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Herpesvirus Infection and Immunity in Neurocognitive Disorders

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Abstract

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Herpesviruses have co-speciated with several vertebrate and invertebrate animals throughout the history of evolution. In the immunocompetent human host, primary infection is usually benign, whereafter the virus is brought into life-long latency. Viral reactivation can however cause severe disease in immunocompromised, and rarely also in immunocompetent, patients. The overall aim of this thesis was to study the immunologic effects of cytomegalovirus (CMV) and herpes simplex type 1 (HSV-1) infection in neurocognitive disorders.

CMV is known to promote T-cell differentiation towards a more effector-oriented phenotype, similar to what is seen in the elderly. We have addressed the frequency of CMV-specific CD8+ T-cells in Alzheimer's disease (AD). Furthermore, we have investigated whether AD patients present with a different CMV-specific immune profile, overall CD8 phenotype or inflammatory cytokine response to anti-CD3/CD28 beads, CMV pp65 and amyloid beta. Subjects with AD presented with a lower proportion of CMV-specific CD8+ T-cells compared to non-demented (ND) controls, but no differences in overall CD8 differentiation were seen. Overall, AD subjects presented with a more pro-inflammatory peripheral blood mononuclear cell (PBMC) phenotype. When PBMCs were challenged with CD3/CD28-stimulation, CMV seropositive AD subjects presented with more IFN-γ release than both CMV seronegative AD subjects and CMV seropositive ND controls.

For effective screening of humoral herpesvirus immunity, both in research and in clinical practice, efficient immunoassays are needed. We have addressed the methodology of multiplex herpesvirus immunoassays and related bioinformatics and investigated antibody levels in AD patients and ND controls. Subjects with AD presented with lower levels of human herpesvirus 6 (HHV-6) IgG. However, there was no difference in HHV-6 DNA levels in PBMCs between the groups.

Herpes simplex encephalitis (HSE) is a devastating disease, where antiviral treatment has greatly decreased mortality but not eliminated the associated long-term neurocognitive morbidity. We have investigated the correlation between N-Methyl-D-Aspartate Receptor (NMDAR) autoimmunity and recovery of neurocognitive functions after HSE. Approximately one quarter of all HSE cases developed NMDAR autoantibodies within 3 months after onset of disease. Antibody development was associated with an impaired neurocognitive recovery during the two year follow-up and could become an important therapy guiding factor in the future.

Keywords: Alzheimer's disease, Herpes simplex encephalitis, Herpesvirus, Cytomegalovirus, CMV, HSV-1, HHV-6, CD8 T-cells, Cellular immunity, Immunosenescence, Autoimmunity, NMDA receptor

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Westman G, Lidehall A-K, Magnusson P, Ingelsson M, Kilander L, Lannfelt L, Korsgren O, Eriksson B-M. (2013) Decreased Proportion of Cytomegalovirus Specific CD8 T-Cells but No Signs of General Immunosenescence in Alzheimer's Disease. *PLoS ONE,* 8(10)

II Westman G, Berglund D, Widén J, Ingelsson M, Korsgren O, Lannfelt L, Sehlin D, Lidehall A-K, Eriksson B-M. (2014) Increased Inflammatory Response in Cytomegalovirus Seropositive Patients with Alzheimer's Disease. *PLoS ONE,* 9(5)

III Westman G, Blomberg J, Elfaitouri A, Yun Z, Ingelsson M, Lannfelt L, Eriksson B-M. Application of a Multiplex Herpesvirus Immunoassay in Alzheimer's Disease. *Manuscript submitted*

IV Westman G, Studahl M, Persson B, Eriksson B-M, Rönnelid J, Schliamser S, Aurelius E. N-Methyl-D-Aspartate Receptor Autoimmunity Affects Cognitive Performance in Herpes Simplex Encephalitis. *Manuscript in preparation*

Contents

Abbreviations

Aβ	Amyloid β
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APC	Allophycocyanin
APOE	Apolipoprotein E
APP	Amyloid precursor protein
CD	Cluster of differentiation
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
Cy	Cyanine
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
E	Early
EBV	Epstein-Barr virus
ECDF	Empirical cumulative distribution function
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FACS	Fluorescence-activated cell sorting
FSC	Forward scatter
FITC	Fluorescein isothiocyanate
GCS	Glasgow coma scale
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
HHV	Human herpesvirus
IE	Immediate-early
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
IRP	Immune risk profile
L	Late
MBAA	Multiplex bead array assay
MDRS	Mattis dementia rating scale
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
ND	Non-demented
NIH	National Institutes of Health
NFT	Neurofibrillary tangles
NMDAR	N-methyl-D-aspartate receptor
NSAID	Nonsteroidal anti-inflammatory drug
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
pp65	Phosphoprotein 65
RNA	Ribonucleic acid
SSC	Side scatter
TCR	T-cell receptor
T_{EMRA}	T effector-memory CD45RA+
TNF	Tumor necrosis factor
VZV	Varicella zoster virus

Introduction

Human herpesviruses

Herpesviruses have co-speciated with humans and several other vertebrate and invertebrate hosts throughout the history of evolution[1]. The related diseases were known by even the earliest recorded practitioners of medicine, although the word *herpes* has been used also in reference to non-viral diseases[2]. Based on growth characteristics and tissue tropism, the nine species known to infect humans can be divided into three taxonomic subfamilies - alpha-, beta- and gammaherpesviruses. They are all globally spread and cause a substantial burden of disease (Table 1). After primary infection, a latent persistent infection is established that can reactivate if immunological conditions allow[3,4].

All members of the herpesviridae group are spherical virions, with a complex genome of double-stranded DNA (dsDNA) of approximately 130-250 kbp in size. The genome is contained in an icosahedral capsid, surrounded by a tegument and lipid bi-layer envelope embedded with glycoproteins (Figure 1). A set of core proteins, involved in viral morphogenesis and replication, are shared among all human herpesviruses[5].

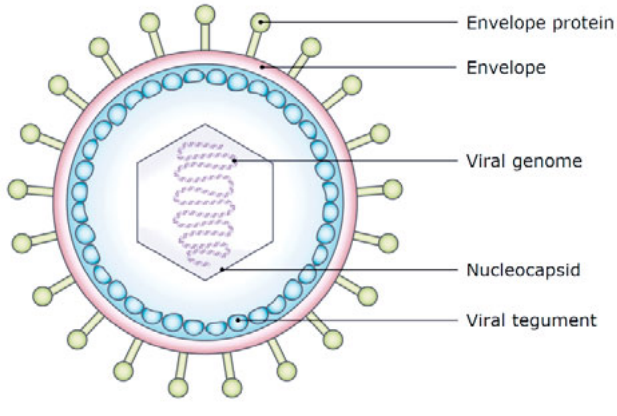


Figure 1. Schematic overview of the herpesvirus structure. Adapted from an original image released into the public domain.

Transmission and cell tropism

The mode of transmission and site of latency differs between the human herpesviruses, reflecting the cell tropism and paths of reactivation. All human alphaherpesviruses are neurotropic, establishing latency in sensory ganglia[6]. Herpes simplex virus (HSV) type 1 and 2 generally transmit through direct or indirect contact with blistered epithelium, but viral shedding is also common in the absence of symptoms[7,8]. Varicella zoster can be transmitted through epithelial contact, but in the acute phase of disease, large amounts of viral particles are shed from the respiratory tract[9].

Table 1. *Overview of human herpesviruses.*

Virus	Group	Clinical presentation	Site of latency
HSV-1	α	Cold sores, encephalitis	Neuronal ganglia
HSV-2	α	Genital sores, myelitis, meningitis, encephalitis*	Neuronal ganglia
VZV	α	Chickenpox, shingles, cerebellitis, meningitis	Neuronal ganglia
EBV	γ	Mononucleosis, encephalitis*, meningitis*, post-transplant lymphoproliferative disease*	B-cells
CMV	β	Mononucleosis-like disease, encephalitis*, myelitis*, retinitis*, colitis*, hepatitis*	Hematopoetic progenitor cells, monocytes
HHV-6A	β	Fever, encephalitis*, pneumonitis*	Hematopoetic progenitor cells, monocytes?
HHV-6B	β	Exanthema subitum, encephalitis*, pneumonitis*	Hematopoetic progenitor cells, monocytes
HHV-7	β	Exanthema subitum, encephalopathy*	Monocytes, T-cells, salivary glands
HHV-8	γ	Kaposi's sarcoma*	B-cells

* Mainly in immunocompromised individuals

The human betaherpesviruses - human cytomegalovirus (hCMV, hereafter referred to as CMV) and human herpesvirus 6A, 6B and 7 (HHV-6A, HHV-6B, HHV-7) - transmit through saliva, breastfeeding, sexual contact, blood transfusion and transplantation. Vertical transmission during pregnancy or delivery is also among known modes of transmission, and HHV-6 can under certain conditions integrate into the genome of germinal cells, thus breaking the boundary between pathogen and host[10]. The latency sites of betaherpesviruses include hematogenic progenitor cells, monocytes and salivary glands, but are not completely characterised for HHV-6A and HHV-7[11-14].

Human gammaherpesviruses - Epstein-Barr virus (EBV) and human herpesvirus 8 (HHV-8) - share several properties and transmission routes with the betaherpesviruses but rather establish latency in B-lymphocytes[15,16].

Immunoevasive mechanisms

As a consequence of the long relationship between human herpesviruses and their hosts, delicate viral immune evasive mechanisms have evolved. At the price of the large proportion of viral genome coding capacity used, these mechanisms can affect both native and adaptive immune response and effectively hinder the clearance of infection[17]. Firstly, there are viral proteins that inhibit complement activation from the mammalian pattern recognition receptors that allow the cells in the innate immune system to identify non-self epitopes and present viral antigens[18,19]. Other evasive strategies that target fundamental immunologic mechanisms include virus-encoded high-affinity decoy chemokine receptors that block pro-inflammatory signalling by capturing chemokines before they reach their intended receptor[20]. A more advanced strategy, also blocking pro-inflammatory signalling, is the production of viral anti-inflammatory cytokines such as IL-10[21].

A plethora of mechanisms allow herpesviruses to avoid the adaptive immune system, dependent on the presentation of non-self antigens by MHC class I and II molecules. These include the blocking of immunoproteasome formation, blocking of degradation within the proteasome, degradation of host mRNA including MHC-I mRNA, blocking of peptide loading onto the MHC molecules, direction of MHC class I molecules to lysosomes and shutdown of host protein production including both MHC-I and MHC-II molecules[18,22,23].

Cytomegalovirus

CMV is the largest of the human herpesviruses, carrying a linear dsDNA genome of approximately 235 kbp[24]. It is widely spread globally, with a seroprevalence ranging between approximately 30-90 % depending on age, ethnicity, breastfeeding practises, attendance levels in day-care, sexual habits and socioeconomic conditions[25-27]. Similar to other betaherpesviruses it causes a life-long persistent infection, and establishes latency in undifferentiated hematogenic progenitor cells and monocytes[14,28].

Replication cycle

CMV exhibits a large variety in cellular tropism including fibroblasts, endothelial cells, epithelial cells, monocytes and macrophages, neutrophils, smooth muscle cells, stromal cells, neuronal cells, and hepatocytes[29,30]. Dur-

ing initiation of infection, CMV glycoproteins B and M bind to heparan sulfate proteoglycans on the cell surface[31]. The onward mechanisms of adhesion and cell entry are not completely clear, as the wide spectrum of permissive host cells has complicated the identification of a single host cell entry receptor. It could be that CMV cell entry is mediated through different mechanisms depending on the cell type. Upon fusion, the DNA-containing protein capsid and the tegument proteins are released into the cell cytosol and transported to the nucleus.

Depending on the cell type, the virus can either enter a lytic or latent phase of replication. In lytic infection, occurring in a wide array of cell types and tissues, viral transcription is extensive and follows the classical temporal cascade of the herpesviruses with immediate-early (IE), early (E) and late (L) genes. This leads to the assembly and release of infectious virions and lysis of the host cell. The latent life cycle however, restricted to cells in the myeloid linage including CD14+ monocytes and their CD34+ progenitors, is associated with a much more limited viral transcription program, reduced splicing and a lack of complete virion assembly[32].

Clinical aspects

In the immunocompetent host, primary infection can be subclinical or cause mononucleosis-like disease, whereafter the virus is brought into latency and kept under strict immunosurveillance[33]. CMV rarely reactivates systemically without endogenous or iatrogenic immunosuppression but CMV DNAemia has been found in intensive care patients, where it was associated with prolonged hospitalisation and mortality[34]. Also, several studies have shown local reactivation with detectable quantities of CMV DNA in saliva, breast milk, urine, semen and cervical secrete consistent with the known routes of between-host transmission[35-37].

In patients with immune suppression related to haematopoietic stem cell transplantation, solid organ transplantation, chemotherapy or manifest AIDS, CMV is a common cause of severe disease affecting both graft and patient survival[38-40]. Another vulnerable group are pregnant women, as CMV infection of the foetus is now the most common cause of infection-related congenital neurological handicap. Foetal abnormalities include sensorineural hearing loss, mental retardation and chorioretinitis[41,42].

Immunosenescence and cognitive decline

CMV is known to inflict a deep imprint in the host T-cell compartment that is characterised by an age-related oligoclonal expansion of differentiated CD8 (CD27-CD28-) cells and a corresponding decrease in proportion of naïve cells[43-46]. Also, the degree of differentiation in the CD4 compartment has been shown to correlate with levels of CMV IgG[47]. Whether this is in

fact promoting immunosenescence in CMV infected individuals, or merely mimicking the shifts in phenotype that are seen in the elderly, is not completely understood[48]. CMV has also been suggested as a promoter of inflammation, but a recent longitudinal study showed that CMV seropositive subjects did not have any increase of systemic pro-inflammatory cytokines compared to non-infected subjects over a 10 year follow-up[49].

