and the overall sympathovagal balance. This technique is used in **study I** to assess the autonomic activity in RA patients compared to controls.

HRV is measured in two different domains, the time domain and the frequency domain, each giving rise to several parameters reflecting specific aspects of the sympathovagal balance. The resulting parameters and their interpretation are highly complex since many parameters are related to each other. To assist analysis international guidelines regarding definition of HRV parameters, ECG recording equipment standards and standards of presenting HRV results were put together in the mid 90's [206]. Although very helpful in standardizing HRV measurement and interpretation, the guidelines do not cover all aspects of HRV. For instance, the circumstances which the ECG is to be recorded under is not discussed and it is up to each researcher to decide what conditions best fit their setting. This has led to ECGs being recorded under many different conditions making it challenging to directly compare HRV between published studies.

The HRV measurement is a robust strategy that has proven useful in a clinical setting e.g. to identify patients at risk of cardiac mortality or diagnosing neuropathies in diabetic patients and is also in a research setting providing substantial insight about autonomic regulation in health and disease[206]. HRV heavily rely on correct identification of individual heart beats. In **study I** the ECGs were therefore visually inspected and corrected manually to ensure that each heart beat is identified properly. Furthermore, ectopic heart beats and areas of interference was excluded which would otherwise influence the HRV outcome.

In **study I** HRV measurements were calculated from 24h ambulatory ECG recordings, a setup used previously by our group[222], which has the advantage that it minimizes the risk of detecting only occasional variations in heartbeat. Study participants were instructed to lead their lives as normal during the ECG recording sessions but to refrain from strenuous physical activity. Since everyone has a different life routine this may increase the variability of the HRV measures in our study, but in using this setup we would also get the HRV results giving the most truthful reflection of each person's individual HRV measures. However, we have previously demonstrated that this method showed comparable results on HRV in for example RA with earlier studies[222].

2.3.5 Mass Spectrometry (MS)

In **study III** two different MS based approaches were employed, a label-based and a label free approach, providing a wide protein detection range to identify as many TNF-blockade associated proteins in CSF of arthritis patients as possible.

Mass spectrometry has emerged as a useful tool for analysis of complex samples such as biological fluids. It has undergone a rapid evolution in recent years greatly improving its

performance rates, including resolution and development of adequate strategies for efficiently coping with false discovery rates[243]. Like any other biological fluid, CSF is a complex mixture of proteins, some more abundant and some more rare. Compared to fluids like serum, CSF has a considerable lower protein concentration which may influence the number of proteins detectable and biologically important low abundance proteins may therefore undergo detection. However, in **study III** we compared our MS results with published data on CSF protein concentrations revealing that we are able to detect proteins with a concentration as low as 4 ng/ml in our material.

It is always important to find ways to validate MS results. Since our CSF samples have been stored for a long time they have inevitably been subjected to some extent of protein decay. This is not a problem in MS since samples are trypsinated as part of the preparation process. However, it makes validation by common antibody based techniques such as sandwich based assays unreliable. In **study III** extensive literature studies were therefore performed comparing our findings to results of other human CSF proteomic studies as well as experimental data, ultimately confirming the reasonability and supporting the validity of our findings.

2.3.6 Vagus nerve stimulation (VNS)

Studying the mechanism and properties of the CAP in animals has been essential for understanding its anti-inflammatory potential and led to the development of VNS-devices for trial in human use in the treatment of chronic inflammatory diseases[177]. In this thesis the anti-inflammatory properties of CAP has been studied via VNS in endotoxaemic mice (**study IV and V**).

When studying the inflammatory responses in mice (or other organisms) it is important to remember that there are a number of aspects that can influence their inflammatory responses. For instance strain differences exist, making it important to choose the appropriate strain for the experimental setup. In **study IV** we chose to work with C56BL/6 mice. C57BL/6 mice are widely used in immunologic research and cover many well established disease models for numerous chronic inflammatory diseases including arthritis[244]. These mice are prone to a Th1 rather than a Th2 directed response which is better reflecting human arthritis considered being a Th1 driven disease[69,244]. Since prostaglandin deficiency in connection to immune regulation was to be studies in **study** V we chose to work with DBA/1lacJ mice because in this strain there was an in-house prostaglandin deficient mPGES-1 ko model validated in previous research[245]. Like C57BL/6 mice the DBA/1lacJ strain is commonly used in immunologic research and inflammatory disease models are well established.

Also external factors exist which may influence the inflammatory responses of the mice during experimentation. In **study IV and V** animals undergoing surgery were anesthetized by inhalation of a controlled isoflurane breathing mix. Isoflurane is known to reduce inflammatory responses[246]. To control for isoflurane effects all animals, including WT

controls, always underwent similar periods of anaesthesia during experimentation ensuring the validity of our results. Additionally, NSAIDs and other pain reducing drugs are known to influence the inflammatory response via effects on prostaglandin production. Since we are studying prostaglandin involvement in the CAP (**study** V) we cannot therefore administer these types of drugs to our animals undergoing surgery. For ethical reasons this means we cannot keep the animals after experimentation for extended periods of time thus limiting us to study short term effects in *in vivo* and *ex vivo* settings.

Importantly, using this setup we have previously reported clear effects of LPS on cytokine production that is significantly attenuated following VNS in line with reports on VNS in severe endotoxaemia[168,247]. In addition, we have also tested this VNS setup in different mouse strains including C57BL/6 and DBA/1lacJ showing similar immune responses, indicating that this VNS setup in our studies are not influenced by strain differences.

2.3.7 Flow Cytometry

Flow cytometry is a versatile tool that has become a widely used technique in research as well as hospital settings aiding e.g. with diagnosis. **Study IV** is primarily based on flow cytometric analysis investigating the effects of CAP on immune cells and in **study V** flow cytometry is used to assess adrenergic receptor expression on $CD4^+$ T cells.

By staining a selection of antigens either on the cell surface or in the intracellular milieu by fluorescently labelled antibodies targeting the antigens of choice, flow cytometry allows recognition of multiple targets in a complex mixture of cells. Each cell in the sample is assessed individually for its pattern of emitted light as it passes through an array of laser beams and light detectors. Flow cytometry thus enables identification and subsequent analysis of multiple targets on the same cell as well as several parameters including relative number (or exact number if count beads are included in the sample) of specific subtypes or phenotypes of cells and their expression levels of selected antigens.

Flow cytometry has greatly contributed to the advancement of our understanding particularly of the field of immunology. However, there are some technical challenges that should be considered for optimal performance. These include planning your antibody panel to avoid spectral overlap and usage of control samples to ensure proper compensation and gating. In most cases there will likely be some spectral overlap which has to be compensated for. In **study IV and V** single stained samples with clear positive/negative population distinction was used in each run to generate a high quality compensation matrix. Furthermore, to assist with gating fluorescence minus one (FMO) samples were always included for antigens with an expected pattern of continuous expression. Additionally, to limit unspecific binding samples were always incubated with Fc receptor blocking antibodies prior to staining. S**tudy IV and** V consist of pooled data obtained on different dates. The experiments were therefore carefully setup so that on each date there was always one sample per study group.

2.3.8 Immunofluorescence (IF)

Like flow cytometry IF utilises fluorochrome coupled antibodies reacting to a light source. Similar pitfalls exist for IF regarding antibody panel selection, however, since the number of combined antibodies that can be used at once is greatly limited in IF, panel setup is less complicated. The complication may instead lay in limited availability of appropriate antibodies, non-specific binding and photostability of the fluorochromes used.

Immunofluorescence has a lot in common with flow cytometry wherefore many challenges and limitations to consider are similar. Contrary to flow cytometry, IF allow assessment of individual cells in its parent tissue enabling visualisation of cell interaction and co-localisation of expressed antigens of interest. Furthermore, IF allows assessment of cell types with complex morphology such as neurons as well as providing a useful alternative to studying antigens lacking appropriate antibodies for flow cytometry as is the case for ChAT.

IF is used in **study V** to visualize and assess the *in vitro* expression of ChAT in splenocytes.

2.4 STATISTICAL CONSIDERATIONS

During the course of the PhD, the student learns about statistical methods and the importance of their appropriate use. Through hands on data analysis it is soon discovered that choosing the appropriate statistical method is not always straight forward and that sometimes different statistical approaches may present equally suitable tests.

To be able to choose an appropriate statistical approach it is vital to know the characteristics of the data set to be analysed. This is important since different statistical tests base analysis on certain assumptions about the data set that needs to be met for correct analysis.

A variety of different statistical tests, reflecting the different requirements of the hypotheses and data set characteristics, have been utilised in the studies encompassed by this thesis.

In **Study I** group differences were assessed by repeated measures analysis of variance (ANOVA) (Bonferroni corrected) or Mann Whitney U test. Associations between parameters were investigated with partial correlate analysis (normally distributed data) or Spearman correlation (non-normally distributed data). Distribution of parameters in the data set was assessed by Shapiro Wilk's test of normality and visual inspection of Q-Q plots.

In **Study II** group differences and the effect of pollen season was investigated by means of mixed effect models where TSPO genotype and sex were always taken into account as covariates. Normality of distribution was assessed by visual inspection of histograms.

In **Study III** both univariate and multivariate analysis strategies were employed. Univariate analysis consisted of Wilcoxon signed rank test comparing arthritis CSF at baseline and after infliximab treatment. Mulitvariate approaches consisted of unsupervised principal component analysis (PCA) and assisted partial least squares discriminant analysis (PLS-DA). Furthermore, associations between proteomics and clinical data were assessed by Spearman correlation.

In **Study IV** group differences were assessed by either Wilcoxon signed rank test (related data) or Mann-Whitney U test (un-related data). Normal distribution was assessed by Shapiro Wilk's test of normality and visual inspection of Q-Q plots.