Whether chronically persistent viral infections can promote cognitive decline in immunocompetent hosts is an issue of debate. Epidemiological studies have shown an association between Alzheimer's disease (AD), Apolipoprotein E ε4 (*APOE ε4)* genotype and infection with HSV-1[50]. Furthermore, a cumulative cognitive effect of infection with multiple agents in the herpes virus family, including CMV[51] has been suggested. Also, a connection between CMV IgG levels, neurofibrillary tangle density, IFN-γ and amyloid load has been described in AD patients[52]. However, as the risk of infection with herpes viruses can be closely related to lifestyle factors, the direct causality between infection and disease is not clear.

Pharmacotherapy and prophylaxis

In the case of CMV disease in an immunocompromised host, there are several drugs available that can block viral replication. The guanosine analogue ganciclovir (Figure 2) and later valganciclovir, a valine ester derivative of ganciclovir providing increased oral bioavailability, have remained the drug of choice for more than 20 years although the search of novel substances now have rendered more promise[53-55].

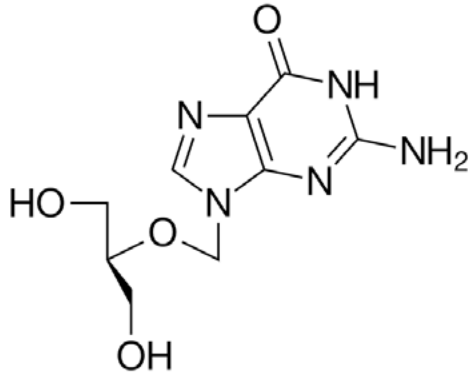


Figure 2. Structure of ganciclovir. Adapted from an original image released into the public domain.

Development of viral drug resistance towards ganciclovir is not uncommon in cases with high viral load and long duration of treatment, and dosage can

be limited by side-effects such as neutropenia[56,57]. In such cases, second line treatment with foscarnet, an inorganic pyrophosphate analogue that directly inhibits the DNA polymerase, can be considered[58].

The search for a CMV vaccine has so far not resulted in any substances reaching registration for clinical use, but phase 2 trials has shown a protective effect of a vaccine candidate containing recombinant glycoprotein B. Progress has also been made using other epitopes to induce broad CMV immunity in rhesus macaques[59,60]. The benefit of a safe and efficient CMV vaccine is obvious to address the problem of congenital CMV, but a more general vaccination strategy could be motivated if health benefits also later in life are shown.

Herpes simplex virus type 1

HSV-1 contains a 152 kbp dsDNA genome with approximately 80 protein encoding regions and several sequence repeats and inversions[61]. Seroprevalence increases with age and is with few exceptions globally high, often above 90 % in the elderly[62]. A majority of those infected become asymptomatic carriers after primary infection[63]. Similar to other alphaherpesviruses it establishes latency in neuronal cells, in the case of orally transmitted HSV-1 predominantly in trigeminal ganglia[6].

Replication cycle

HSV-1 attaches to the host cell surface through glycoproteins B and C[64,65] and triggers endocytosis or fusion by the interaction of glycoproteins D, B, H and L with entry receptors[66,67]. Capsids and tegument proteins are released into the cytosol, taking advantage of the microtubule-based transport system to reach the cell nucleus. There, viral DNA is released and passes through nuclear pores to the nucleoplasm[68,69].

The IE phase of transcription is initiated by an enhancer complex that together with additional transcription factors bind to enhancer-promoter regions of IE genes. Proteins expressed during the IE phase, most notably infected cell polypeptide 4, then promotes the encoding of E and L phase proteins[70]. Host cell DNA synthesis is down-regulated, relocating the use of DNA precursors to the production of viral DNA[71].

Both initiation and termination of genome packaging into procapsids is mediated by packaging elements in the viral DNA, together with specific viral proteins[72,73]. Several alternative pathways for virion release has been suggested, where the viral envelope is either acquired during passage though the nuclear membrane, or in the endoplasmatic reticulum, Golgi apparatus or *trans*-Golgi network[74,75]. Due to mechanisms not completely understood, the HSV replication cycle is thought to transform into a latent chromatinised

state where expression of viral DNA is almost completely suppressed, except for the latency-associated transcripts[76]. As viral mechanisms are severely limited in the latent state, reactivation is most likely a result of changes in cellular processes such as reaction to stress. Alternatively, it has been suggested that rather than establishing latency, HSV is forced into a state of slow persistent infection by constant supervision by CD8+ T-cells[77]. This would be compatible with the findings of frequent asymptomatic viral shedding[63]. As these hypotheses are not mutually exclusive, HSV could be harboured in both latent and chronically persistent cycles simultaneously depending on the cell type.

Clinical aspects

Primary HSV-1 infection is usually confined to mucocutaneous disease in the oral cavity, lips and face, but can be complicated by fever and pharyngitis-associated nutritional deficiency[78]. Neonates and immunocompromised hosts can suffer severe, sometimes life-threatening, disease[79]. The most common clinical presentation of HSV-1 reactivation, affecting approximately 30 % of all HSV-1 carriers, is cold sores in the mouth and on the lips. Symptoms usually start with a prodromal phase of pain or tingling sensations and most often lesions disappear within a week. If local or systemic antiviral treatment is initiated early, the duration and severity of symptoms can be further limited[80].

Herpes simplex encephalitis

A far more serious clinical presentation of primary or reactivated HSV-1 infection is herpes simplex encephalitis (HSE), affecting 2-4 individuals per million each year[81]. The clinical presentation is characterised by fever, headache and alterations in cognitive performance, level of consciousness and personality. A prodromal phase with fever and headache may precede the onset of neurological symptoms. In severe cases, the disease is further complicated by seizures and brain oedema. Without effective treatment, mortality exceeds 70 %[82]. Although outcome can be significantly improved with high-dose intravenous aciclovir, current treatment strategies still leave many patients with severe neurologic disabilities[83].

In addition to the cytopathic effect of HSV-1 infection in neuronal cells, the human immune response also causes cellular damage and death due to its necessary but imprecise attempts to control infection. Based on results from animal models and retrospective clinical studies suggesting a beneficial effect of immunotherapy, attempts have been made to further improve clinical outcome by supporting the antiviral treatment with corticosteriods[84,85]. As there are currently no data available from prospective randomised clinical

trials on the effect of adjuvant corticosteroid treatment in HSE, there is a need for further studies[86].

Pharmacotherapy and prophylaxis

The replication of HSV-1, HSV-2 and VZV, and to some extent also CMV and EBV, can be effectively blocked by treatment with aciclovir, penciclovir (Figure 3) or their ester prodrugs valaciclovir and famciclovir that provide better oral bioavailability[87-89]. Aciclovir and penciclovir are guanosine analogues that are selectively activated by herpesvirus-specific thymidine kinase, effectively targeting only infected cells. The antiviral effect is exercised through a selective inhibition of herpesvirus DNA polymerase, where the nucleoside analogues act as false substrates and block viral DNA synthesis [88]. Aciclovir has been considered first-line treatment in HSE, as it has been shown to reduce both morbidity and mortality compared to older compounds such as vidarabine[90]. For optimal clinical efficacy, treatment should be initiated as early as possible when viral replication is still ongoing. Alternatively, in cases of frequent episodes of reactivation, prophylactic use of antivirals can be considered[91].

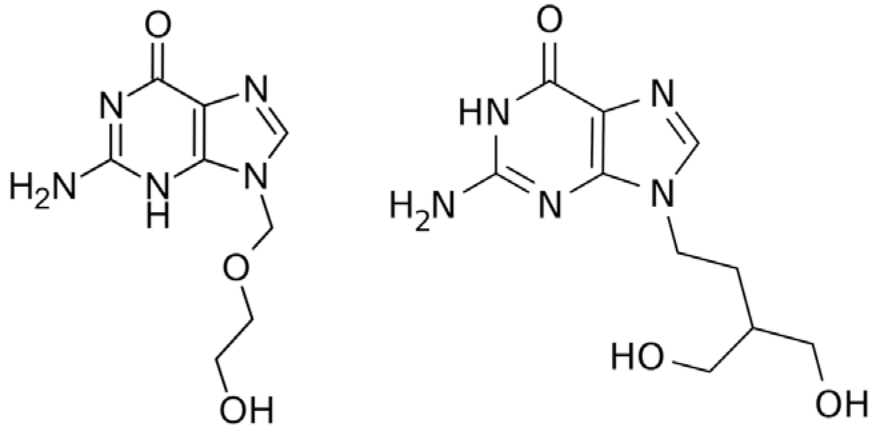


Figure 3. Structure of aciclovir (left) and penciclovir (right). Adapted from original images released into the public domain.

Ideally, an effective HSV vaccine could be used in naïve populations to create protective immunity and in already infected individuals to prevent viral reactivation. However, although there has been an effective live attenuated VZV vaccine available for over 40 years[92,93], vaccine development for the other human alphaherpesviruses has proven much more difficult. So far no HSV vaccines are commercially available, but candidate vaccines have shown limited preventive effects in clinical trials[94,95].

Alzheimer's disease

It is estimated that 35.6 million people are currently (2013) living with dementia[96]. Late-onset sporadic AD is the most common form of disease, causing approximately 60-70 % of all dementia cases. Multiple genetic, vascular and psychosocial risk factors have been identified, with *APOE ε4* genotype being the strongest known genetic predictor[97,98]. Due to demographic changes the disease burden on society is projected to grow at an alarming rate, with the number of cases expected to double during the next 20 years[96].

According to the amyloid cascade hypothesis on the pathogenesis, the disorder starts by increased aggregation of amyloid β (Aβ) in certain brain areas such as the entorhinal cortex. As the disease progresses, the hippocampus and neocortex is also affected. The intracellular changes associated with AD are thought to be secondary to the initial amyloid pathology[99].

Clinically, AD is characterised by a progressive deterioration of cognitive and functional capacity and is usually diagnosed using cognitive performance tests such as the Mini-mental state examination (MMSE)[100]. To differentiate from other dementing disorders, the diagnostic workup can be extended to include different modalities of tomographic brain imaging and sampling of cerebrospinal fluid (CSF). Commonly used CSF biomarkers include the 42 amino acid form of amyloid β (Aβ42), tau and phosphorylated tau, which taken together can show a characteristic pattern supportive of a clinical AD diagnosis[101].

Neuropathology

The neuropathologic hallmarks of AD are progressive synaptic and neuronal loss, extracellular plaques mainly consisting of Aβ and intracellular neurofibrillary tangles (NFT) of the microtubule-associated protein tau. Affected brain regions also show a state of local inflammation[102,103].

As mutations in genes related to the generation and processing of Aβ and its precursors have been shown to cause early-onset familial disease[104,105], Aβ has been suspected to have a primary role in pathogenesis of AD. However, it is still unclear exactly how Aβ pathology causes synaptic and neuronal loss. Both human and animal studies suggest that changes in Aβ metabolism and aggregation precedes cytoskeletal changes and neuron damage[106-108]. Large soluble pre-fibrillar forms of Aβ, so-called protofibrils, have been shown to be more neurotoxic than monomers[109,110].

Autopsy studies of AD patients in different stages of disease have shown that the neuropathologic changes related to AD are first seen in the entorhinal cortex and surrounding areas of the temporal lobe. As the disease progresses over time it is spread to adjacent cortical areas, consistent with clinical disease progression as the spectrum of affected cognitive functions is extended[111,112]. Although separated from vascular dementia, AD often pre-

sents with vascular alterations and deposition of Aβ in the vessel walls, which is most pronounced in *APOE* ε4 allele carriers[113].

Immunology

Aβ immunity has been investigated both in animal models and in clinical cases of AD. APP transgenic mice show Aβ hyporesponsiveness in both humoral and cellular immunity[114]. Moreover, AD subjects present with an impaired lymphocyte proliferation rate when peripheral blood mononuclear cells (PBMCs) are stimulated with Aβ[115].

It has been hypothesized that humoral immunity plays a role in counteracting Aβ and its harmful effects on the CNS, as natural antibodies against Aβ can be detected in both AD patients and healthy subjects. Most studies analysing the free fraction of endogenous antibodies against monomeric Aβ have shown decreased levels in AD subjects[116-120], while studies attempting to measure total levels of anti-Aβ generally show increased levels[121-124]. It has also been shown that CNS clearance is indeed affected in AD[125], but whether this is a causal disease mechanism is not clear.

Whether AD is associated with an overall increase in systemic inflammation is controversial. Increased levels of pro-inflammatory cytokines have been shown in some studies of AD subjects, but not in others[103]. In addition, offspring with a family history of AD present with a more pro-inflammatory cytokine profile than those without[126]. If inflammatory responses related to infection can promote AD is not clear, but patients with AD seem to deteriorate in cognitive capacity when challenged with inflammatory events[127].

Pharmacotherapy

Currently, there is no treatment that can affect the long term prognosis of AD. Most commonly used medications are the acetylcholinesterase inhibitors rivastigmine, galantamine and donepezil, which target the impaired cholinergic signalling seen in AD. Effects are only symptomatic, and overall small[128]. Another pharmacological target is the N-methyl-D-aspartate receptor (NMDA) receptor, since it is believed that overactivity in glutamate signalling may contribute to AD pathology[129]. Memantine, initially developed as an anti-influenza agent, acts as an competitive receptor inhibitor and provides protection against excitatory neurotoxicity[130].