In **Study V** group differences were assessed by one way ANOVA (Tukey's post-hoc test) or student's t-test.

2.5 ETHICAL CONSIDERATIONS

Throughout the PhD education we are taught the importance of considering the ethical implications of our work. The work that has gone into this thesis has encompassed both human and animal studies wherefore I find it essential, as it should be to any researcher, to address the respective ethical considerations.

2.5.1 Human studies

Although humans and animals share many physiological processes, there are also substantial differences that should be taken into account when translating research from animals to humans. Most research performed, this work included, is ultimately designed to benefit humankind. I therefore believe it is only fair that as much research as possible is performed on human subjects. Both because humans have the ability to actively choose to consent to a study and because the risk of studying processes and responses that are only present in animals is eliminated.

Having stated this, proper care must always be taken to ensure the health and safety of the test subjects, and to ensure that individuals are never coerced or exploited in research. In the wake of the second world war global guidelines, the Helsinki declaration, was established in order to protect study participants in human research from unethical practices and misconduct. The experimental setup of any study using human participants is subjected to rigorous scrutiny regarding ethical considerations, necessity and potential gain of knowledge or benefit by an ethical committee before being allowed (or rejected).

When recruiting participants to the studies, it is important to ensure that the individual considering participation is given sufficient information to be able to make a well informed decision before signing the consent form. Ensuring proper information and understanding of what participation in the study entails is also beneficial for the researcher as I believe this minimizes mid-study drop outs.

Importantly, participants are always instructed that they can abort each test and withdraw from the study whenever they want to. Ultimately care is always taken to ensure that the risks of the study are outweighed by the importance of the study.

Another thing to reflect upon is how to handle any abnormal findings of the participants which might need further investigation in the regular health care system. In such situations we made it our responsibility to refer any such individuals to experts for further testing, thereby enabling early discovery and treatment of disease conditions.

To ensure the well fare of the participants in our studies experimental procedures were always performed by trained and experienced personnel. To ensure that no unnecessary experimentation was performed, procedures such as genotyping and quick assessment of CRP was performed before allowing the participant to be tested.

All studies in this work using human study participants or human samples (**Study I, II, III and IV**) are following the Helsinki declaration and was approved by the regional ethics committee of Stockholm, Sweden. Study subjects gave written informed consent to participate in these studies.

2.5.2 Animal studies

The work in **study IV and V** is predominantly based on investigations in mice. Since mice are not able to consent to their participation in our experimentation, considerable care is and must be taken to ensure the best possible well fare of the research animals. To safeguard animal well fare stringent systems have been put in place where all experimentation involving research animals must be approved by an ethical board following national and international ethical guidelines on animal research. All animal experimentation in this work was approved by the local ethics committee in Stockholm, Sweden.

In addition, when planning the experiments the guidelines of the three R's (replacement, reduction and refinement) are always kept in mind to minimize the number of animals used and to reduce the suffering they are subjected to. In both **study IV and V** we perform VNS, a procedure that require surgery, as well as using i.p. administered LPS to be able to study the mechanism of the CAP and its effect on acute inflammatory responses. The cholinergic inflammatory pathway is a complex structure involving many different organ systems and cell types [176]. This makes it difficult to study the entirety of this pathway without using research animals, thus limiting us in the R of Replacement. Efforts have instead been made in the R's of reduction and refinement to ensure maximal well fare of our animals. Even though the VNS surgery protocol was developed in consultation with the animal facility veterinarian and is performed by a skilled researcher considerable suffering for the animals is still associated with this technique. Firstly, to study VNS in the context of inflammatory responses, mice are subjected to LPS induced inflammation causing fever and flu-like symptoms in the animals causing them some discomfort. Secondly, we are not able to administer pain relief to the animals after surgery since NSAID's and other drugs are known to interfere with the inflammatory responses and would thus compromise our studies. To reduce animal suffering experiments are always acute and animals are never kept beyond 6h after surgery. Moreover, before starting routine VNS experimentation the LPS dosage was titrated down in order to discover the lowest usable dose with a maintained readout quality to further reduce the discomfort of our animals. This illustrates our work to continuously refine our experimental models for the benefit of the research animals. Reduction can be illustrated in **study V** where VNS is mimicked by *in vitro* treatment with NA and other substances to study splenocyte responses in PGE_2 deficient mice. This allows us to spare animals from the suffering associated with VNS.

The animals are kept at the animal facility at the Karolinska University hospital, Stockholm, Sweden in cages meeting stringent KI standards following the EU regulations regarding cage size, availability of food and water as well as bedding and nesting materials. Animals are cared for by highly trained staff and any researcher expected to work with animals are required to undergo extensive training. Further measures are taken such as use of protective gear and allowing animals to acclimatise to their new environment for at least one week before experimentation can start to ensure health and low stress levels in animals.

As a researcher, it is then my responsibility to make sure that the animals I use are always handled with the care and respect they deserve. Therefore, I always try to handle them calmly, considerately, competently and quickly.

Despite extensive guidelines being in place both for animal and human studies, ethical standards will always be subjective to the current societal norms. As society evolves ethical practices will evolve with it, and what is accepted as ethical today may very well not be so in the future.

3 RESULTS AND DISCUSSION

3.1 STUDY I AND II

Symptoms that can be attributed to the CNS are frequently reported in both RA and allergic rhinitis patients and may persist in spite of controlling the disease with medications, representing a considerable burden for the patient. In RA the most important CNS related symptoms consist of fatigue and altered pain processing, but also include altered autonomic activity[186,189,205]. In allergy the most important CNS related symptoms are related to fatigue [191]. Although the mechanisms behind these symptoms are largely unknown, a growing amount of evidence point toward an ongoing inflammatory response in the CNS in these diseases, which may drive the associated CNS related symptoms.

In RA patients, we have previously shown elevated CSF levels of the pro-inflammatory cytokine IL-1β that furthermore revealed an association to fatigue measurements, indicating a possible connection between the two[197]. In allergic rhinitis, studies of CNS involvement is limited to animal investigations showing e.g. elevated levels of central IgE and a CNS gene expression pattern altered towards inflammation, indicating substantial CNS involvement also in allergic inflammation[214,215]. Interestingly, microglia activation in the spinal cord in response to inflammation has been shown to associate with pain sensitisation in animal models of arthritis[217,248] and allergy[249].

In light of this, a hypothesis was put forward suggesting that compared to controls RA patients and allergic subjects during pollen season would show increased microglial activity in the CNS. We additionally sought to investigate relations between microglial activity and peripheral inflammation as well as autonomic activity and CNS related symptoms.

3.1.1 No evidence of brain glial activation in either RA or allergic subjects

To test this hypothesis a study was initiated exploring *in vivo* glia activation as measured by PET using the radiotracer [11C]PBR28 in RA patients and allergic subjects compared to controls. Allergy patients and their respective controls were studied both in and out of pollen season. [11C]PBR28 is binding to TSPO, a membrane protein which has been shown to be upregulated on activated glial cells in chronic inflammatory settings in both animal and human studies[125,131,241]. It should however be noted that PET scan using TSPO as a ligand is complex and provide several challenges. For example, the ligand binding of [11C]PBR28 is genetically regulated, warranting comparisons between high, mixed and low affinity binders respectively[241].

Contrary to our hypothesis, as shown in **figure 11** no increase of TSPO expression was discernible in whole brain or selected brain regions of interests for either RA patients or allergic subjects either in or out of season as compared to controls in our studies ($p>0.05$).

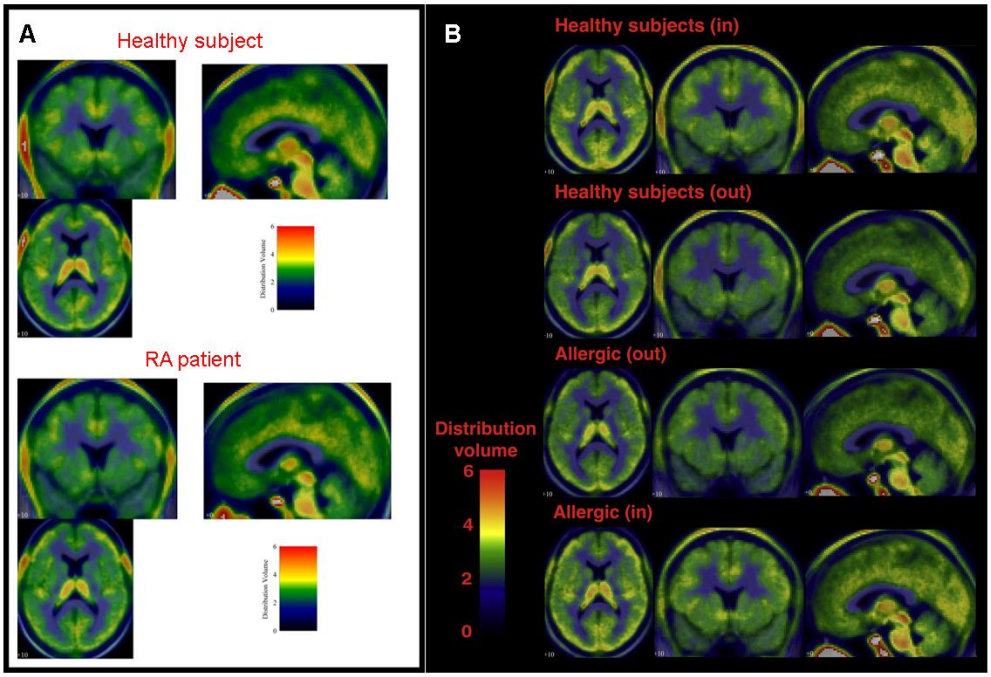


Figure 11 Brain TSPO levels in A) representative RA patient and respective healthy subject and B) representative allergic and respective healthy subjects both in and out of pollen season.

It has become evident that the retest performance of PBR28 is relatively high[250,251] and with the additional need to control for variation of genetically high or mixed PBR28 binding affinity[226] large study cohorts may be needed to generate reliable results. It may thus be argued that our study cohort was underpowered. However, we found similar results of no increased TSPO binding in RA as well as seasonal allergy, suggesting that in both these chronic inflammatory diseases brain glial activation, as measured by TSPO expression, may not be a prominent feature. A further possible confounding factor is that TSPO has been reported to not be exclusively expressed by microglia, but has also been detected in other immune responsive cells such as astrocytes and macrophages[252]. Interestingly, recent reports reveal that TSPO upregulation may be more complex than previously thought. For example, it has been shown that there is a discrepancy in the level of TSPO expression between different activation states of murine microglia with higher levels displayed in pro- than in anti-inflammatory microglia[253]. Furthermore, in some human inflammatory settings microglia reveal no pro-inflammatory upregulation of TSPO[254]. Even decreased TSPO expression has been reported in human pro-inflammatory macrophages, including macrophages derived from RA synovium[255]. Since the phenotype of microglia and central macrophages are unknown in RA and allergy this complicates the interpretation of the lack of TSPO increase here reported. It is however plausible that there may exist a skewed central microglial and/or macrophage response. In support of this animal studies have reported the ability of infiltrating immune cells to be able to affect microglia polarization[256]. In further support, we have previously reported that the IL-1 system is activated in RA CSF[197] which together may create a polarizing microenvironment for driving microglia differentiation toward a certain phenotype. In line with this and with our current observations, PET studies investigating glial activation via TSPO has reported lower TSPO levels in schizophrenia, a neurologic condition with expected central inflammatory activation[126,257]. Finally, there are

other radiotracers in use, and an ongoing discussion on the comparable performance and sensitivity for detecting significant activation of microglia in different parts of the brain[258].

In conclusion, we found no increased TSPO-binding in RA or seasonal allergy which could indicate that brain microglia activity is not upregulated in these diseases. However, with the known other neuro-inflammatory features of chronic inflammatory disease and potential considerations with the [11C]PBR28 tracer, investigations with alternative ligands should be performed before excluding a role of microglia activation in central nervous mechanism of RA and seasonal allergy.

3.1.2 Markers of peripheral inflammation are associated with measures of autonomic function in RA

In RA patients **(Study I)** the peripheral cytokine levels as well as the CNS related features of altered autonomic activity as measured by HRV and VAS assessed fatigue was investigated. In **study I**, RA patients displayed increased peripheral levels of TNFα, IFNγ, IL-6 and IL-10 compared to controls as shown in **table 2**, in concurrence with RA being considered an inflammatory disease[259]. RA patients also displayed increased levels of fatigue compared to controls.

Table 2 Differences in cytokine serum levels between RA and HC tested with Mann Whitney U test. Values are presented as mean ± SD.

	RA	HC	p
IL-6 (pg/ml)	4.3 ± 9.4	0.5 ± 0.3	0.021
IL-8 (pg/ml)	8.0 ± 3.2	8.0 ± 2.0	0.993
IL-10 (pg/ml)	0.7 ± 0.5	0.4 ± 0.3	0.014
IFNγ (pg/ml)	10.4 ± 6.0	6.5 ± 4.1	0.027
MCP-1 (pg/ml)	307.9 ± 72.1	299.3 ± 89.0	0.782
TNFα (pg/ml)	24.0 ± 30.6	2.1 ± 0.3	0.001

HC, Healthy control; RA, Rheumatoid arthritis

Furthermore, several significantly ($p<0.05$) decreased measures of autonomic activity, primarily in the time domain features known to be more robust during long term (i.e. 24h) ECG recordings, was demonstrated in RA patients in accordance with literature[206,212,260]. Contrary to our initial hypothesis, no relation was shown between brain TSPO expression levels and either measures of HRV, peripheral inflammation or fatigue.

Interestingly, circulating levels of the pro-inflammatory cytokine IFNγ was shown to inversely correlate to root mean square of successive differences (RMSSD), a time domain HRV measure reflecting vagal activity (**figure 12**). With the close connection between the vagus nerve and peripheral inflammation as highlighted by the inflammatory reflex and its efferent cholinergic anti-inflammatory pathway[261] a relation between RMSSD and IFNγ is thus not surprising. In fact, inverse relation between another pro-inflammatory cytokine, IL-6, and RMSSD has previously been reported in RA[212,223]. Since IFNγ is primarily produced by

Th1 cells and to some extent by NK cells as well as microglia a question regarding their connection to the vagus nerve and CAP ability to influence their activity is raised, something that is beginning to be investigated in an animal setting in **study IV**.

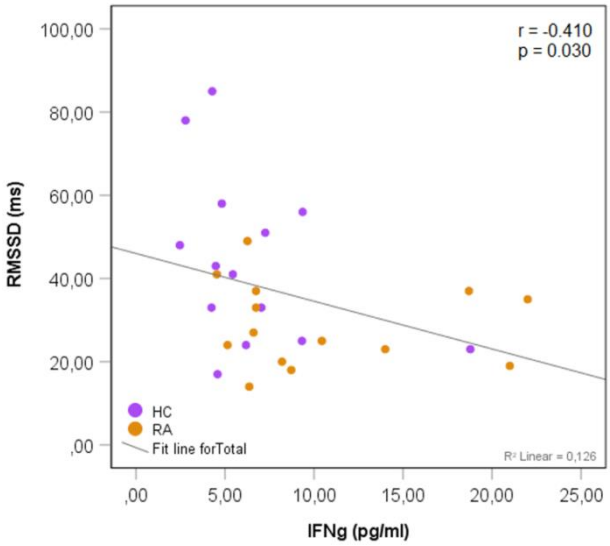


Figure 12 Correlation plot showing inverse correlation between RMSSD measured by 24h HRV and IFNγ assessed by multiplex sandwich assay in RA patients and matched controls.

Taken together, while brain TSPO expression on microglia may not be associated with autonomic dysfunction, fatigue or peripheral inflammation in RA, we provide further evidence that there is a strong link between autonomic activity and peripheral inflammatory levels. Together with substantial evidence pointing toward central neuro-immune involvement in CNS-related features of RA, this indicates that alternative neuro-immune mechanisms independent of microglia activation may be at play.

3.1.3 Markers of peripheral inflammation are elevated in allergic subjects both in and out of pollen season

In **Study II** the peripheral cytokine levels, measures of sleep as well as the CNS related symptom of fatigue as measured by the validated multidimensional fatigue inventory (MFI-20)[262], was investigated both in and out of pollen season in allergic and healthy subjects. Allergic subjects displayed increased serum levels of TNFα and IL-5 during pollen season compared to controls as shown in **figure 13,** confirming the chronic inflammatory nature of allergy as described previously[263]. Interestingly, IL-5 serum levels were also elevated in allergic subjects compared to controls out of pollen season. Allergic subjects also reported increased sleep disturbance during pollen season, in line with litterature[263], as well as shorter sleep time irrespective of season.

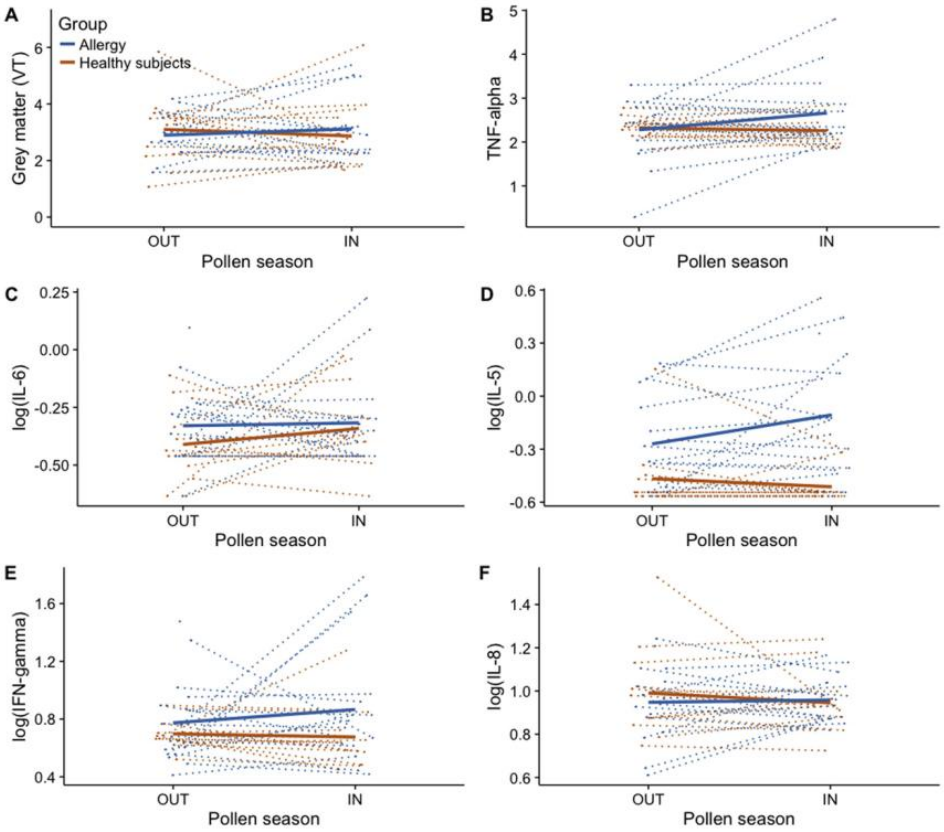


Figure 13 Cytokine serum levels in allergic (blue) and healthy (red) subjects in and out of pollen season. Dotted lines represent paired individual measures and solid lines represent group average.