Based on the findings of Aβ aggregation and increased inflammation in AD, several attempts have been made to target these mechanisms through pharmacotherapy. Long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) has been epidemiologically correlated to a reduced risk of AD[131] but several randomised placebo-controlled prevention trials have failed to show any protective effect[132-134]. Similarly to the case of NSAID, animal models and early human trials provided promising results of biopharmaceu-

tical immunotherapy. However, treatment with intravenous immunoglobulin, Aβ vaccination and passive immunisation with Aβ-specific monoclonal antibodies have all been unsuccessful in larger placebo-controlled clinical trials[135-137].

The aging immune system

After reaching a peak in early mid-life the human immune system deteriorates in a process named *immunosenescence*, affecting both innate and adaptive immune responses. Adaptive mechanisms are generally more affected, as decreased levels of hematopoietic stem cells and thymic involution affects both generation and differentiation of lymphocytes[138,139].

Clinical implications for the elderly include an impaired vaccine response[140] and increased incidence of severe bacterial and viral infections which contributes significantly to mortality in these age groups[141]. In Swedish octogenarian and nonagenarian cohorts[142,143] a defined immune risk profile (IRP) consisting of a decrease in CD4/CD8 ratio was associated with increased morbidity and mortality, but later studies conducted in different epidemiological settings have shown somewhat conflicting results[144].

Inflammaging

In 2000, Franceschi et al coined the term *inflammaging* to describe an age-related pro-inflammatory state and hypothesized a connection to the development of several age-related diseases[145]. Since then, several studies have strengthened this theory. Increased levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, have been associated with an age-related risk of developing atherosclerosis, cognitive impairment and general frailty[146-149]. Moreover, studies of coronary heart disease have identified a correlation between systemic CRP levels and risk of future acute coronary events[150].

As many infectious agents are known to induce inflammation through the human immune response, it has been suggested that an accumulation of chronically persistent infections throughout life could contribute to the increase in system inflammatory biomarkers seen in the elderly. A correlation between infection, systemic inflammation and risk of age-related diseases has been shown for some chronic infections such as HIV[151] but whether this is also applicable to more common infections, such as human herpesviruses, is less clear[49].

NMDAR encephalitis

The NMDA receptor (NMDAR) consists of a heterotetramer of NR1, NR2 and NR3 subunits, which all exist in several isoforms. It mediates signalling in excitatory glutamate synapses, controlling the plasticity that is the foundation of learning and memory formation (Figure 4)[152,153]. While a certain degree of activation is necessary for cell survival and normal function, overactivation induces cell death through excitotoxic mechanisms. Dysregulation of NMDAR-mediated signalling has been seen in several neuropsychiatric conditions such as epilepsy, schizophrenia, AD and substance abuse[154-156].

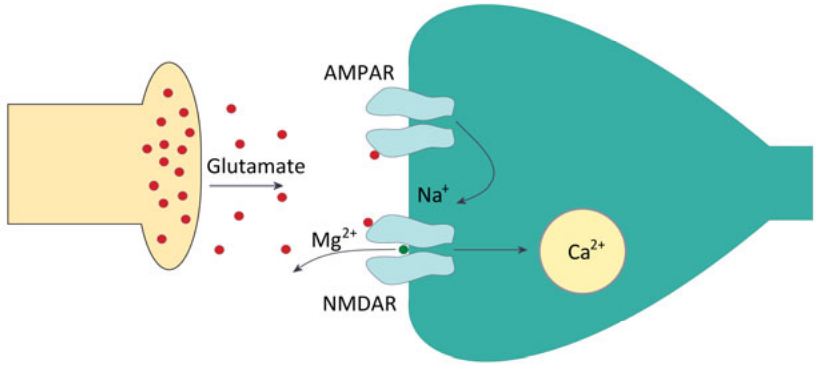


Figure 4. Overview of NMDA receptor function in long-term potentiation. Glutamate first activates AMPA receptors that unblocks the NMDA receptor through a sodium-dependent mechanism. When activated, the NMDAR release calcium into the cytosol for further enzymatic signal propagation.

NMDAR encephalitis is an autoimmune disease where specific autoantibodies against the receptor induce inflammation and affect neurocognitive mechanisms negatively. Common presentations of disease include headache, low-grade fever and a wide variety of psychiatric symptoms, such as anxiety, agitation, paranoid delusions, hallucinations and catatonia. Neurologic symptoms are also frequent and include memory loss, dyskinesia, autonomic instability, hypoventilation and seizures[157]. Several cases of encephalitis of previously unknown origin have now been identified as NMDAR encephalitis[158,159]. The disease is more common in young patients than in the elderly. It was first described as a paraneoplastic syndrome, but in the majority of cases there is no known underlying disease[157,160-162].

Recently, there have been several case reports of NMDAR autoantibody development is association with HSE[163-166]. Also, in a retrospective study of

HSE patients in various stages of disease, NMDAR antibodies of different isotypes were found in approximately 30 % of the 44 patients[167]. The temporal development of this autoimmune process, and the clinical impact of anti-NMDAR in HSE, is not yet fully understood.

Aims

Overall aim

The overall aim of this thesis is to characterise the immunological relationship between herpesvirus infection and neurocognitive disorders such as AD and HSE.

Specific aims

I In Paper I, the aim was to investigate the frequency of CMV-specific cytotoxic CD8+ T-cells in AD, and whether patients present with a different CMV-specific immune profile or different overall CD8+ phenotype, compared to non-demented (ND) controls.

II In Paper II, the aim was to investigate the inflammatory response of PBMCs from AD patients and ND controls when challenged with CD3/CD28-stimulation, CMV pp65 antigen and Aβ protofibrils.

III In Paper III, the aim was to investigate the relationship between antibody levels and neurocognitive disease in patients with AD and ND controls by applying a novel multiplex herpesvirus immunoassay.

IV In Paper IV, the aim was to investigate NMDAR autoimmunity in HSE and the correlation to recovery of neurocognitive performance.

Materials and Methods

Ethics

Informed consent was obtained in writing from all study participants, together with written consent from a close relative if there was any uncertainty on whether the subject was capable of providing informed consent him- or herself. Patient identity and clinical status was coded, allowing blinding during all laboratory work. The studies were approved by the Regional Ethical Review Boards in Uppsala (Papers I-III) and at Karolinska Institutet (Paper IV).

Study groups

Papers I-III

A group of AD subjects were compared with ND controls. In the AD group, 51 patients were recruited from the Memory Disorder Unit at Uppsala University Hospital. All had undergone an extensive clinical work-up and were diagnosed in accordance with the NINCDS-ADRDA[168] and DSM-IV criteria. Thus, all patients presented with a clinical picture of AD as well as a CT or MRI scan consistent with the diagnosis. In the ND control group, 52 age-matched subjects were recruited from a database of listed volunteers from the same geographical area as the AD group. Subjects in the ND control group were initially recruited via local advertising and did not have any subjective cognitive impairment.

Paper IV

A total of 47 patients with PCR-verified HSE were included during 2001-2009 at Karolinska University Hospital, Sahlgrenska University Hospital, Skåne University Hospital and Uppsala University Hospital. All subjects were previously included in the NCT00031486 clinical trial, investigating the effect of long term treatment of HSE with valaciclovir, hence fulfilling all inclusion and exclusion criteria of the original study[169]. All study subjects received standard of care treatment with iv aciclovir 10-15 mg/kg for 14-21 days, after which they were randomised to receive either oral valaciclovir or placebo for 3 months. Serum and cerebrospinal fluid samples were acquired

at onset of disease, at the end of iv treatment and after 3 months of follow-up. At onset of disease, subjects were evaluated using the NIH stroke scale[170] and a more extensive batch of neurocognitive tests, including the Mattis Dementia Rating Scale (MDRS)[171], Glasgow Coma Scale (GCS)[172] and MMSE[100], was performed at the beginning of the follow-up period and after 3, 12 and 24 months.

Quantification of CMV-specific T-cells

The absolute number, or relative proportion, of CMV-specific T-cells can be analysed by several techniques, all with different advantages and disadvantages. Both enzyme-linked immunospot (ELISPOT)[173] and intracellular cytokine staining (ICS)[174] relies on the detection of the functional response to CMV-specific antigens. In ICS, cytokine vesicles are locked inside the cells by the use of brefeldin A or monensin, after which responsive cells are stained and quantified through flow cytometry. In the ELISPOT protocol, cytokines are captured in the matrix surrounding the cells on a coated microplate and detected by direct fluorescence staining. Both methods can be time-consuming, and rely on the T-cells being in a functional state that is relevant to the *in vivo* biology.

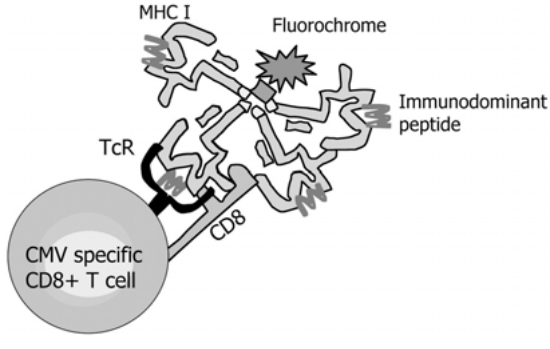


Figure 5. Schematic drawing of CD8 T-cell binding a MHC-I tetramer. Reproduced with permission from Dr. Anna-Karin Lidehäll.

Quantification of antigen specific T-cells can also be performed through the T-cell receptor (TCR) MHC-I restriction, by cell surface staining using fluorochrome labelled MHC-I tetramers and flow-cytometric analysis (Figure 5). CMV-specific MHC-I tetramers were developed by Altman and co-workers in 1996[175]. Due to the HLA specificity of the MHC tetramers, one specific set is needed for each HLA allele present in the sample. The full set of tetramers used in Paper I is listed in Table 2.

Table 2. *List of CMV-specific MHC-I tetramers used in the study.*

HLA restriction element	CMV peptide sequence	Peptide origin
A*0101	YSEHPTFTSQY	pp65
A*0201	NLVPMVATV	pp65
A*2402	QYDPVAALF	pp65
B*0702	TPRVTGGGAM	pp65
B*0801	ERLRKMMYM	IE1
B*3501	IPSINVHHY	pp65

Flow cytometry

Introduction

The technology behind the instruments we today call flow cytometers were developed in the 1950s by Wallace H Coulter. The *Coulter principle* has ever since been the name for the change in electric impedance that can be detected when particles pass through an orifice over which an electric current is applied[176]. Soon thereafter, Mack Fulwyler developed this technique further, adding the possibility of sorting cells by their volume[177]. Since then, the application of multi-colour lasers, optical refractors, fluorescence detectors and conjugated antibodies - designed to flag specific surface markers of the particles passing through the detector - has made flow cytometry and fluorescence-activated cell sorting (FACS) one of today's most important techniques in biomedicine.

The principles of flow cytometry (Figure 6) has long remained the same. Particles from a sample are carried by a sheath fluid through a thin nozzle, forming a hydrodynamically focused stream of liquid. Travelling in a single stream, the particles are then excited by one or several laser beams, and surrounding detectors will register wavelength and intensity of light in different directions. The basic parameters, forward scatter (FSC) and side scatter (SSC), corresponds to particle size and granularity, and through the use of antibody-conjugated fluorochromes, multiple surface molecules can be detected simultaneously. Recently, the introduction of mass spectrometric detection in flow cytometry has changed the landscape as the total number of signals that can be analysed simultaneously has increased at least tenfold[178].

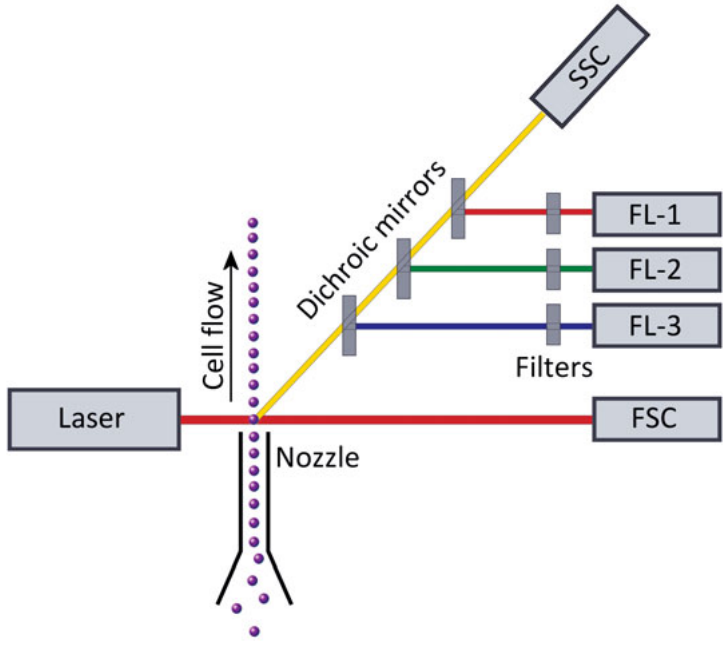


Figure 6. Schematic overview of a flow cytometer. The laser beam passes through a particle flow whereafter the spectrum is separated using dichroic mirrors. Forward scatter (FSC), side scatter (SSC) and different wavelengths of fluorescence (FL-1, FL-2 and FL-2) are detected in photomultiplier tubes.

During data acquisition, all parameters are registered for each particle passing through the detector cell. If a cell sorting mechanism has been attached to the flow cytometer, output consists not only of detector data but also of cell fractions separated by one or several flow cytometric parameters[177].