In concordance with previous studies on allergy and fatigue[202], allergic subjects reported elevated levels of fatigue during pollen season, primarily driven by general fatigue. However, no relations were shown between brain expression levels of TSPO and peripheral cytokine levels, sleep as well as measures of fatigue, either in or out of pollen season, also in **study II**.

While the exact mechanism of fatigue is unknown several possible immune-to-brain communication routes, including vagal signalling and HPA-axis engagement, favours peripheral and/or central inflammation as a highly potential driving factor[264]. For example, associations between peripheral inflammation and fatigue in cancer survivors are frequently reported[264]. Contrastingly, no relation between measures of fatigue and peripheral levels of cytokines was observed for allergy patients (in or out of season) in **study II**. However, the multidimensional aspect of fatigue is rarely taken into account in studies exploring relation to inflammation and different aspects of fatigue is likely affected differently by inflammation[264]. If fatigue is associated with central rather than peripheral inflammation, it is possible that such relations go undetected in this study as glial TSPO levels may not reflect the full spectrum of all central inflammatory events.

In conclusion, also in seasonal allergy brain levels of TSPO may not be a feature associating with CNS-related symptoms (fatigue), peripheral inflammation or sleep. In light of numerous reports of a possible central neuronal inflammatory component in fatigue, together with suggestions of serotonin system involvement in allergic inflammation[263], other aspects of brain neuro-immune involvement than microglia activation should also be explored.

3.2 STUDY III

To investigate the relationship between possible central nervous inflammation in arthritis and CNS related symptoms from a different perspective, the proteome of CSF samples from a small but unique cohort of patients (n=10) with different arthropaties were analysed using MS. In this cohort, patients are assessed and sampled once at baseline (BL) and once eight weeks after initiating the anti-TNF therapy infliximab (IFX). By exploring this unique cohort we are thus able to ascertain IFX related changes of individual proteins detected by MS. This further enabled us to infer information about central inflammation and how it is affected by treatment, additionally shedding light on new candidate proteins to study more closely in relation to arthritis, inflammation and CNS related symptoms.

3.2.1 Proteins affected by anti-TNF therapy in CSF are predominantly related to inflammatory processes

Following eight weeks of infliximab treatment patients displayed significantly lower measurements of DAS28, levels of pain as measured on a visual analogue scale (VAS-pain) and peripheral inflammation (C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)) indicating a positive treatment response in accordance with literature[265-268].

In line with these observations univariate analysis of data from two independent MS experiments revealed 35 candidate proteins, a majority with immune related function, that were reduced in CSF of the arthritis patients following anti-TNF therapy. Further multivariate analysis identifies 11 of the 35 significantly reduced proteins as additionally significantly contributing to the separation of samples at BL and after treatment. Interestingly, as shown in **table 3** a majority of these 11 proteins have neuro-inflammatory properties. In concordance with this observation, anti-TNF treatment is additionally used as a treatment strategy in the neuroinflammatory Behcet's disease, where lower levels of IL-6 are reported both in the periphery and in CSF following treatment[269,270]. Importantly, studies of anti-TNF therapy in experimental models of arthritis also indicate beneficial central effects on e.g. pain[218,271].

It is currently unknown if BBB function is concomitantly attenuated in arthritis. In our cohort of arthritis patients BBB integrity was assessed by albumin ratio and found to be intact. However, as elevated levels of circulating pro-inflammatory cytokines are able to affect the CNS via alternate routes such as the vagus nerve or via interaction with the endothelial cells of the BBB[111,177] significant central responses are thus plausible even with an intact BBB.

Table 3 Proteins important for separation between patients before and after infliximab treatment. Proteins were identified in CSF of polyarthritis patients using label free proteomics and uni- and multivariate data analysis.

			Univariate analysis			*Multivariate analysis*			
Protein Name	Gene Symbol	Accession	Fold change†	Z score	p value	VIP	p(corr)	Process	Suggested function(s)*
Cell Adhesion Molecule 3	CADM3	Q8N126	-0.68	-1.992	0.046	2.0	0.7	Cell adhesion	Overexpressed in murine microglia after bacterial challenge and may be involved in development of depressive symptoms following immune challenge. [46]
Insulin Like Growth Factor Binding Protein 7	IGFBP7	Q16270	-0.50	-2.201	0.028	1.6	0.7	Cell adhesion	Upregulated in spinal cord during EAE and suggested to be a regulator of oligodendrocyte differentiation. [57]
Protein Tyrosine Phosphatase, Receptor Type N	PTPRN	Q16849	-0.49	-2.201	0.028	1.6	0.6	Cell signalling	Important for proper secretion of hormones (insulin) and neurotransmitters [58]
Apolipoprotein H	APOH	P02749	-0.32	-1.992	0.046	1.7	0.8	Coagulation	May be associated with brain atrophy in healthy individuals [59]. Is the main antigen in antiphospholipid syndrome and may be associated with CNS related disease in these patients [60]
Fibrinogen gamma chain	FGG	P02679	-0.61	-2.201	0.028	1.5	0.5	Immune response, Acute phase protein	Important for proper T cell functioning and neutrophil pathogen clearance [40]. Regulator of microglia activation which may be important in pathogenesis of experimental autoimmune encephalomyelitis [61]
Alpha-1-B Glycoprotein	A1BG	P04217	-0.39	-2.201	0.028	2.6	0.7	Immune response, Acute phase protein	-
Beta-2-Microglobulin	B2M	P61769	-0.44	-1.992	0.046	1.7	0.8	Immune response, Adaptive immuntity	Increased in circulation in chronic fatigue syndrome [62] and identified as important in CSF of female chronic widespread pain patients [63]. CSF levels of B2M is suggested to reflect immune activation and lymphoid cell turnover in the CNS [64]
Complement C7	C7	P10643	-0.48	-2.201	0.028	2.1	0.5	Immune response, Innate immunity	-
Complement Factor B	CFB	P00751	-0.38	-1.992	0.046	1.7	0.6	Immune response, Innate immunity	Differentially expressed in AD CSF [65]
Complement C4B (Chido Blood Group)	C4B	P0C0L5	-0.37	-2.201	0.028	2.1	0.5	Immune response, Innate immunity	Differentially expressed in CSF of AD patients [65] and elevated in CSF of MS pateints with active disease [66]
Hemopexin	HPX	P02790	-0.33	-1.992	0.046	1.7	0.7	Oxidative stress protection	Neuroprotective in stroke and intracerebral haemorrhages [67]. Increase in CSF following yeast-induced inflammation [68]

† Fold change is calculated as "(sample after infliximab - baseline sample)/baseline sample" * References are refering to reference list in study III manuscript.

3.2.2 CSF candidate proteins affected by IFX associate with clinical measures of inflammation and disease activity

Although, all the infliximab reactive proteins are of considerable interest to explore further for their role in inflammation and arthritis, a selection was made to form the initial focus of investigation. Of the 11 aforementioned proteins, seven were selected based on their significant contribution in multivariate analysis, significant reduction by IFX and having known associations to arthritis for closer scrutiny with regards to relations to clinical data. Of these, fibrinogen gamma chain (FGG), complement factor B (CFB), cell adhesion molecule 3 (CADM3) and contactin-1 (CNTN-1) were found to have significant correlations with clinical measures of inflammation, functionality and pain. The respective correlations for FGG and CFB were very similar and closely followed each. The fold changes in FGG and CFB each correlated to the fold change in ESR as shown in **figure 14** as well as fold changes in health assessment questionnaire score (HAQ score), a questionnaire measuring the functional abilities of the patients.

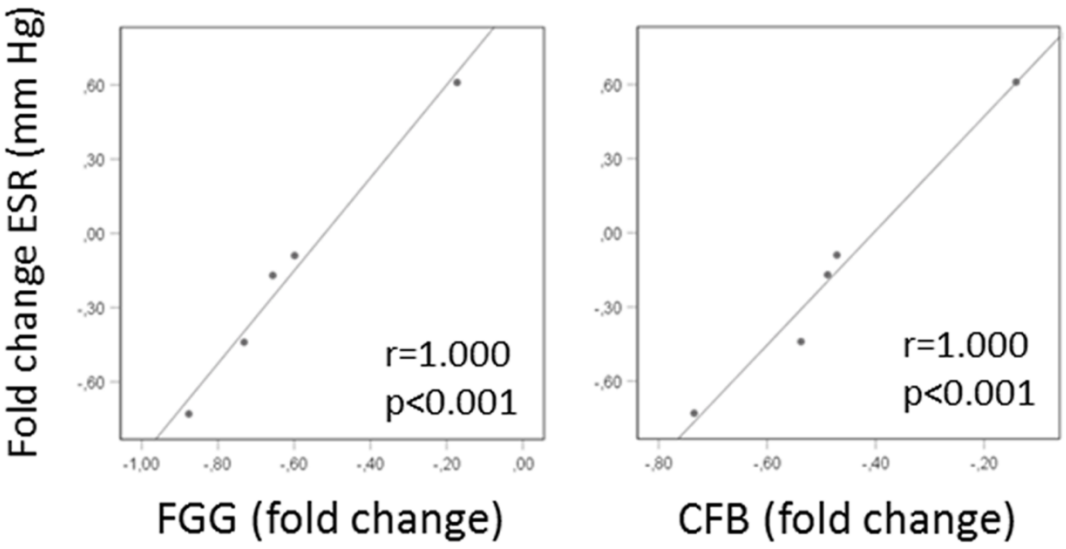


Figure 12 Correlation plots between selected CSF candidate proteins FGG and CFB affected by anti-TNF treatment and ESR in arthritis patients assessed at baseline (BL) and after eight weeks of infliximab treatment.

Fibrinogen is normally involved in coagulation, but is recognized as a marker for inflammation in several chronic inflammatory diseases including RA, Alzheimer's disease and multiple sclerosis[272]. In line with our observation of decreased FGG in arthritis CSF and correlation between FGG and ESR, FGG reduction has been reported in plasma of an arthritis patient after anti-TNF therapy[273]. Moreover, a neuro-immunoregulatory role for FGG, mediated via interaction with the macrophage antigen complex-1 (Mac-1) present on immune cells such as microglia, is suggested by animal experimental data[274,275]. The modulation of microglial function by blocking Mac-1 has been reported to be neuroprotective by inhibition of microglial H_2O_2 and PGE_2 production respectively[276,277]. In addition, blockade of CFB has been reported to have neuroprotective effects in a murine experimental model of experimental autoimmune encephalomyelitis (EAE)[278]. CFB is one of the key players in the alternative complement pathway and its expression has been reported

to be regulated dose dependently by TNFα in circulating human immune cells[279]. Thus, both FGG and CFB in CSF may play a possible role for central inflammatory responses and may therefore be involved in CNS related symptom pathology.

Interestingly, correlations to VAS-pain were observed for both CNTN1 and CADM3. CNTN-1 and CADM3 are both involved in cell adhesion and has both been reported in relation to depressive states in experimental settings[280,281] demonstrating their potential for central effects. Importantly, in line with our observations of CNTN-1 correlation to pain, CNTN-1 has been reported to play a role in the sodium channel $Na_v1.3$ mediated neuronal pain signaling[282]. CNTN-1 is additionally reported to be increased in the CSF of neuropathic pain patients following analgesic spinal cord stimulation, further supporting the involvement of CNTN-1 in pain processing although the exact nature of involvement remains to be elucidated.

Although using a small material the unique samples enabled investigation of the proteome in arthritis patient CSF for the first time, revealing that anti-TNF effects extend to the CSF which may help explain the beneficial treatment effects on select CNS related symptoms. Furthermore, we here identify FGG and CFB as potential mediators of central nervous inflammation, and CADM3 and CNTN-1 with possible roles in pain processing which should be investigated further.

3.3 STUDY IV AND V

The pronounced anti-inflammatory potential of VNS together with an inability of current treatment strategies to sufficiently reduce disease activity in a number of patients suffering from chronic inflammatory diseases, including RA, swiftly initiated clinical trials to harness the anti-inflammatory power of CAP in such patients[230]. However, the exact mechanism of action for CAP is still being discovered and the full extent of CAP mediated effects is rarely studied. In **study IV and V** investigations into the inner workings of the CAP is initiated to contribute to a more complete understanding of vagally mediated neural inflammatory regulation. Because, by understanding the mechanism better, we enable CAP activation to be used more efficiently and to its full potential as a treatment strategy in patients likely in a plethora of different inflammatory settings.

3.3.1 VNS decrease CD69 expression in CD4+ T cells and NK cells in spleen of endotoxaemic mice without apparent effects of cell trafficking (study IV)

In our setup of VNS in endotoxaemic mice, a clear anti-inflammatory effect was observed with significantly decreased levels of TNFα as well as IFNγ in VNS compared to Sham animals in accordance with the pivotal CAP study by Borovikova et.al.[168]. In the present experimental setup cells were collected 6h after VNS or Sham surgery limiting us to study the early inflammatory responses.

The bacterial endotoxin LPS, is known to induce a clear activation of immune cells resulting in avid cytokine production both in mice and men[283]. In accordance, we observe a clear increase in CD69 in both NK and all T cell subsets in groups receiving LPS compared to control groups not receiving LPS, indicating a LPS driven immunoreactivity in our model.

Populations of different immune cell types of both innate and adaptive origin were identified by commonly used extra- or intracellular markers. While both relative and absolute cell numbers remained largely constant in identified cell populations indicating little effect of VNS on cell trafficking, CD69 expression was readily reduced by VNS in both splenic and circulating $CD4^+$T cells. As shown in **figure 15**, when dissecting the contribution of different $CD4^+$T cell phenotypes or subtypes to this observation, it was revealed that VNS mediated decrease in CD69 extend to T cells of both memory and naïve phenotype but not regulatory T cells or Th17 cells although a tendency was observed in Th1 cells. In blood CD69 reduction in VNS groups is largely reflecting observations is spleen. Interestingly, also splenic NK cells displayed reduced CD69 expression in the VNS group compared to sham, an effect that was not extended into blood (**figure 16**). It will thus be interesting to explore what CAP mediated CD69 reduction means in terms of functionality of the affected cells.

Interestingly, we report similar tendencies of decreased CD69 expression in T cells also in the mesenteric lymph nodes (MLN). In contrast to the observations in spleen and blood a significant increase in relative $CD4^+$ T cell numbers was observed in MLNs indicating that cells T cells may be sequestered there and illustrating that CAP effects may be organ specific.

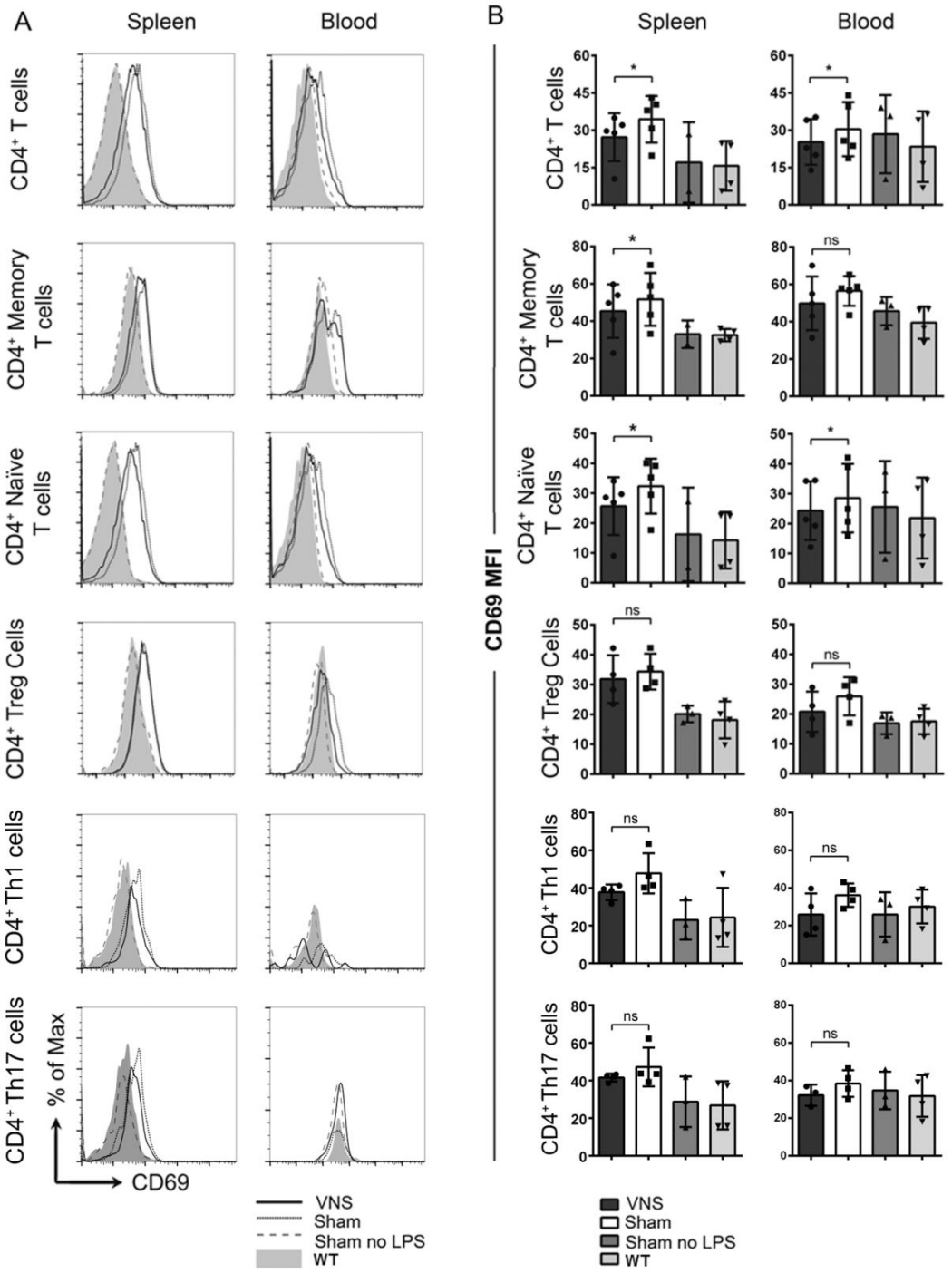


Figure 13 CD69 surface expression as measured by flow cytometry on T cell subsets from C57BL/6 endotoxaemic mice 6h following VNS or Sham surgery. Sham treated animals not receiving LPS (Sham no LPS) and untreated animals (WT) were used as control groups. A) Representative histograms of CD69 staining in spleen (left) and blood (right). B) Geometric mean fluorescence intensity (MFI) of CD69 in CD4 T cells ($CD4^+$), CD4 memory T cells ($CD4^+$, $CD44^{high}$, $CD62L^{low}$), CD4 naïve T cells ($CD4^+$, $CD44^{low}$, $CD62L^{high}$), regulatory T cells ($CD4^+$, $FoxP3^+$), Th1 cells ($CD4^+$, $IFN\gamma^+$) and Th17 cells ($CD4^+$, $IL\text{-}17^+$). Each dot represents one animal. * indicates $p<0.05$.