The set of surface markers and fluorochromes used in Paper I is listed in Table 3. Fluorochromes with low emission intensities should be used to stain highly expressed surface markers, and vice versa, to minimize differences in signal strength between channels. Tandem fluorochromes have a donor and acceptor molecule in close proximity, allowing transfer of energy between them. This allows excitation at the excitation wavelength of the donor molecule, and emission at the emission wavelength of the acceptor molecule, creating separation from signals emitted by single fluorochromes[179].

Table 3. *List of fluorochromes conjugates used in Paper I.*

Surface marker	Fluorochrome	Type	Emission peak (nm)
CD8	BD Horizon V450	Single	448
CD4	BD Horizon V500	Single	500
CD45RA	FITC	Single	519
CMV MHC-I	PE	Single	578
CD28	APC	Single	660
CD27	PerCP-Cy5.5	Tandem	695
CD19	Alexa Fluor 700	Single	719
CD3	APC-H7	Tandem	785
CCR7	PE-Cy7	Tandem	785

Spectral overlap compensation

In multi-colour flow cytometry, the wavelengths emitted by the fluorochromes often overlap and cause incomplete separation between signals (Figure 7). This issue is managed in a process called *spectral compensation*[180]. First, each individual emission spectrum is captured by conjugating the single antibody-fluorochromes to carrier beads with affinity for the antibody Fc-region. These clean spectra are then fed into a compensation algorithm, to estimate spill-over between fluorochrome channels for any specific combination. When the colours are used simultaneously in the final assay, the compensation factors are then applied to estimate the corresponding clean signal intensity in each detection channel.

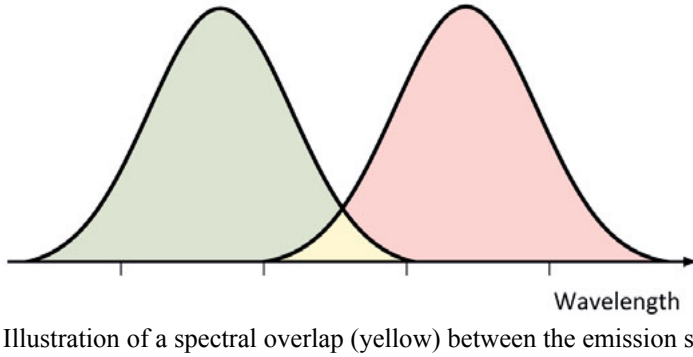


Figure 7. Illustration of a spectral overlap (yellow) between the emission spectra of two theoretical fluorochromes (green and red).

Gating of flow cytometry data

During data acquisition and analysis, the single cell readings (events) are separated in a process called *gating*. In the acquisition phase, this makes it possible to record only the relevant events, for example excluding FSC/SSC areas where only debris or noise is present. This process is then refined during data analysis, where the events are usually displayed as two-dimensional plots using one fluorochrome per axis, in a step-wise identification of the cell populations of interest (Figure 8).

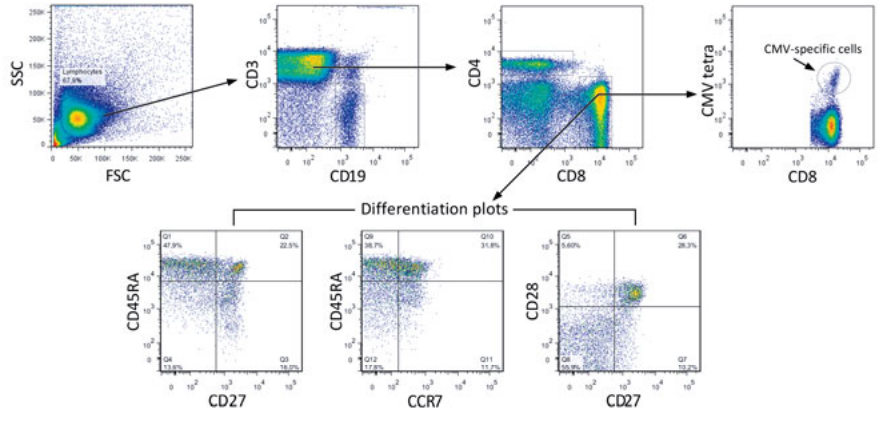


Figure 8. Schematic overview of the gating protocol in Paper I. Based on a preliminary FSC/SSC gate, lymphocytes are identified and further divided into a CD3+CD8+ subset where the proportion of CMV positive cells can be calculated. Differentiation plots are created from the total CD3+CD8+ subset.

Analysis of T-cell differentiation

The differentiation in the human CD8+ cell linage, although still not completely characterized, can be described by the corresponding change in surface markers. Naïve cells express a multitude of surface markers, which are sequentially lost during the process of differentiation. In early memory cells, the CD45RA is replaced by other CD45 isoforms[181]. On the onward path from intermediate memory, late memory to effector-memory cells, the chemokine receptor CCR7 and co-stimulatory receptors CD28 and CD27 are lost. A subset of late memory cells, so-called T effector-memory RA (T_{EMRA}) cells, regain their CD45RA expression (Figure 9)[182].

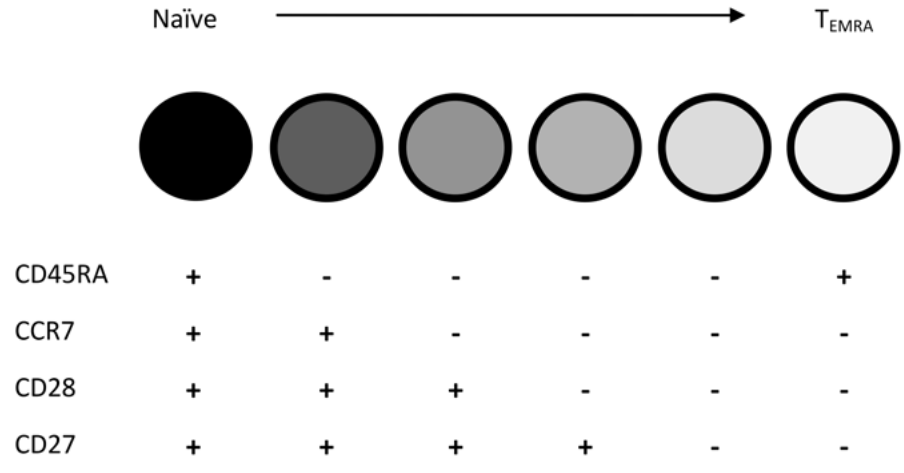


Figure 9. Overview of the successive loss of surface markers during CD8 differentiation from naïve to T effector-memory CD45RA+ (T_{EMRA}) cells.

Multiplex bead array assays

Introduction

The enzyme-linked immunosorbent assay (ELISA)[183,184] was developed from the previous radioimmunoassay[185], and has ever since been the golden standard method for many analytical applications including cytokine and antibody detection. However, this method has the inherent disadvantage of handling only one analyte at a time and a relatively large amount of sample is needed if multiple analytes are to be handled separately.

With the advent of flow cytometric techniques, customized antibodies and further development in conjugate fluorochromes and fluorescence detectors, several multiplexed applications of ELISA has been developed, allowing simultaneous detection of tens or even hundreds of multiple analytes.

Luminex® xMAP

The Luminex® xMAP (Multi Analyte Profiling) technology is one of the most commonly used multiplex bead array assays (MBAAs) available for commercial use. It is based on 5.6 μm polypropylene microspheres, internally labelled with different concentrations of red and infrared fluorophores to create a unique signature for each bead type. Beads are conjugated with a primary antibody or antigen to capture the intended analyte, after which a secondary biotinylated detection antibody is added. The secondary antibodies are then stained with a fluorescent with biotin affinity such as strepta-

vidin-phycoerythrin (SA-PE) to allow flow cytometric detection of beads and their respective intensities of captured analyte[186,187].

The xMAP system has been used in Paper II with cytokine-antibody conjugated beads, and in Paper III with beads conjugated to herpesvirus antigens. The complete set of antibodies and antigens is listed in Table 4.

Table 4. *Bead-conjugated cytokine antibodies and herpesvirus antigens used in the studies.*

Paper II
Anti-GM-CSF
Anti-IFN-γ
Anti-IL-1ß
Anti-IL-2
Anti-IL-4
Anti-IL-5
Anti-IL-6
Anti-IL-8
Anti-IP-10
Anti-TNF-α

Paper III	**Strain**
HSV-1 viral lysate	MacIntyre
CMV viral lysate	AD169
EBV gp125	P3HR1
VZV viral lysate	Rod
HHV-6A viral lysate	GS

The performance of MBAAs has been compared to monoplex assays, such as ELISA, in several studies. Results are generally consistent between single analyte and multiplex methods when analytes are in non-complex matrices such as a PBMC supernatant. In complex matrices such as human plasma, where interindividual variations in serum lipids, proteins, heterophilic antibodies and immune complexes cause interference between signals, matrix effects are more pronounced[188-190]. These limitations explain why there are still only a few clinical applications of MBAAs protocols, although this technique has greatly improved scientific applications such as high-throughput cytokine profiling.

Polymerase chain reaction

History

Although the history of the polymerase chain reaction (PCR) is somewhat disputed, with some legal processes still pending, it is widely accepted that the complete concept regarding this DNA amplification protocol was developed by Kary Mullis in 1983[191]. His invention, awarded the Nobel prize in 1993, was based on previous work regarding DNA sequence replication by Kornberg, Sanger, Khorama, Kleppe, Molineux and others in the preceding decades[192]. The DNA polymerase originating from *Escheria coli* that initially was used, was later replaced with the more thermo-stable polymerase from the heat-tolerant bacterium *Thermus aquaticus* (Taq)[193]. Through sequential improvements during the latest decades, PCR has evolved into one of the most important tools in biomedical research.

Quantitative PCR

Most modern applications of PCR use a real-time protocol, allowing quantification of the initial amount of genetic material. This is accomplished by the use of continuous detection of PCR product in-between cycles of the amplification process, making the number of cycles needed for detection an estimate of quantity[194]. Amplification in real-time protocols follow the same principles as in conventional PCR (Figure 10). Detection can be performed with non-specific DNA-binding dyes, specific DNA hybridization probes or by a combination of these techniques[195].

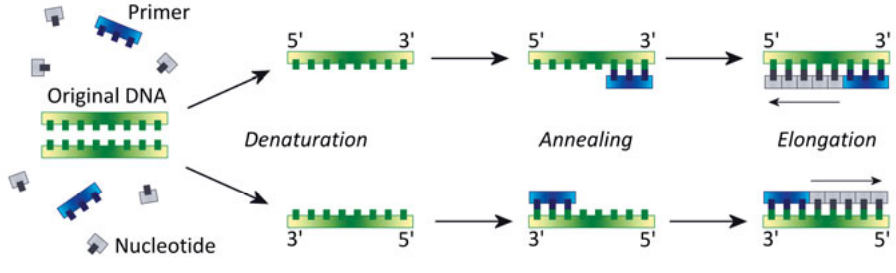


Figure 10. Basic principles of PCR. In the denaturation phase, the DNA strands are separated at 94-96°C, followed by the annealing where lowering of the temperature allow primers to bind their anti-sense targets. During elongation, the temperature is optimized to allow the DNA polymerase to elongate the new strand in the 5' to 3' direction.

Applications in infectious diseases

PCR has evolved into one of the most useful clinical tools in the field of infectious diseases, allowing rapid detection of both viral and bacterial genome sequences[196]. As PCR methodology have evolved - genotyping of specific segments can now often predict the microbial phenotype in terms of drug resistance mechanisms - it has replaced several older assays using antigen detection or *in vitro* isolation of the infectious agent. In the management of HIV, viral hepatitis and other chronic virus-related diseases, quantitative PCR has made it possible to monitor both disease activity and treatment response[197].

NMDA receptor antibody assay

The autoantibody assay used in Paper IV is based on human embryonic kidney 293 (HEK293) cells, immortalised by the adenovirus E1 gene[198]. For the purpose of this assay, these cells have been transfected with the human NMDA NR1 type glutamate receptor and are commercially available as a biochip slide kit (Euroimmun, Lübeck, Germany). In brief, the cells, dispersed on biochip slides, are exposed to patient serum in room temperature for 30 minutes. After rinsing, the slides are stained with fluorescein isothiocyanate (FITC) labelled anti-human IgG. After another washing step, the presence of NMDA receptor antibodies are determined using a fluorescence microscope at 200-400x magnification. All samples were evaluated independently by two analysts and discrepancies handled by consensus rating.

Neurocognitive tests

Glasgow Coma Scale

Initially developed as an objective tool for assessing level of consciousness in patients with head injury, the Glasgow Coma Scale (GCS) has become a standard tool for assessing consciousness also in other patient groups. The GCS consists of three components - eye opening (1-4), verbal response (1-5) and motor response (1-6) - creating a total score in the interval of 3 to 15 points, where 3 is completely unresponsive and 15 is a normal healthy response[172,199]. However, the variability between different observers can be substantial, and simpler measures for describing level of consciousness has showed comparable performance[200,201].

NIH stroke scale

The National Institutes of Health Stroke Scale (NIHSS) provides an 11 item scoring system, covering level of consciousness, cranial nerve function, motor function, coordination, sensory function, language, speech and extinction/inattention. The total score ranges between 0 and 42, where a score of 0 is considered normal and all scores above 21 are consistent with severe stroke. The NIHSS has been shown to provide a good prognostic estimate to outcome after both ischemic and hemorrhagic lesions in the brain[170,202].

Mini-mental state examination

In 1975, Folstein and co-workers presented the Mini-mental state examination (MMSE) as a simplified method for estimating cognitive impairment. The MMSE protocol was intended to allow testing of orientation, registration, attention and calculation, recall and language, repetition and complex commands within a time period of only 5-10 minutes[100]. The maximum total score is 30, which is considered normal. Several score intervals has been suggested for dementia staging, and a stratification of 26-29 for questionable, 21-25 for mild, 11-20 for moderate and 0-10 for severe dementia has been shown to correlate fairly well with more advanced scoring algorithms[203].

Mattis dementia rating scale

The Mattis dementia rating scale (MDRS) was developed to assess cognitive performance in patients "with known cortical impairment, particularly of the degenerative type"[171]. It consists of five subscales - attention, initiation-perseveration, construction, conceptualization and memory - rendering a maximum total score of 144. MDRS, and the updated test named MDRS-2 has been thoroughly evaluated and validated in the setting of Alzheimer's disease, Parkinson's disease dementia and related disorders[204,205].

Statistical analysis

Mann-Whitney U-test

This non-parametric test, attributed to Wilcoxon[206], Mann and Whitney[207], is used in Papers I-IV to compare continuous and ordinal variables between groups. As the U-test relies on rank rather than the numerical distribution, this test is less sensitive to non-gaussian distributions and outliers than the

independent samples Student's T-test at a price of only a few percent in power in a worst case scenario[208].

Fisher's exact test

In Paper IV, Fisher's exact test[209] is used to compare categorical values between groups. This test belongs to a group of exact tests, where the test statistic is calculated exactly and does not rely on approximations that will only become exact when the groups are of infinite size. Although known to be conservative in certain situations, Fisher's exact test is one among the recommended tests for comparing contingency tables when group sizes are small, as the chi-squared test statistic then becomes less applicable[210].

Multiplicity correction strategies

When using multiple outcome variables at a certain level of significance, the risk increases of type I errors. For example, there is a 5 % risk of a false positive at the 0.05 significance level for each variable that is tested. This can be managed by several adjustment strategies, aimed at keeping the intended total risk of a type I error at the original significance level. The simplest of these is the Bonferroni correction where the significance level is divided, or p-values are multiplied, by the number of parallel outcome variables[211].

A refined method based on the Bonferroni correction, the Holm-Bonferroni method, was presented by Sture Holm in 1978[212]. This method is uniformly more powerful than the Bonferroni correction without increasing the risk of type I errors above the intended significance level. Simply put, instead of multiplying all p-values with the number of parallel statistical tests (N), only the test with the lowest p-value is multiplied by N. Then the second lowest value is multiplied by N-1 and so on, leaving the highest p-value in the set of tests without any need for correction. In Paper III, a Bonferroni-Holm-like permutation based step-down procedure accounting for the correlation between the test statistics is used[213].

Results

CMV-specific cellular immunity in Alzheimer's disease (Papers I and II)

In Paper I, the CMV-specific cellular immunity was compared between AD subjects and ND controls. Using multi-colour flow cytometry, the CD3+CD8+ lymphocyte subset in PBMCs was analysed with CMV MHC-I tetramers. Based on the individual HLA types, one or several of tetramers A01, A02, A24, B07, B08 and B35 were used. In a total of 197 flow-cytometric analyses, approximately 20 million 11-dimensional data points were recorded. Data from CMV IgG positive subjects were analysed, both with respect to the total proportion of CMV-specific cells including all available tetramers and the proportion of cells limited to the most common allele HLA-A02. Furthermore, the CD4/CD8 ratio was calculated.

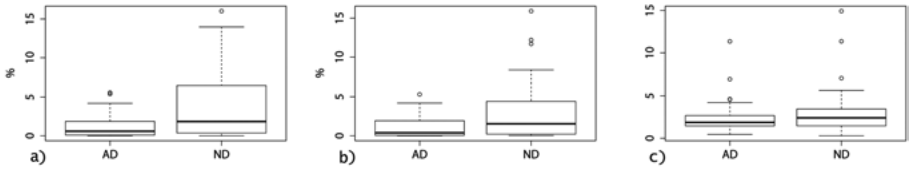


Figure 11.

a) *All CMV-specific CD8.* Comparing total cell count for all HLA-types, there was a clear difference with significantly lower proportions of CMV-specific CD8 cells in AD compared to ND group; 1.16 % versus 4.13 % (p=0.0057).

b) *HLA-A02 restricted CMV-specific cells.* Comparing only subjects with HLA-A02 tetramer data, there is a trend towards lower proportions of CD8 cells in the AD compared to the ND group; 1.26 % versus 3.07 % (p=0.066).

c) *CD4/CD8-ratio.* No difference in overall CD4/CD8-ratio between groups.

Among CMV seropositive subjects, patients with AD presented with significantly lower proportions of CMV-specific CD8 T-cells compared to ND

controls, 1.16 % vs. 4.13 % (p=0.0057). When comparing only HLA-A02 restricted cells between AD and ND groups, the results were similar (1.26 % vs. 3.07 %), but not significant on a 0.05 level (p=0.066). Taken together these data suggest that the CMV-specific subset of cytotoxic T-cells is reduced in AD, and that there are no signs of total CD8 inflation affecting the CD4/CD8-ratio.

To further investigate the CD8 phenotype in CMV positive AD subjects, the differentiation of the total CD8 subset was determined using antibodies targeting the following surface markers: CD27 (TNF-receptor), CD28 (CD3 co-receptor), CCR7 (C-C chemokine receptor type 7, lymphoid homing receptor) and CD45RA (protein tyrosine phosphatase receptor type C, isoform RA). After visual identification of positive and negative clusters, identical gating cut-off levels were applied to all samples. CD8 differentiation was then illustrated by CD27/CD45RA, CCR7/CD45RA and CD27/CD28-projections of the four-dimensional CD27/CD28/CCR7/CD45RA phenotypic space. No difference in CD8 differentiation was found between AD and ND groups (Figure 12). Accordingly, we could not find a link between CD8 T-cell immunosenescence and AD.

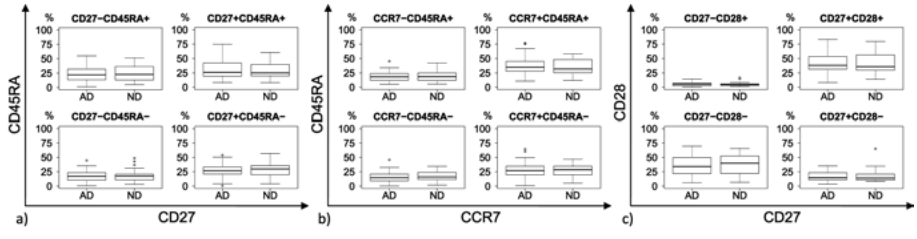


Figure 12.

a) *CD27 vs. CD45RA differentiation plot.* Similar CD8 differentiation in AD and ND groups in terms of CD27 and CD45RA expression. Upper right: Naïve. Lower right: Memory. Upper left: Effector.

b) *CCR7 vs. CD45RA differentiation plot.* Similar CD8 differentiation in AD and ND groups in terms of CCR7 and CD45RA expression. Upper right: Naïve. Lower right: Central memory. Upper left: Effector. Lower left: Effector-memory.

c) *CD27 vs. CD28 differentiation plot.* Similar CD8 differentiation in AD and ND groups in terms of CD27 and CD28 expression. Upper right: Early memory and naïve. Lower right: Intermediate memory. Lower left: Late memory.

To investigate the effect of CMV infection on CD8 differentiation and validate the biological relevance of the differentiation assay, data for all subjects regardless of dementia status was stratified according to CMV serostatus.

CMV seropositive subjects presented with markedly lower proportion of naïve CD8 cells and a higher proportion of effector-oriented CD8 cells compared to seronegative subjects (Figure 13).

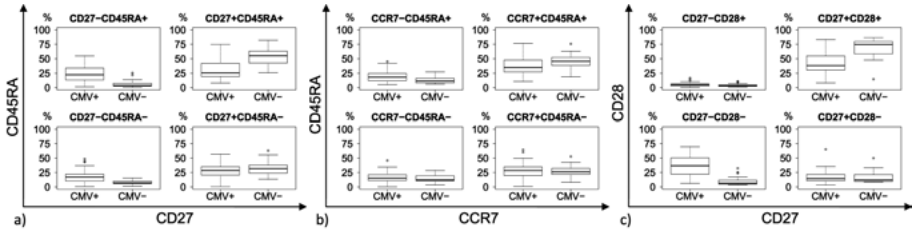


Figure 13.

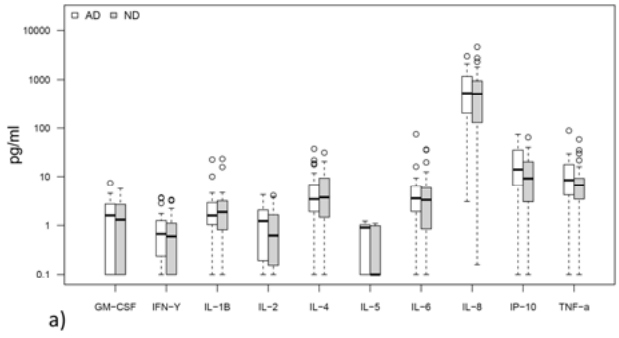
a) *CD27 vs. CD45RA differentiation plot.* Significant shift from the CD27+CD45RA+ naïve (p=8.13E-05) to CD27-CD45RA+ effector (p=1.76E-06) and CD27-CD45RA- (p=1.17E-04) subsets with CMV status. Upper right: Naïve. Lower right: Memory. Upper left: Effector.

b) *CCR7 vs. CD45RA differentiation plot.* Non-significant trend in shift from the CCR7+CD45RA+ naïve (p=0.062) to the CCR7-CD45RA+ effector (p=0.13) subset with CMV status. Upper right: Naïve. Lower right: Central memory. Upper left: Effector. Lower left: Effector-memory.

c) *CD27 vs. CD28 differentiation plot.* Significant shift from the CD27+CD28+ early memory and naïve (p=3.19E-05) to the CD27-CD28- late memory (p=2.73E-07) subset with CMV status. Upper right: Early memory and naïve. Lower right: Intermediate memory. Lower left: Late memory.

In Paper II, a multiplex Luminex xMAP assay was used to quantify PBMC cytokine response to antigen stimuli in HLA-A02 positive AD (N=30) subjects and ND (N=35) controls. The cytokine panel was chosen to cover both Th1 and Th2 cytokines and included GM-CSF, IFN-γ, IL-1ß, IL-2, IL-5, IL-5, IL-6, IL-8, IP-10 and TNF-α. Samples were handled in four aliquots and cytokine profiles were analysed without antigen stimulation and after stimulation with anti-CD3/CD28 beads, CMV pp65 peptide mix and Aβ protofibrils.

When comparing CMV seropositive AD (n=26) and ND (n=30) groups, cytokine release at baseline and after Aβ protofibril stimulation was similar. CMV seropositive AD subjects presented with higher IFN-γ levels after stimulation with both anti-CD3/CD28 and CMV pp65, compared to CMV seropositive ND controls (Figure 14).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IP-10
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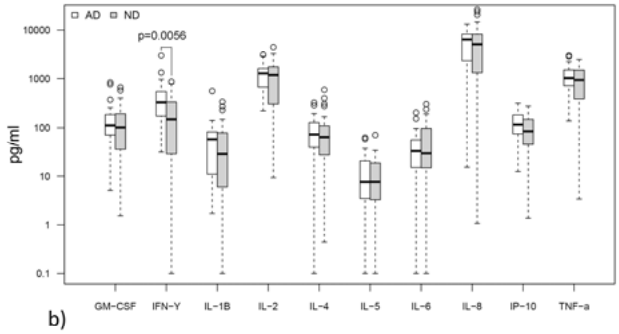
a)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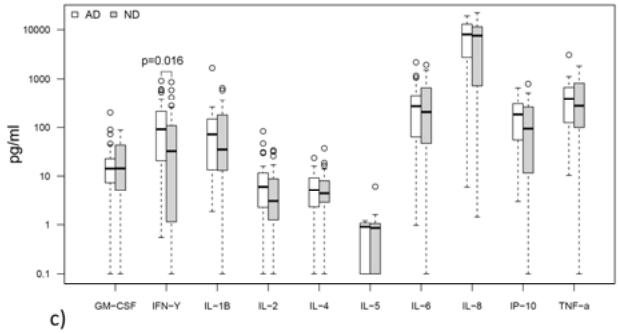
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GM-CSF
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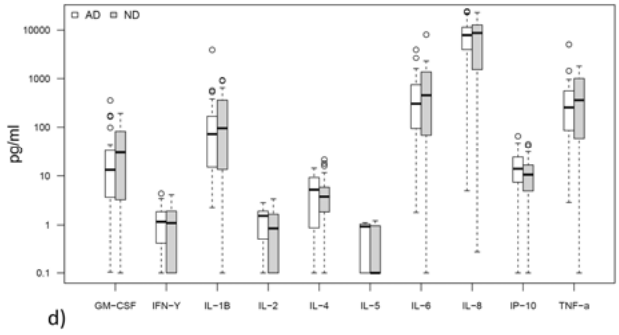
IL-1B
IL-2
IL-4
IL-5
IL-6
IL-8
IP-10
TNF-a

c)


AD
ND
pg/ml
10000
1000
100
10
1
0.1
GM-CSF
IFN-Y
IL-1B
IL-2
IL-4
IL-5
IL-6
IL-8
IP-10
TNF-a

d)

Figure 14 (opposite page).

a) *AD vs. ND, negative control.* No significant differences in baseline cytokine secretion between AD patients and ND controls.