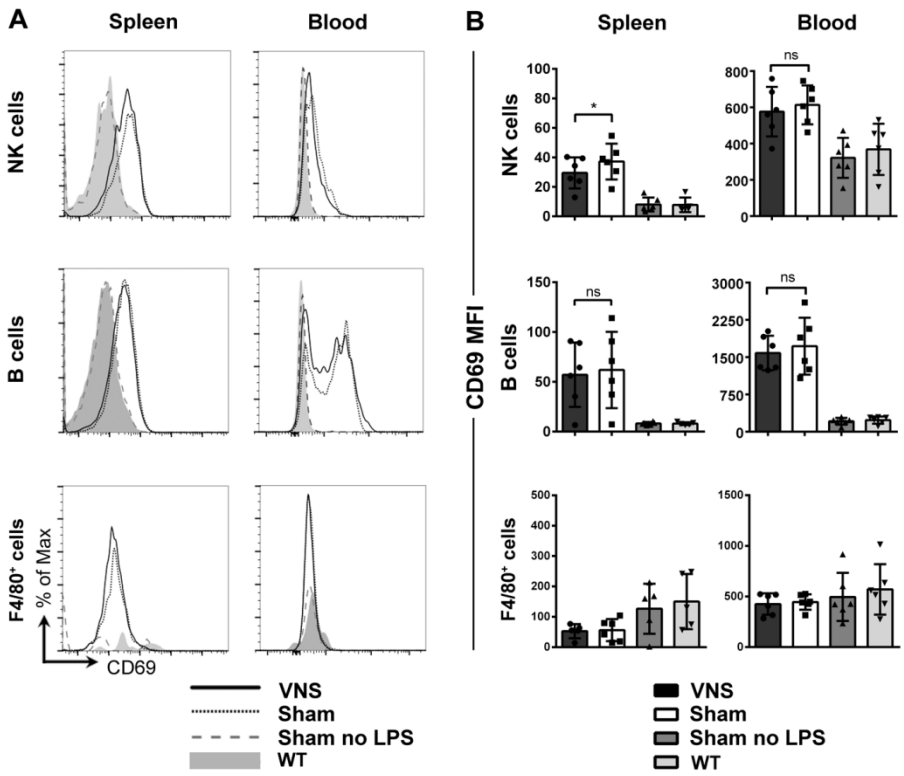


Figure 14 Surface expression of CD69 as measured by flow cytometry on non-T cells from C57BL/6 endotoxaemic mice 6h following VNS or Sham surgery. Sham treated animals not receiving LPS (Sham no LPS) and untreated animals (WT) were used as control groups. A) Representative histograms of CD69 staining in spleen (left) and blood (right). B) Geometric mean fluorescence intensity (MFI) of CD69. Each dot represents one animal. * indicates $p<0.05$.

The efficacy of VNS to limit pro-inflammatory cytokine production is well established in both human and animal models[168,230]. However, only Mihaylova and co-workers have so far investigated the effect of VNS on immune cells in a broader perspective, reporting no effects on cell trafficking[284], further supporting our observations. Contrasting to our observations of VNS mediated reduction of CD69, Mihaylova et.al. detects no effect of VNS on cell activation[284]. Several reasons for this discrepancy may be related to differences in experimental setup between our two studies. First, Mihaylova's study is conducted in rats and although mice and rats are closely related, species differences may exist. Secondly, a higher dose of LPS is used in Mihaylova's study, which may overpower the VNS ability to fine tune the immune response. Third, and most importantly, whereas Mihaylova and colleagues use CD134 as a marker of activation, CD69 is used in our study. While CD69 upregulation is reported to be detectable already 1h after activation with full expression plateauing around 6h, CD134 upregulation after activation may take up to 12h, but can be re-expressed in memory subsets as early as 1h after activation[285,286]. The different temporal dynamics in expression pattern after activation, may thus explain why we are able to observe VNS effects on activation in contrast to Mihaylova's study.

Finally, **study IV** show for the first time that CAP effects extend also to NK cells. NK cells are not only important for cell mediated killing, but may also be key players in directing both T and B cell responses and have gained interest as mediators in several autoimmune diseases including rheumatoid arthritis[287]. In RA an accumulation of primarily cytotoxic NK cells are

found in the synovial fluid, where these cells are reported to express increased levels of CD69 and produce greater amounts of pro-inflammatory cytokines compared to their circulating counterparts[287]. Accumulation of such NK cells may thus be contributing to the pro-inflammatory environment in the arthritic joint. This is further supported by an experimental animal model of arthritis reporting beneficial effects of NK cell depletion on bone erosion and arthritis severity[287]. However, it is important to note that the nature of NK cell involvement may be beneficial since NK depletion has also been reported in experimental arthritis to exacerbate disease and autoantibody production[287].

In light of this, activation of CAP to may therefore be beneficial in inflammatory conditions where dysactivation of NK cells is driving pathology.

3.3.2 The anti-inflammatory potential of CAP is dependent on functional PGE_2 synthesis in murine and human immune cells subjected to endotoxaemia

In a previous study from our group it was revealed that CAP in PGE_2 deficient mPGES-1 ko mice were unable to reproduce the evident anti-inflammatory effects seen in mPGES-1 wt counterparts[245]. There are several steps in splenic events of the CAP response where PGE_2 involvement may play an important role, including NA release, β_2AR expression and synthesis of ACh. In **study V** PGE_2 involvement is therefore investigated in each of these steps.

The first two steps of splenic events following initiation of CAP is NA release into the spleen mediated by splenic nerve activity and engagement of β_2AR on ACh producing T cells of memory phenotype[177]. Signaling in the splenic nerve has been shown to be initiated also by peripheral LPS and to depend on central PGE_2 involvement[158]. Since mPGES-1 deficiency display reduced PGE_2 levels in the CNS, NA release in the spleen may be affected in mPGES ko mice although anti-inflammatory signaling in the splenic nerve in this study is mainly attributed to VNS. PGE_2 is also an important modulator of T cell activation, differentiation and function[288], wherefore PGE_2 deficiency may affect splenic T cell β_2AR expression or functionality. However, as shown in **figure 17** NA release was found to be increased after VNS in both mPGES-1 ko and wt mice. Furthermore, β_2AR expression as well as relative cell numbers of $CD4^+$ T cell memory, naïve and effector subsets were normal following VNS in both mPGES-1 ko and wt mice. PGE_2 mediated NA and β_2AR deficiency as the reason for impaired CAP in mPGES-1 ko mice was thus ruled out. It is important to point out that while β2AR expression was normal on splenic $CD4^+$ T cells, we are not able to disclose if its function is normal in mPGES-1 ko mice.

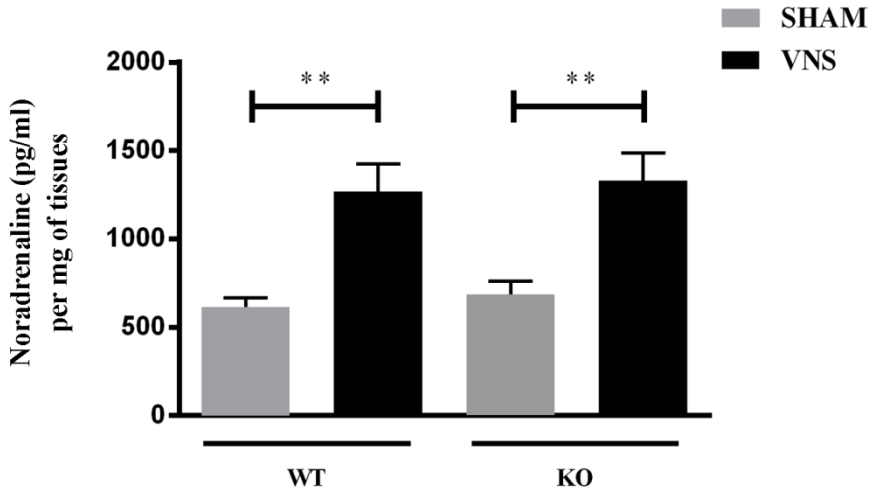


Figure 15 Noradrenaline measured in spleen extract of mPGES-1 ko and wt mice subjected to VNS or sham surgery following i.p. LPS (2mg/kg).

In vitro studies of splenocytes from mPGES-1 ko and wt mice reveal intact ability of both ko and wt splenocytes to produce pro-inflammatory cytokine in response to LPS. However, the anti-inflammatory effect of NA treatment, mimicking splenic nerve signaling, was observed in mPGES-1 wt splenocytes but impaired in mPGES-1 ko splenocytes. Thus, it is indicated that PGE_2 dependence is mediated at a cellular level. With the knowledge that immune cells must depend on upregulation of the ChAT enzyme for their ACh production[289] and LPS treatment is known to induce ChAT expression in immune cells[290] PGE_2 may affect this process. As shown in **figure 18** mPGES-1 ko and wt derived splenocytes displayed similar levels of ChAT expression during unstimulated conditions. However, when splenocytes were stimulated with LPS an increase of ChAT expression was seen in mPGES-1 wt but not ko derived splenocytes. Thus the PGE_2 dependent step in the anti-inflammatory response of CAP is attributed to ChAT induction. In accordance, studies of a human T cell line report induced ACh synthesis via ChAT expression instigated by engagement of prostaglandin receptor EP4[291]. This illustrates that a role for PGE_2 in mediating a proper anti-inflammatory CAP response upon VNS could be important also in a human setting.

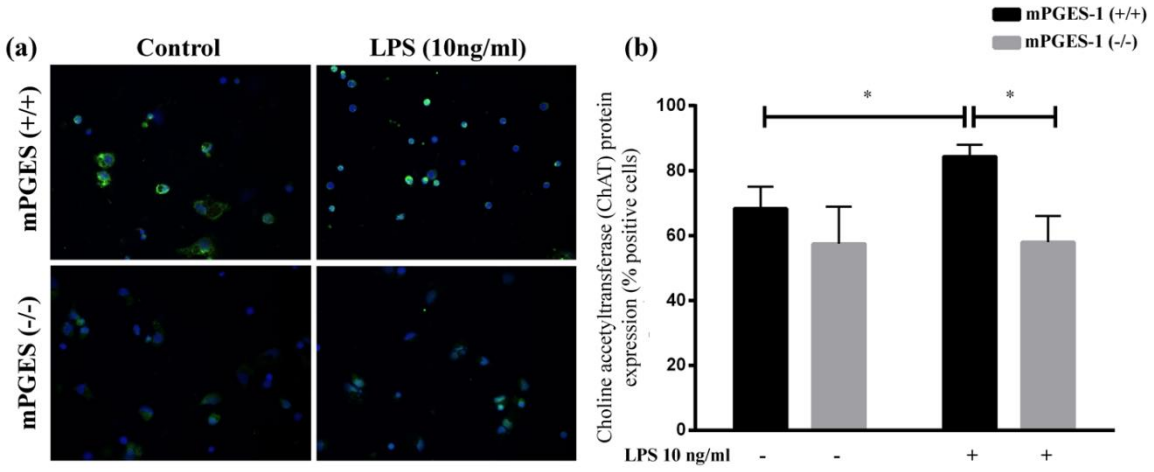


Figure 16 ChAT expression in splenocytes from mPGES-1 ko and wt mice after 20h *in vitro* exposure to LPS (10ng/ml). A) Representative images of immunofluorescent staining of ChAT (green) and DAPI (blue) of cultured mPGES-1 ko and wt splenocytes. B) Quantification of ChaT expression in LPS treated or unstimulated mPGES-1 ko or wt splenocytes.

Importantly, engagement of the cholinergic receptor α7nAChR is not only an integral part of the CAP, but has been shown to diminish pro-inflammatory cytokine production of immune cells in both human and animal settings in its own right[292,293]. In line with this, a nicotine mediated inhibition of pro-inflammatory cytokine production in response to LPS was demonstrated both in mPGES-1 wt splenocytes and human PBMCs (**figure 19**). This anti-inflammatory response was not seen in mPGES-1 ko splenocytes and was abolished in human PBMCs after pharmacologic blockade of PGE_2 synthesis.

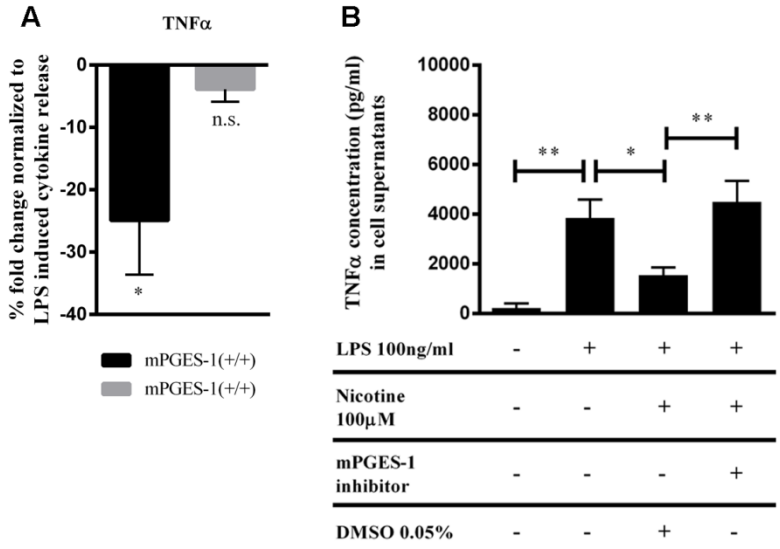


Figure 17 TNFα release in splenocytes treated with nicotine under endotoxaemic condition. A) Percent decrease of TNFα in nicotine pre-treated condition vs LPS only in mPGES-1 ko and wt mouse splenocytes. B) TNFα release measured in supernatant from cultures of human PBMCs pre-treated with nicotine and stimulated by LPS in conditions with and without mPGES-1 inhibitor.

Together, these results indicate that PGE_2 may be important for proper CAP-mediated anti-inflammatory regulation at the level of ACh production as well as α7nAChR dependent pro-inflammatory cytokine regulation. These results furthermore illustrate that the PGE_2 dependence is also present in humans warranting careful consideration of NSAIDs in persons depending on the anti-inflammatory properties of CAP.

4 GENERAL DISCUSSION

Several routes whereby the CNS can be made aware of peripheral inflammation, including afferent vagus nerve signalling and cytokine meditated prostaglandin production at the BBB interface, has been discovered in recent years[147,177]. Together with the discovery of active neuronal control of inflammation via the vagus nerve in the form of the CAP[177] it was established that the CNS plays an important role in directing inflammatory responses. Intriguingly, animal studies of vagotomy show reduction in the production of pro-resolving mediators and a delay of resolution[294] further strengthening the importance of functional vagal signalling to properly control inflammation. In light of this, it is interesting to note that autonomic activity is often reported to be altered in different chronic inflammatory conditions which may contribute to a dysregulated immune response perpetuating inflammation. In RA patients, measurements of HRV reveal a consistent reduction in parasympathetic (vagal) signalling[211] which was also observed in our patients in **study I**. The close connection between vagal signalling and peripheral inflammatory mediators is illustrated by a frequently reported inverse relationship between HRV measures and peripheral HMGB1, IL-6, IL-8, IL-13 as well as IL-10 levels in inflammatory conditions such as RA[212,223], encephalopathy in newborns[295], sepsis[296], cardiovascular disease[297] and type-1 diabetes[298]. While the HRV relation to IL-6 is reported in multiple conditions, the HRV relationship to other cytokines may be disease specific reflecting the inflammatory nature of the respective diseases. In **study I** we report a similar relationship between autonomic activity and IFNγ. In support of this, data from animal sepsis studies report that LPS administration induced a reduction in HRV which was related to increased peripheral cytokine levels[299]. Interestingly, mouse studies of pulmonary inflammation, known to be associated with altered breathing patterns (i.e. altered autonomic activity), identifies a connection between elevated TNFα and IL-1β levels in lung and elevated IL-1β levels in the NTS, the primary brainstem nucleus controlling vagal signalling [300]. Interestingly, Ek et.al. has previously shown that neurons terminating in the NTS are highly responsive to peripheral IL-1β[301]**.** Together this underscores the potential involvement of central nervous inflammation in autonomic functionality in particular and CNS related symptoms in general.

Supporting this notion, we have previously reported elevated central cytokine levels in both RA patients and experimental arthritis models and shown that CSF cytokine profile differs between RA (IL-1β) and fibromyalgia (IL-8) patients[197,222]. Interestingly, the source of CSF IL-1β was indicated to be locally produced in the CNS since CSF levels was found to exceed plasma levels[197]. The central cytokine profile of allergic subjects remains unknown. However, both IL-6, TNFα and IFNγ were detectable in CSF of allergic patients with different types of cognitive impairment although no differences were observed in cytokine levels compared to non-allergic cognitively impaired subjects[302].

Pain sensitisation which is one of the CNS related symptoms frequently associated with RA and other arthropaties has been found to be connected to microglia as well as astrocyte activation in the spinal cord in several models of experimental arthritis[217,218,248]. It has further

been shown that inflammatory mediators such as prostaglandins and TNFα are closely linked to microglia associated pain[217,218,303]. Interestingly, a recent report by Yamasaki and co-workers suggest that similar actions may be seen also during allergy. They similarly demonstrate an activation of astrocytes and microglia in spinal cord of allergic mice relating to allodynia, furthermore describing a shift in spinal microglia phenotype toward pro-inflammatory M2 in the allergic mice[249]. Activated microglia has been shown in various animal and experimental models to play an important part in CNS cytokine production as well as behavioural changes such as infection driven delirium[304].

Microglial activation detected by PET has been shown to be induced by peripheral inflammation[129] and is reported in a growing number of acute and chronic inflammatory conditions such as stroke[132], Alzheimer's disease[130] and multiple sclerosis[305]. Since activated microglia are a considerable source of cytokines in the CNS[306] they are likely important players involved in CNS-related pathology in chronic inflammatory diseases.

However, we found no relation between HRV and glial activation as assessed by central TSPO levels in either RA **(Study I)** or allergy patients **(Study II)**. Recent contradictory reports of reduced central TSPO levels despite evidence of elevated central cytokine levels in schizophrenia patients initiated a suggestion by Notter and colleagues that TSPO may not accurately reflect low grade central inflammation[126]. Such a theory seems likely considering the high plasticity of microglial phenotypes and their strong dependence on microenvironmental ques to direct differentiation and thus their response. The nature of the potentially disease specific central cytokine profile[222] may therefore also contribute to the discrepancy in microglial TSPO response. This may provide an explanation for the lack of association between central TSPO levels and CNS related symptoms observed in **study I and II**. However, in support of our results, a study in an allergic mouse model report decreased microglial activation and increased neurogenesis in hippocampus of allergic mice[307] suggesting that there may indeed be no association between central glial activation and allergy or RA.

It is however conceivable that also other centrally induced/elevated mediators exert substantial effects on central inflammation and CNS related symptoms. As shown in **study III** several proteins related to inflammatory processes identified in CSF of arthritis patients, including fibrinogen gamma, was shown to be down regulated after TNF-blockade. Fibrinogen gamma was shown to be related to the reduction in peripheral inflammation. The vagus nerve also innervates the liver, the principal site of production of fibrinogen, and has there been reported to exert anti-inflammatory effects after VNS[176]. Whether or not this anti-inflammatory effect extends to fibrinogen production is unknown, however a hint is provided by Carney et.al and Cooper et.al. They report an inverse relationship between HRV and peripheral fibrinogen levels in general population[308] and in coronary heart disease patients with depression[309] respectively. In line with this, elevated levels of circulating fibrinogen are reported in arthritis[310]. Whether elevated peripheral levels of fibrinogen are reflected in the CSF of arthritis patients remains unknown. However, as CSF fibrinogen gamma levels are reducible by TNF-blockade **(study III)** it is conceivable. We suggest a role

for central FGG in modulating central inflammation in accordance with reports of FGG involvement in microglial activation via MAC-1 complex in an EAE model[311].