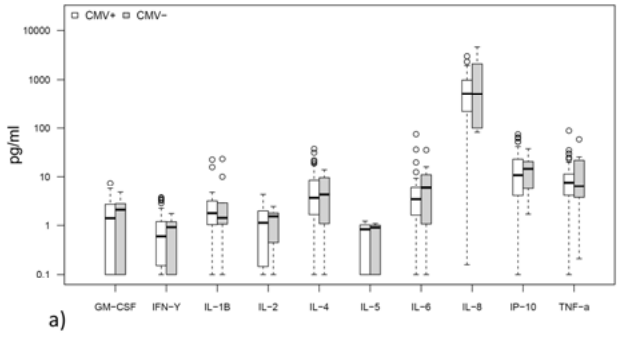
b) *AD vs. ND, anti-CD3/CD28 stimulation.* AD patients showed higher levels ($p=0.0056$ or 0.056 with Bonferroni correction) of IFN-γ upon CD3/CD28 stimulation compared to ND controls.

c) *AD vs. ND, CMV pp65 stimulation.* Higher levels ($p=0.016$ or 0.16 with Bonferroni correction) of IFN-γ upon pp65 stimulation in AD patients compared to ND controls.

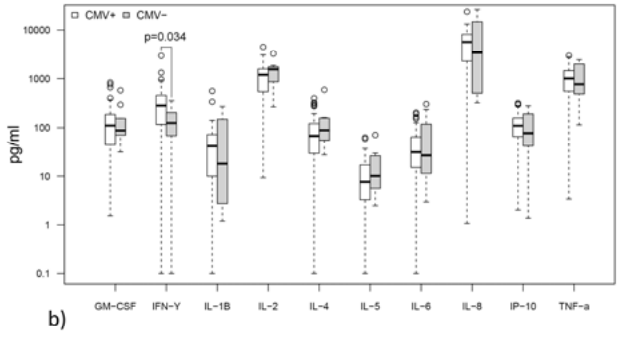
d) *AD vs. ND, amyloid beta protofibril stimulation.* No significant differences in cytokine secretion between AD patients and ND controls upon Aβ protofibril stimulation.

When comparing CMV seropositive (n=56) and seronegative (n=9) subjects regardless of dementia status (Figure 15), there were no differences in baseline cytokine levels or response to Aβ protofibril stimulation. CMV seropositive subjects presented with a higher IFN-γ response upon stimulation with anti-CD3/CD28. Also, stimulation with CMV pp65 resulted in a strong response with a 200-fold increase in levels of IFN-γ, 8-fold increase in IL-2, 30-fold increase in IP-10 and 3-fold increase in TNF-α in the CMV seropositive group compared to the seronegative group, confirming the biological validity and CMV antigen specificity of the assay.

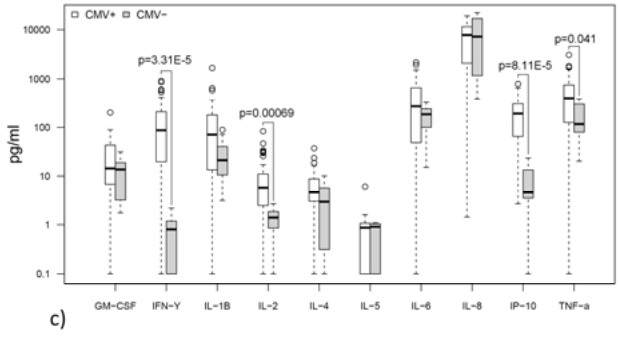
A post-hoc subgroup analysis of IFN-γ response (Figure 16) showed that CMV seropositive AD subjects presented with higher levels after anti-CD3/CD28 stimulation, compared both to CMV seronegative AD subjects and CMV seropositive ND subjects, hence indicating a possible role of CMV as an inflammatory promoter in AD immunology. However, there were no differences in systemic CRP or IL-6 between groups (data not shown), indicating that such a pro-inflammatory state is probably restricted to local tissue.


CMV+ CMV-
pg/ml
10000
1000
100
10
1
0.1
GM-CSF
IFN-Y
IL-1B
IL-2
IL-4
IL-5
IL-6
IL-8
IP-10
TNF-a

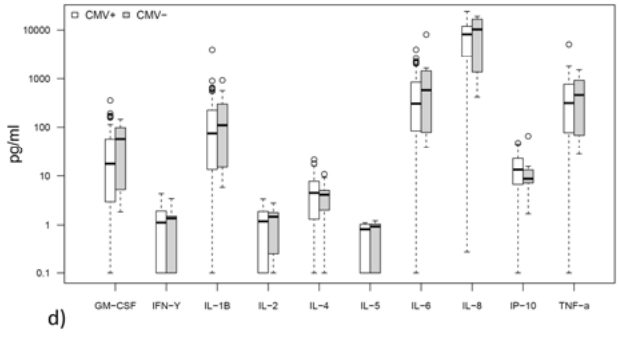
a)


CMV+ CMV-
p=0.034
pg/ml
10000
1000
100
10
1
0.1
GM-CSF
IFN-Y
IL-1B
IL-2
IL-4
IL-5
IL-6
IL-8
IP-10
TNF-a

b)


CMV+ CMV-
p=3.31E-5
p=0.00069
p=8.11E-5
p=0.041
pg/ml
10000
1000
100
10
1
0.1
GM-CSF
IFN-Y
IL-1B
IL-2
IL-4
IL-5
IL-6
IL-8
IP-10
TNF-a

c)


CMV+ CMV-
pg/ml
10000
1000
100
10
1
0.1
GM-CSF
IFN-Y
IL-1B
IL-2
IL-4
IL-5
IL-6
IL-8
IP-10
TNF-a

d)

Figure 15 (opposite page).

a) *CMV+ vs. CMV-, negative control.* No significant differences in baseline cytokine secretion between CMV seropositive and seronegative subjects.

b) *CMV+ vs. CMV-, anti-CD3/CD28 stimulation.* Higher levels (p=0.034 or 0.34 with Bonferroni correction) of IFN-γ in CMV seropositive subjects compared to seronegative subjects, upon CD3/CD28 stimulation.

c) *CMV+ vs. CMV-, pp65 stimulation.* Higher levels of IFN-γ (p=3.31E-5 or 0.00033 with Bonferroni correction), IL-2 (p=0.00069 or 0.0069), IP-10 (p=8.11E-5 or 0.00081) and TNF-α (p=0.041 or 0.41) in CMV seropositive subjects compared to seronegative subjects.

d) *CMV+ vs. CMV-, amyloid beta protofibril stimulation.* No significant differences in cytokine secretion between CMV seropositive and seronegative subjects upon Aβ protofibril stimulation.

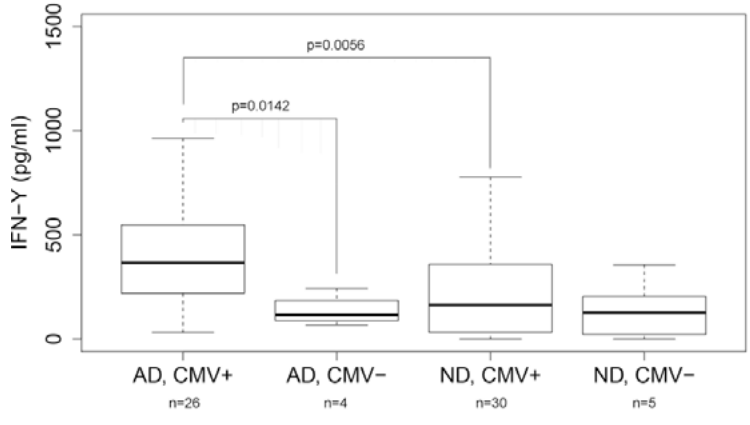


Figure 16. Sub-group analysis of IFN-γ response upon anti-CD3/CD28 stimulation stratified by dementia and CMV status. Significantly higher response in the AD CMV seropositive group, compared to both AD CMV seronegative subjects (p=0.0142 or 0.14 with Bonferroni correction) and non-demented CMV seropositive subjects (p=0.0056 or 0.056). Outliers excluded from plot, but included in the statistical analyses.

Multiplex herpesvirus serology in Alzheimer's disease (Paper III)

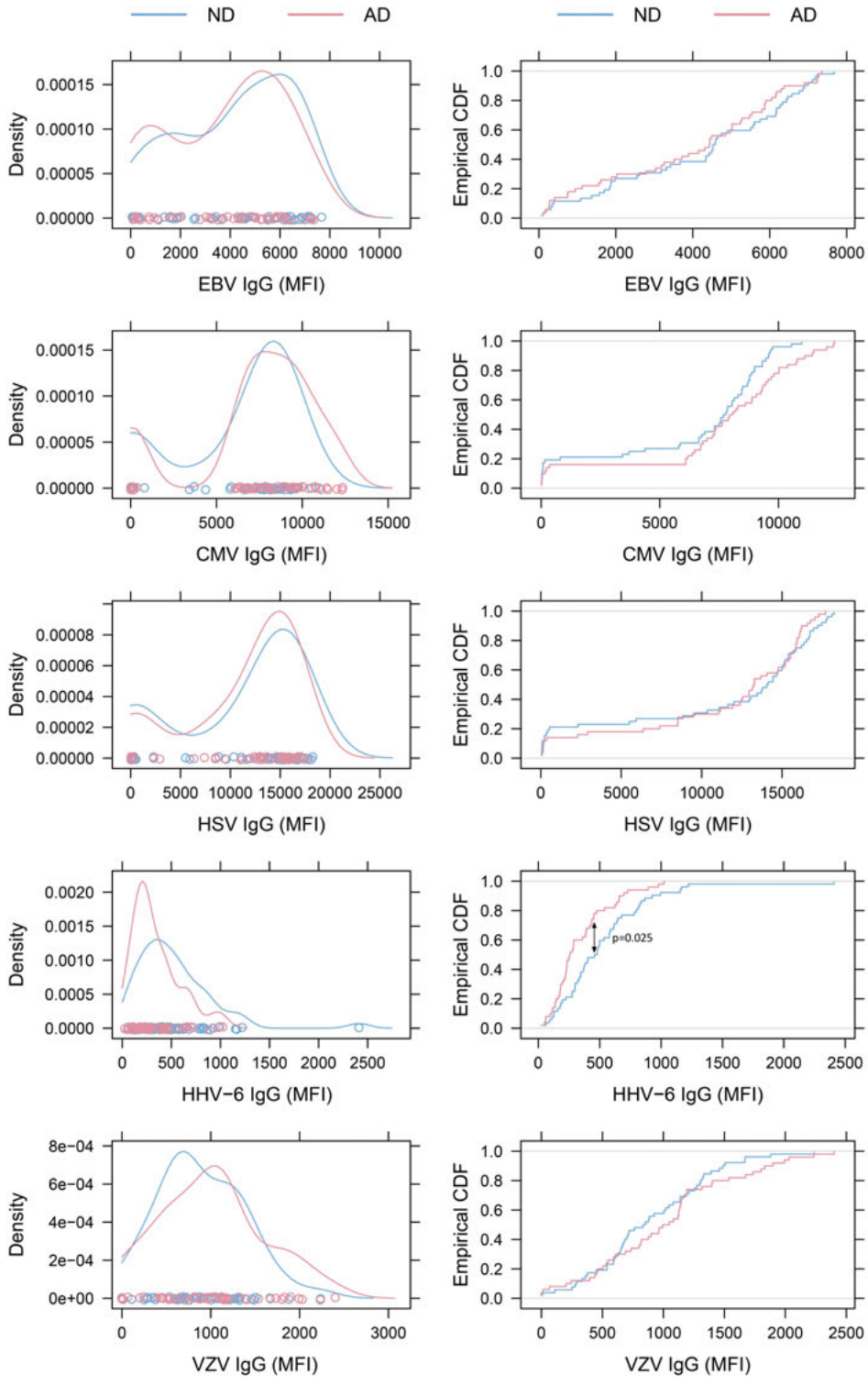
A previously in-house developed multiplex immunoassay[214] was adapted to herpesvirus serologic analyses and applied to serum samples from AD and ND groups. Viral lysate antigens from HSV, VZV, CMV and HHV-6, and purified protein from EBV, was conjugated to colour-coded polystyrene detection beads. IgG was detected using biotinylated protein G and streptavidin-PE, and analyses of median fluorescent intensity (MFI) were performed in a parallel flow-cytometric system. Only a 2.5-5 µl sample volume was needed for a complete multiplex analysis.

As there were no validated cut-off levels between seronegative and seropositive groups, the complete distributions of continuous data were used in the statistical analyses both for identifying data clusters and in comparisons between AD and ND groups. After screening for cross-reactivity, MFI data was visualised through density plots and empirical cumulative distribution functions (Figure 17).

Figure 17 (opposite page). Density plots and empirical cumulative distribution functions (ECDF).

Left: Density plots of virus-specific antibody levels. The distributions for EBV, CMV and HSV-1 illustrate clustering of subjects into seronegative and seropositive populations. No obvious clustering was seen in HHV-6 and VZV plots, as expected when seroprevalence is close to 100 %.

Right: ECDF plots illustrating that AD patients present with lower HHV-6 antibody reactivity than ND controls (p=0.025 with Bonferroni-Holm correction).


ND
AD
Density
Empirical CDF
EBV IgG (MFI)
CMV IgG (MFI)
HSV IgG (MFI)
HHV−6 IgG (MFI)
VZV IgG (MFI)
p=0.025

Clustering into seropositive and seronegative populations was seen for EBV, CMV and HSV, but not for VZV and HHV-6, probably reflecting epidemiological differences between the members of the herpesvirus group. Also, the VZV and HHV-6 signals were overall weaker, possibly affecting the sensitivity of the assay. When comparing antibody MFI distributions, AD subjects presented with significantly lower levels of HHV-6 IgG compared to ND controls ($p=0.025$). To investigate whether the difference in HHV-6 humoral immunity correlated with viral reactivation in peripheral blood, a type-specific HHV-6 PCR was performed on PBMCs from AD and ND groups. There was no difference between groups, the overall rate of PCR-positive samples was low (8 % vs. 7.7 %) and all samples positive for HHV-6 were of subtype B.

NMDA receptor autoimmunity in herpes simplex encephalitis (Paper IV)

In total, 47 subjects with PCR verified herpes simplex encephalitis were included in the study. Along with repeated CSF and serum sampling for N-methyl-D-aspartate receptor (NMDAR) IgG autoantibody development, their neurological level of function and cognitive performance was followed prospectively for 24 months. All subjects were NMDAR antibody negative at baseline, and 11 of 47 subjects developed NMDAR IgG during the first three months of follow-up.

Anti-NMDAR positive subjects presented with significantly less improvement from baseline in MDRS total score compared to negative subjects. However, the differences in change from baseline were numerically similar to an opposite difference in baseline MDRS total score that did not reach statistical significance (Figure 18). Also, antibody positive subjects presented with significantly less improvement in MMSE score from baseline after 2 years, but this was also reflected by a comparable, opposite, but non-significant difference in baseline score between groups. There was no difference in GCS or NIH stroke scale score between groups.

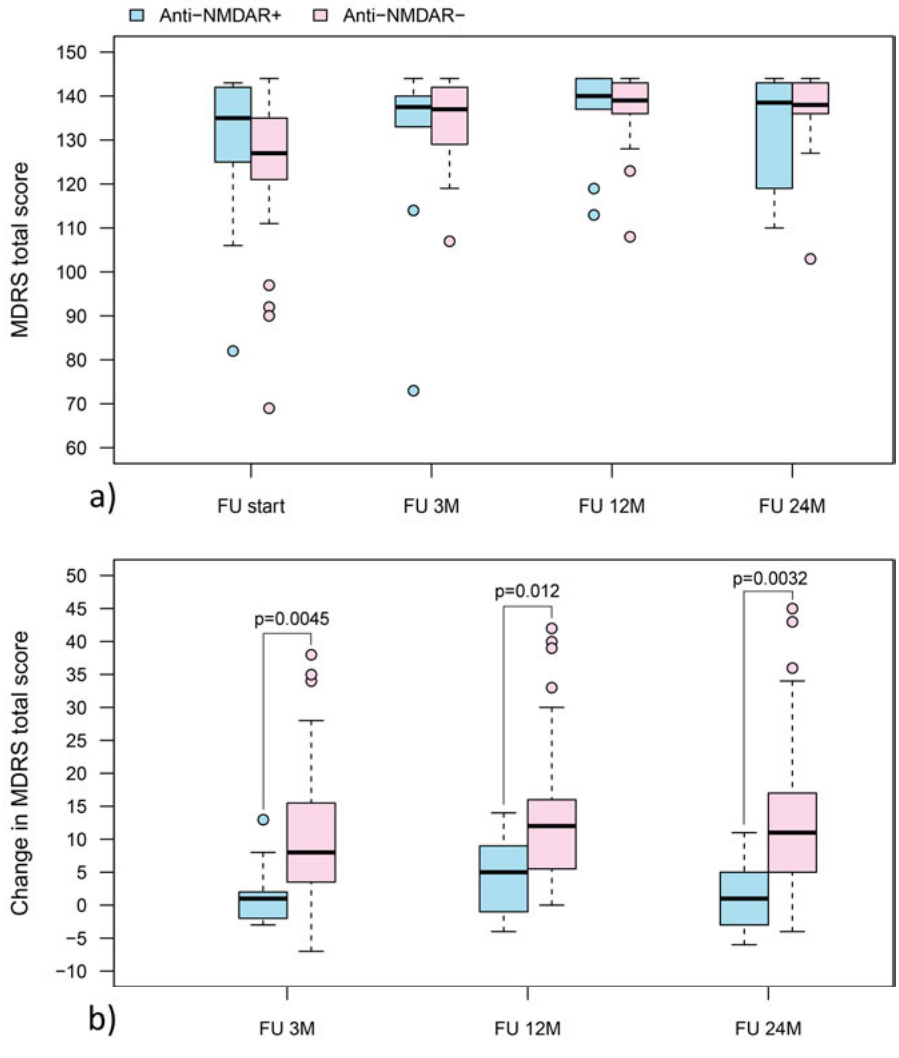


Figure 18. Mattis Dementia Rating Scale total score and change from start of follow-up.

a) No significant differences in MDRS total score between anti-NMDAR IgG positive and negative subjects, but numerically higher scores in the antibody positive group at start of follow-up (FU start).

b) Significantly lower increase in MDRS total score from start of follow-up in anti-NMDAR IgG positive subjects at follow-up after 3, 12 and 24 months (FU 3M, FU 12M, FU 24M). Box plots were defined with boxes containing quartiles 2 and 3 and whiskers displaying quartiles 1 and 4, excluding any outliers outside 1.5 times the interquartile range

Discussion

The interplay between herpesviruses and the mammalian immune system is intricate and has developed during several million years of cospeciation[215]. These viral infections - whether active, chronically persistent or latent - both trigger and regulate the immune response to assure life-long persistence and further transmission, but rarely cause severe disease in the immunocompetent host.

In the research projects of this thesis, we have tried to characterise the human cellular immune response to CMV in AD and the autoimmune mechanisms triggered by HSV-1 encephalitis. Furthermore, we have sought to develop more efficient tools for serologic analyses in herpesvirus infection. In a broader perspective, the concept of viral infections causing cognitive decline is well-established, as illustrated by the neurotropic viruses causing tick-born encephalitis[216,217] and HSV-1 encephalitis[218]. Furthermore, herpesviruses, such as HHV-6A, HSV-1 and EBV, but not CMV, have been detected in higher frequencies in brain biopsies from AD patients than in ND controls[219-221].

In Papers I-II we have found what we believe are significant correlations between CMV immunity, inflammatory response and AD. Previous research have focused mainly on the topic of HSV-1 infection in AD as this virus has been found in the vicinity of amyloid plaques in brain tissue, especially in *APOE* ε4 allele carriers[50,221-223]. Also, HSV-1 IgM levels seem to be elevated in AD subjects, which could indicate more frequent episodes of reactivation[224,225]. However, as HSV-1 is very common both in AD patients and non-demented subjects, these findings *per se* cannot provide a simple and straight-forward explanation of causality in the AD pathogenesis. One hypothesis is that CMV infection, by affecting HSV-1 immune response, could facilitate HSV-1 reactivation or affect the immune response toward a local pro-inflammatory state in the brain tissue[226].

Based on previous reports of highly expanded clones of CMV-specific CD8 cells in the elderly[43-46] we were initially expecting AD patients to present with an increased frequency of CMV-specific cells as a sign of premature aging of the immune system. But as our data indicate the opposite, it does not support this line of argumentation. Neither were there any differences in overall CD8 differentiation between AD and ND groups, as can be seen when comparing CMV positive and negative subjects overall[43-45]. One could instead hypothesise that AD patients, for hitherto unknown reasons,

cannot mobilise an age-adequate cellular immune response to CMV which could lead to more frequent local CMV reactivation as has been suggested by Stowe et al[227]. Also, the induction of an overall more inflammation-prone cellular immune response could promote Aβ production and cognitive decline[127,228].

Associating a highly prevalent virus as CMV with a common disease such as AD is far from simple, especially since the pathophysiological link is most likely indirect. Any correlation between an age-related disease and herpesvirus prevalence is at risk of bias from the lifestyle factors that can be associated with an increased risk of infection, such as smoking and alcohol consumption[229]. During the process of our research the case for CMV involvement in AD has been strengthened by the report from Lurain et al, describing a correlation between CMV IgG levels, IFN-γ in cerebrospinal fluid (CSF) and NFT density that was not seen for HSV-1[52]. These findings are consistent with our own data showing a general pro-inflammatory state in PBMCs from CMV positive AD patients, but neither study does really address the issue of causality. CMV could very well be an innocent bystander in the AD pathophysiological process, if the CMV-specific immune response is affected by more fundamental changes in the AD pathophysiology[48].

One of the biggest obstacles in understanding the potential role of CMV in AD is that the AD pathophysiology is not completely understood. Furthermore, the clinical diagnosis it at best probable, as definite AD is a post-mortem conclusion[168]. As CMV is highly prevalent in the age group where AD is most common, it would be of interest to compare individuals who have acquired infection early in life to those who are seronegative or only have been carrying CMV for a few years. This would allow a comparison between duration of viral exposure with cognitive decline. Such a study could better reveal the immunological cost of controlling the virus and whether this over time translates into an increased risk of age-related conditions such as AD, in individuals with an increased genetic risk of disease.

CMV is frequently spread in the human population, and its genome could almost be seen as an acquired part of human DNA. This raises the question whether there are unknown evolutionary benefits for infected individuals or merely a price that most of us have to pay. It is not impossible that human genotypes that allow complete clearing of herpesvirus infections have been lost in the evolutionary process. A strong immune response can in itself be associated with more severe disease during primary infection, as is seen in VZV and hepatitis B infections[230,231]. Another possibility is that the health of the host or host species directly benefit from the introduction of viral DNA, either through the transcriptional regulation mechanisms introduced by the virus or by the use of virus-encoded translational products. Finally, in the world of bacterial colonisation as well as in viral epidemiology, there is a principle of limited ecological space. Perhaps the human herpesviruses are

occupying a conceptual space that would otherwise be open to other infectious agents, less adapted to coexist with humans.

To better understand the impact of CMV on age-related diseases in general, and AD in particular, it would be of great interest to perform a prospective study following both CMV-infected and non-infected, analysing CMV serostatus, T-cell differentiation, cytokine profile and lifestyle factors together with known genetic risk factors for age-related diseases. The advent of a CMV vaccine could also indirectly provide an interesting control group for comparison, eliminating the bias between lifestyle and risk of acquiring CMV.

In Paper III, we have showed that herpesvirus antibody assays can be multiplexed through the use of bead array assays analysed with flow cytometry. These techniques enable measurement of antibodies to many infectious agents simultaneously with very small sample volumes. Multiplex immunoassays can allow rapid syndrome-centred overview of pathogen antibodies, but the risk of an increasing number of false positives - as with any extensive analytic screening - must be properly managed both from a scientific and clinical perspective. A complete overview of herpesvirus antibodies could even provide better control of false positive reactions due to cross-reactivity, than what can be achieved with a more limited set of single-antigen assays.

It has been previously reported that multiplexing serologic assays can introduce unwanted matrix effects caused by interindividual variations in serum lipids, proteins, heterophilic antibodies and immune complexes[232,233]. However, the suspension matrix immunoassay protocol we have used in conjunction with herpesvirus antigens, employs a lower concentration of carrier proteins and has so far proven robust. In this study we chose to measure only IgG levels, as the relevance of IgM is limited outside the clinical context of paired sampling during the course of a primary infection. The natural cross-reactivity between different members of the herpes virus group due to their structural similarity affects IgM more than IgG[234,235] and false positive herpesvirus IgM reactions are not uncommon in autoimmune disease[236-238]. However, the assay could easily be extended to cover other isotypes such as IgM and IgA.

Bioinformatics tools will become of increasing importance both in medical sciences and clinical practice, as the amount of data continues to grow exponentially. Visualization methods such as scatter plot matrices can be used to screen for unwanted cross-reactivity between signals, and multivariate analysis of variance provides further support for interpretation and quality control. Although data mining strategies can be effective for detecting statistical patterns in large, multidimensional datasets, any hypothesis generated during such a process must then be scrutinized for biological relevance and validated with completely new and uncorrelated data.

Unexpectedly, we found a difference in HHV-6 humoral immunity between AD and ND groups, suggesting that AD subjects present with lower

antibody levels. This difference is in opposite direction to what is seen for CMV and HSV-1, where increased IgG levels have been associated with cognitive decline[50,227,239]. As HHV-6A is known as a rare cause of encephalitis[240] and has been connected to multiple sclerosis[241-243], it is not impossible that this neurotropic virus is implicated in AD. In contrast to what has previously been described by Carbone et al[219], we could however not find any difference in PBMC HHV-6 DNA levels between groups. This incoherence between study results could be explained by differences in the sensitivity of the PCR assays, or more fundamental epidemiologic differences between study groups. Whether HHV-6 is connected to the AD pathophysiology remains a hypothesis for future studies.

In Paper IV, the focus was shifted from CMV to HSV-1 and the topic of autoimmune post-infectious complications after HSE. The NMDAR is a well-characterised glutamate receptor, important for the synaptic plasticity that is essential to memory function[152,153]. Also, the clinical syndrome of NMDAR encephalitis that is associated with development of NMDAR autoantibodies is well described, both as a paraneoplastic phenomenon and an apparently stand-alone disease[157,159,160].

NMDAR antibody development after HSE has been previously described in case reports[164-166], sometimes associated with clinical relapse of neurocognitive or neuropsychiatric symptoms[163]. Also, one previous retrospective study have shown that NMDAR antibodies, if counting IgM, IgG and IgA isotypes, are present in approximately 30 % of HSE subjects[167]. We believe that we are the first to show the temporal development of anti-NMDAR in HSE and that this autoantibody mediated disturbance of glutamate signalling causes an impaired recovery of neurocognitive performance.