Although a successful treatment strategy, TNF-blockade and other biologic treatments are not able to help all patients and symptoms may persist. Together with the conceivable risk of the patient developing anti-drug antibodies[312] there is a need for the development of alternative treatment strategies. One such promising alternative is activation of the CAP via VNS, which has been proven effective in mouse models of e.g. sepsis, RA, colitis and postoperative ileus[229,313-315]. Autonomic dysregulation is described in many patients with different chronic and acute inflammatory disorders including RA[205], systemic lupus erythematosus[316], allergy[317] and sepsis[296]. In RA, autonomic dysfunction is characterised by decreased vagal signalling[205], indicating that the CAP in these patients may also be impaired and may thus contribute to the exacerbated inflammation. Activation of the CAP in RA patients via VNS could then provide an important tool to restore autonomic function to a certain extent and provide increased ability for neuro-immunoregulation. Intriguingly, pilot clinical trials are showing promising results in RA and Crohn's disease with indications also in postoperative ileus[230,231,315]. However, a lot remain unknown regarding the exact mechanism and extent of the CAP mediated anti-inflammatory properties as illustrated by the failure of VNS to reduce inflammation in human endotoxaemia[318]. Therefore it is important to continue to explore the mechanism and effects of CAP to ensure continued safe and optimal use of VNS as a treatment strategy.

As the CAP is increasingly investigated, a growing amount of studies report CAP effects beyond reduced pro-inflammatory cytokine production from macrophages in the spleen. For example, vagal projections to the gut are found to influence muscularis macrophages via enteric neurons in a mechanism independent of the spleen[319]. Furthermore, CAP has been shown to affect expression of the surface adhesion protein CD11b on endothelial cells reducing leukocyte migration[320]. In line with this, in **study IV** we extend the observations of VNS mediated effects to reduction of CD69 expression on NK cells in spleen and effects on $CD4^+$ T cell trafficking in the MLN. Additionally, in **study V** a prostaglandin dependence for intact anti-inflammatory effects on pro-inflammatory cytokine production is demonstrated, together adding to the constantly broadening scope of CAP mediated effects to consider in therapeutic settings.

Emerging evidence point toward an additional role for VNS in central inflammation and the control of cognitive function. Meneses and co-workers for instance describe a role for VNS in neuronal inflammation, where central cytokine levels and microglial activation as measured by Iba-1 expression is attenuated by VNS in a model of intrathecal endotoxaemia[321]. In line with this, VNS has also been shown to reduce inflammatory mediated recruitment of neutrophils into areas of the CNS with BBB fenestration, thus controlling pro-inflammatory cytokine levels in the brain[322].

Together these data highlight the extensive ANS involvement and detrimental impact of ANS dysregulation in the control of inflammatory responses. The potential contribution of neuroinflammation on burdensome CNS-related symptoms and the importance of continued investigation into all these aspects of neuroimmune mechanisms in chronic inflammatory disease conditions.

5 CONCLUDING REMARKS

With chronic inflammatory conditions being a considerable burden for society as well as the patient it is essential to make every effort to better understand the underlying disease pathology. The work of this thesis has investigated the role of central inflammation in disease pathology and connection to CNS related symptoms as well as explored mechanisms of neuroimmune regulation.

Based on evidence pointing toward presence of central inflammatory involvement in both RA and allergy pathogenesis, we sought to confirm this hypothesis in **study I and II** by investigating microglia activation in the CNS of RA patients and allergic subjects using PET. We were not able to confirm this hypothesis as no difference in microglia activation was detected between either RA patients or allergic subjects and their controls. Furthermore, no relation was found between central microglia activation and peripheral inflammation or fatigue in RA or allergy, autonomic dysfunction in RA patients or sleep measures in allergic subjects. However, it remains to be noted that low grade central inflammatory events may not be reflected by increased glial activation as measured by TSPO expression leaving the question of central inflammation in RA and Allergy and relations to CNS related symptoms to be conclusively answered in future investigations. In **study I** we provide further evidence of the close relation between ANS dysfunction and circulating levels of inflammatory mediators. In **study II** we provide evidence that allergen exposure is associated with increased levels of not only circulating IL-5 but also TNFα and that being allergic is associated with higher IL-5 levels even when not exposed to allergen.

In **study III** we sought out to investigate central inflammation and relation to CNS related symptoms from a different angle in arthritis patients. This was done by exploring proteomic changes in CSF of arthritis patients treated with biologic blockade of TNFα since this treatment strategy is known to be able to ameliorate certain CNS related symptoms. We demonstrate for the first time that TNF-blockade exerts considerable effects on the proteome in CSF of arthritis patients and identify 35 CSF proteins decreased by treatment. Of these proteins, the majority show involvement in inflammatory processes, thus adding to the growing expanse of circumstantial evidence supporting central inflammatory involvement in arthritis. Furthermore, we identify fibrinogen gamma and complement factor B as a likely important players in central nervous inflammation, as well as contactin-1 and cell adhesion molecule 3 as potentially involved in pain sensitisation/regulation which should be investigated further.

Since one of the CNS related features associated with RA is reduced vagal activity, VNS is tested as a feasible treatment strategy to restore parasympathetic, i.e. CAP, function although much remain to be understood about CAP mechanism of action. In **study IV** we therefore explore the extent of CAP mediated effects on the immune system and in **study V** investigate mechanistic involvement of PGE_2 in the CAP. We here provide the first evidence that CAP mediated effects extend to NK cells. Additionally, we provide evidence that CAP effects are not limited to the spleen and that those effects may be organ specific. We also

demonstrate for the first time that a functional mPGES-1 and subsequent PGE_2 production is essential for ChAT upregulation following engagement of β_2AR as well as for inhibition of pro-inflammatory cytokine release following engagement of α7nAChR in splenocytes subjected to endotoxaemia.

Although not confirmed in **study I and II** we provide further evidence of central inflammatory involvement in arthritis in **study III**, pinpointing several proteins likely involved in this central inflammatory response and CNS related symptoms. Using experimental murine models in **study IV and V** we extend our mechanistic insight into the anti-inflammatory properties of the CAP important to consider when applying VNS as an anti-inflammatory treatment strategy in human chronic inflammatory diseases.

6 FUTURE PERSPECTIVE

A big step forward for a PhD student is a small step forward for the scientific community towards understanding neuroimmune involvement in chronic inflammatory diseases. Thus, even though this thesis has led to an advancement of our current understanding of neuro-immune mechanisms it has also pin-pointed several questions in wanting of answers from future research projects.

For example, so far only few studies have considered altered HRV in allergy patients hinting at withdrawal of sympathetic activity and increased vagal activity[210,317]. Could allergy patients then have an abnormally active CAP? Engagement of β_2AR on immune cells is known to promote a Th2 environment[156,157]. Together with the fact that PGE_2 also has the capacity to shift an immune response toward not only Th2 but also B cell IgE production[143,152] and our report of a functional CAP depending on PGE_2 **(study V)**, could an overactive CAP thus provide an environment suitable for initiation/sustention of allergen reactive Th2 and B cells? Could a side effect of chronic VNS be increased predisposition to allergy? Could allergy itself be treated by sympathetic stimulation or inhibition of vagal or β_2AR activity? How is PGE_2 involved in allergy? Do allergy patients have altered PGE_2 levels and in such a case would that relate to peripheral inflammation, central inflammation and symptoms?

Furthermore, CAP effects were shown in **study IV** to be extended to NK cells and possibly Th1 cells, both important sources of IFNγ and both indicated in RA disease pathology. Together with our reports in **study I** of increased peripheral levels of IFNγ in RA patients and an overall inverse relation between IFNγ and vagal activity raise questions about the status of circulating Th1 and NK cells in RA patients and possible relations to autonomic as well as disease activity. Interestingly , in **study III** one of the proteins identified as down regulated in CSF of arthritis patients after TNF-blockade was NCAM-1, which is also known as CD56, the primary surface marker used to identify human NK cells [30]. Is there an inflammatory driven influx of NK and other immune cells into arthritis CSF? Could such an influx explain generation of CNS related symptoms?

Data sets and a number of parameters collected from **study I, II and III** are considerably large and the work of analysing all aspects of these data sets has only just begun. With HRV measurements recorded also from allergy patients and their controls in and out of pollen season, investigations are currently underway to address some of the questions raised regarding HRV involvement in allergy. Furthermore, potential associations between additional aspects of fatigue measurements and microglia activity in brain regions of interest in RA remain to be investigated. **Study I and II** additionally included flow cytometric analysis data of blood cells, which is currently investigated to address some of the questions regarding circulating immune cells and relations to disease symptoms and activity in RA and allergy. Furthermore, a project to investigating immune cell status in CSF in RA has been initiated.

Regarding the proteins identified as decreased in CSF of IFX treated arthritis patients (**study III**) only a minority has so far been thoroughly investigated and continued efforts with the rest of the identified proteins will surely reveal many more interesting candidates. And much more work remains to further investigate these in arthritis and inflammatory models to pinpoint their specific function and contribution to arthritis, inflammation and CNS related symptoms.

Answering all of these questions and more inevitably raised along the way will go far towards understanding disease initiation and progression of disease pathology as well as indicating new and improved treatment strategies in chronic inflammatory conditions.

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