It is clear that the anti-NMDAR positive group presented with an impaired recovery rate compared to the negative group, but there was no difference between positive and negative groups when comparing the MDRS and MMSE total scores at the end of the follow-up period. This phenomenon could be due to a randomly skewed distribution of case severity between antibody positive and negative groups, which would lead to the conclusion that NMDAR autoimmunisation plays a significant and causative role in hindering neurocognitive recovery in HSE. Alternatively, the differences in neurocognitive performance at the beginning of follow-up could be explained by unknown mechanisms in the pathogenesis or epidemiology, associating less severe forms of HSE with the development of NMDAR antibodies. Our data, indicating a trend towards lower CSF cell count, and the findings of Prüss et al that anti-NMDAR development was associated with a longer duration between onset of symptoms and start of antiviral treatment[167] could support the second line of argumentation. In either case, the cognitive measurements during recovery could be affected by the limitations in dynamic range of the MDRS as many subjects reach the top scores. This could

to some extent be controlled for through statistical modelling, but the validity of such approaches depends on the size of the study groups.

The mechanism behind the NMDAR autoimmunisation is not known, but two different models have been suggested[244]. It could be that there are virus-specific antigens that have enough homology with NMDAR for antigenic mimicry to occur, causing cross-immunisation. If this process is restricted to the CNS immune compartment it could explain why HSE, but not primary HSV-1 infection, is associated with anti-NMDAR development. Another hypothesis is that the cytolytic HSV-1 infection exposes neuronal and synaptic antigens in an environment where the activated immune response provides an adjuvant. Previous findings that other types of autoantibodies (anti-D2R, anti-neuropil) can be found in parallel to anti-NMDAR post HSE[166,167], could support the latter hypothesis.

Our findings regarding anti-NMDAR in HSE could have several clinical implications. As autoimmunity seems to affect approximately one quarter of all HSE cases, CSF screening for anti-NMDAR IgM and IgG could be considered at cessation of aciclovir treatment and possibly also at a three month follow-up examination. This would allow closer clinical monitoring of antibody positive subjects and also gives the possibility of initiating immunotherapy at the first sign of clinical relapse. In confirmed cases of post-HSE clinical relapse, and in all cases of encephalitis with unknown aetiology, anti-NMDAR CSF testing should be considered. In cases of anti-NMDAR IgG positive, HSV-1 DNA negative clinical relapse, potent immunotherapy in combination with iv aciclovir should be considered. In relapses where HSV-1 DNA is positive however, the potential negative effects of immunosuppression in HSE must be considered[245]. Hopefully, future studies addressing the effect of adjuvant corticosteroid treatment in HSE can be stratified by anti-NMDAR IgG status in CSF, to investigate whether both subgroups can benefit from immunotherapy.

To put the findings in Paper IV into context, more clinical data is needed. Most obviously, known prognostic cofactors such as time from onset of symptoms to start of antiviral treatment, adjuvant corticosteroid treatment and MRI lesion distribution should be included in the analysis. If NMDAR antibody status remains an independent prognostic factor for neurocognitive recovery in HSE, this could be one result of the studies in this thesis that is of direct benefit to patients.

Conclusions

- AD subjects present with a lower proportion of CMV-specific CD8+ T-cells compared to ND controls. CMV infection induces dramatic shifts in CD8 differentiation overall, but no shifts in CD8 differentiation are seen when comparing AD and ND groups.

- CMV seropositive subjects with AD present with a more pro-inflammatory PBMC phenotype than both CMV seronegative AD subjects and CMV seropositive ND controls, when quantified as release of IFN-γ upon stimulation with anti-CD3/CD28 beads.

- Herpesvirus antigens can be used in a multiplex bead-based immunoassay, allowing effective quantification of specific IgG in small sample volumes. Furthermore, a difference in HHV-6 humoral immunity was found when comparing AD patients and ND controls. No difference in HHV-6 DNA levels in peripheral blood was seen between groups.

- Approximately one quarter of all HSE cases develop NMDAR IgG within 3 months after onset of disease. Antibody development is associated with an impaired neurocognitive recovery.

Future perspectives

We are only in the beginning of the process of understanding how infections affect us throughout life, not only by the acute diseases they cause but also through the immunological changes that may persist long after the infectious agent is controlled or cleared. Most likely, future research will provide new links between infection and autoimmune or age-related diseases.

Aging seems to be an unavoidable part of biological life, as it creates the very foundation for the evolutionary process that leaps forward from one generation to the next. However, the bar between normal and disease has constantly risen, as we expect longer and healthier lives than ever before. While there seems to be a general agreement in the scientific community regarding the positive health effects of carrying a rich, and preferably antibiotic naïve, bacterial flora, the same does not necessarily apply to viral infections. From an evolutionary perspective, it is reasonable to assume that we are not genetically adapted to the changes in lifestyle that has been the result of the rapid growth in global population and technological inventions that has occurred in the last century. Humans now live physically closer to each other than ever before, while jet-fuelled transportation effectively carries communicable diseases around the world.

Herpesviruses have long walked side by side with our own path of evolution, but as modern medicine treat an increasing number of diseases and patients with immunosuppressive drugs and life expectancy continues to rise, their negative effects on human health could be increasing. In this perspective we could find ourselves vaccinating against HSV, CMV and EBV in the future, provided that safe and cost-effective vaccines are developed. There are already many examples of successful vaccination programs against viral infections, including polio, measles, mumps, rubella, human papillomavirus, hepatitis B and to some extent VZV.

Although promising, the AD research community has so far not been able to clearly unveil the pathophysiological mechanisms behind the disease. The amyloid cascade hypothesis has been successful in pre-clinical research and has provided clinical tools for AD diagnostics, but this has so far not been translated into effective treatment. Although animal models and early human trials with immune modulating interventions have provided positive results, large-scale clinical trials with non-specific intravenous immunoglobulin, active immunisation against Aβ and passive immunisation with Aβ-specific monoclonal antibodies have so far been largely unsuccessful. It could very

well be that we need a better understanding of AD before effective drugs can be designed. Similarly, a better defined pathophysiology is needed to untangle the epidemiological correlations between HSV-1, CMV and AD. Perhaps AD is not one disease but several more well-defined entities, joined by a similar neuropathological picture and clinical presentation of cognitive decline.

Moving on to the topic of HSE, there are many questions unanswered on the mechanisms behind NMDAR autoimmunity. If immunisation occurs as a result of synaptic antigen exposure during inflammatory conditions, this could have implications beyond the scope of HSE. It would be interesting to investigate whether similar processes can occur after traumatic brain injury or other CNS lesions where an inflammatory component is present. On the other hand, if antigenic mimicry is to blame, could there be a connection between subclinical HSV-1 reactivation and other forms of NMDAR encephalitis that are currently considered unrelated to infection?

It is almost impossible to speculate on where the rapid technological development in data acquisition and analysis will take us in the future, but through the use of multiplex and high throughput methods the total amount of scientific data will probably continue to rise exponentially. Consequently, this will increase the need for scientific application of data-mining and pattern-recognition algorithms, where computers are not only providing answers to pre-defined questions but also formulate new hypotheses. There are already medical expert systems in use and several more are in development, dimming the borders between automated data processing, machine-based learning and creative science. The role of future human scientists might not only be to ask and answer scientific questions, but also to understand and control the path of discovery and development that is driven by artificial intelligence.

Sammanfattning på svenska

Herpesvirus har funnits hos flera olika djur under stora delar av evolutionens gång. De nio virus som vanligtvis infekterar människa kan delas upp i grupperna alfa-, beta- och gammaherpesvirus. Dessa DNA-virus är idag spridda i stora delar av befolkningen över hela världen och kan orsaka välkända sjukdomar såsom vattkoppor, körtelfeber och herpesblåsor på hud och slemhinnor. De första symptomen på infektion efter smittoöverföring är ofta milda, men därefter stannar dessa virus kvar i kroppen under hela livet och kräver kontinuerlig immunologisk övervakning.

I samband med nedsättningar av immunförsvaret orsakade av exempelvis HIV, benmärgssjukdom, cellgiftsbehandling eller andra svåra sjukdomstillstånd, kan herpesvirus reaktiveras och orsaka allvarlig sjukdom. Reaktivering kan också ske trots ett i övrigt välfungerande immunförsvar, och beroende på vilket virus som reaktiveras kan sjukdomar som bältros, herpesblåsor eller inflammation i hjärna och hjärnhinnor uppstå. I de vetenskapliga arbeten som ingår i denna avhandling har vi främst studerat hur infektioner med cytomegalovirus (CMV) och herpes simplex typ 1 (HSV-1) påverkar immunförsvaret hos patienter med kognitiva sjukdomar.

Det är sedan tidigare känt att CMV påverkar utmognaden av den grupp vita blodkroppar som benämns T-lymfocyter och utgör en del av kroppens försvar mot bland annat virusinfektioner och cancer. Vid infektion ändras balansen mellan omogna och mogna celler på ett sätt som liknar det som kan ses vid stigande ålder. Det är inte helt klarlagt huruvida immunsystemet verkligen åldras i samband med infektion eller om förändringarna endast imiterar de åldersrelaterade förändringarna, men förändringar i T-cellspopulationen har tidigare associerats med risk för både sjukdom och död.

Vi har därför studerat T-lymfocyterna hos CMV-infekterade patienter med Alzheimers sjukdom i jämförelse med kontrolldeltagare utan demens. Studiedeltagare med Alzheimers sjukdom uppvisade en lägre andel virusspecifika T-lymfocyter än vad som återfanns hos kontrolldeltagare utan demens. Vi har också kunnat visa att cirkulerande immunceller från patienter med Alzheimers sjukdom reagerar med högre utsöndring av den inflammationsbefrämjande cytokinen IFN-γ, när cellerna stimuleras med antikroppar mot receptorerna CD3 och CD28 eller med proteinet pp65 från CMV. Däremot sågs ingen skillnad vid stimulering med amyloid beta, ett protein som lagras in i de amyloida plack som finns i hjärnan hos Alzheimerpatienter.

Sammanfattningsvis finns en samvariation mellan CMV-infektion, immunförsvaret och Alzheimers sjukdom, som skulle kunna tyda på ett orsakssamband. En tänkbar förklaring är att CMV-infektion förändrar immunsvaret mot HSV-1, som återfinns i hjärnvävnad i högre grad hos demenspatienter än hos icke-dementa. För att med större säkerhet kunna säga om CMV-infektion påverkar risken att insjukna i demens behövs studier där man följer både smittade och icke smittade individer utan demens under en längre tid och följer insjuknanderisken över tid i båda grupperna.

I ett separat projekt har vi anpassat en tidigare utvecklad mätmetod, baserad på mikrometerstora plastkulor som är märkta med fluorescerande färgkoder, till att nu mäta antikroppsnivåer för fem herpesvirus parallellt. Detta kan ske i en mycket begränsad provvolym (2,5-5 mikroliter), men kräver statistisk databearbetning för att säkerställa att de samtidiga mätningarna inte stör varandra. Patienter med Alzheimers sjukdom visade sig ha lägre antikroppsnivåer mot humant herpesvirus 6 (HHV-6) jämfört med kontrolldeltagare utan demens. För att utröna om denna skillnad i antikroppsnivåer kunde vara kopplad till reaktivering av latent virus i cirkulerande vita blodkroppar, analyserade vi mängden HHV-6 med PCR-teknik. Vi kunde inte påvisa någon skillnad i virusreaktivering mellan grupperna, och inte heller någon skillnad i fördelningen mellan virussubtyp A och B. För övriga i studien ingående herpesvirus - herpes simplexvirus, varicella zostervirus, Epstein-Barr-virus och CMV - sågs ingen skillnad i antikroppsnivåer mellan Alzheimerpatienter och kontrolldeltagare. Huruvida HHV-6 kan vara en riskfaktor för utvecklande av Alzheimers sjukdom kvarstår som hypotes inför framtida studier.

I det fjärde delarbetet har vi undersökt patienter med hjärninflammation orsakad av HSV-1 (HSE). Denna herpessjukdom kan uppkomma både vid primärinfektion och reaktivering av virus och drabbar uppskattningsvis 2-4 individer per miljoner invånare och år. Utan behandling är dödligheten ca 70 %, men även om moderna virushämmande läkemedel förbättrat prognosen får många patienter allvarliga och bestående funktionsnedsättningar. Det är tidigare känt att HSE-patienter kan utveckla antikroppar mot N-methyl-D-aspartat-receptorn (NMDAR). Dessa antikroppar har bland annat återfunnits hos patienter som fått återfall av hjärnhinneinflammation. NMDAR är ett protein som deltar i signalöverföringen mellan hjärnans celler och är av stor betydelse för minne och inlärning.

I en studie som inkluderade 47 patienter från olika delar av Sverige har vi kunnat visa att ca 25 % av patienterna som genomgår HSE utvecklar NMDAR-antikroppar och att dessa vanligen uppstår mellan 14 och 90 dagar efter sjukdomsdebut. Vi har också upptäckt att den patientgrupp som utvecklar antikroppar återhämtar sin hjärnkapacitet sämre under en två år lång uppföljningsperiod. Dessa fynd skulle, om de kan bekräftas, i framtiden kunna göra det möjligt att ge immunhämmande behandling riktat till de patienter som har störst risk för antikroppsrelaterad försämring av sjukdomsbilden.

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