Discrimination between CMV infection and Alzheimer's disease as driving forces for immune senescence

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David Goldeck

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Contents

Chapter 1

Introduction

1.1   Types of dementia and their diagnosis

Dementia is defined as a loss of memory and other cognitive functions relevant to daily life which persists for more than six months. The cause of the disease is the destruction or death of brain cells. In order for dementia to be diagnosed, at least two of the following four cognitive functions should be impaired:

> *"(1) memory; (2) ability to speak or understand language; (3) capacity to plan, make sound judgments, and carry out complex tasks; and (4) ability to process and interpret visual information"*

[70]. Clinically dementia can be classified into different categories shown in Figure 1.1.
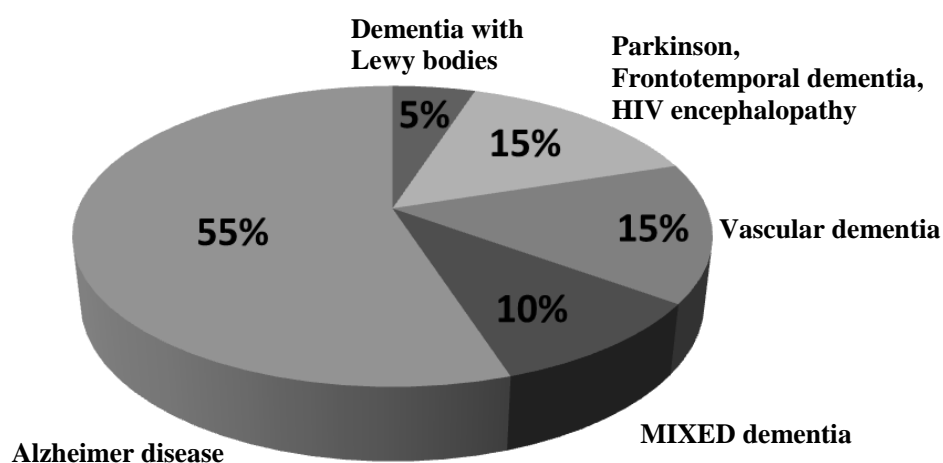
Figure 1.1: **Forms of dementia; after Bosser, 1992 (modified) [17]**

Table 1.1: Risk factors for dementia

category of dementia	factor	reference
cognitive decline	sex	[1]
cognitive decline	age	[1]
cognitive decline	education	[1]
dementia	income	[1]
PD[3]	no cigarette smoking	[97]
neuronal damage	no consumption of nicotine and caffeine	[97]
cognitive decline	chronic health conditions	[1]
cognitive decline	inflammatory markers (CRP,[2] cytokines)	[1]
AD, PD[3]	no nonsteroidal anti-inflammatory drugs	[97]
AD, PD[3]	infections (influenza? *Borelia*? others?)	[97]
cognitive decline[1]	serum antibodies to CMV, HSV-1, HSV-2	[146]
cognitive decline	maybe high anti-CMV IgG antibody levels	[1]
cognitive decline	HSV-1 + positive for *ApoE-E4* allele	[1]
AD	*ApoE-E4* gene mutation / ApoE status	[70]
AD	*PS1* / *PS2* gene mutation	[58]
AD	family history of AD	[70]
AD	Down Syndrome	[70]
PD[3]	excess weight / bmi[4] in midlife	[97]
AD	obesity	[70]
PD[3]	high caloric intake	[97]
AD	hypercholesterolemia	[70]
AD	high homocysteine	[70]
AD	hypertension	[70]
AD	type 2 diabetes	[70]
AD	metabolic syndrome	[70]
AD	heart disease	[70]
AD	cerebrovascular disease	[70]
AD	folate or other B-vitamin deficiencies	[70]

[1] in elderly Finish with vascular disease
[2] C-reactive protein
[3] Parkinson's disease
[4] body mass index

1.1.1 Mild cognitive impairment (MCI) and the difficulties of its diagnosis

Mild cognitive impairment (MCI) is a brain disorder associated with a decline in cognitive function that does not interfere with daily life [90]. It is considered a transitional state that in many cases leads to dementia [9]. There are many subtypes of MCI with amnestic MCI (aMCI) being the most prominent. aMCI is diagnosed when cognitive decline is accompanied by memory impairment [112]. Both types can, but must not necessarily, progress to the different types of dementia, including Alzheimer's Disease (AD) [53, 174, 43]. Dubois *et al.* studied a cohort of people with amnestic mild cognitive impairment. Of those which - according to the criteria of that time - were clinically identified to have progressed to dementia only 70% met the neuropathological criteria for AD. Therefore a question is posed as to when MCI can be considered to have progressed to AD and where to draw the defining line in what appears to be a continuous process [32]. In order to identify predictive markers for this progression, Tabert and colleagues analysed 148 patients and 63 age-matched controls in a follow-up study. The main conclusion was that *"deficits in verbal memory and psychomotor speed/executive function abilities strongly predicted conversion to AD"* [148]. Other risk factors for dementia are listed in Table 1.1. For diagnostic visualisation of the conversion from MCI to AD, MRI (Magnetic Resonance Imaging) patterns of hippocampus atrophy were proposed [165] or in a more recent study a *"Short-form Everyday Technology Use Questionnaire"* that can be used as a screening tool for evaluating the use of everyday technology (e.g. using a telephone or household equipment, managing finances and transportation) [72]. Using MRI it is possible to analyse loss of grey matter, while questionnaires can assess memory function, but neither technique is able to explain the origin of disease. Another idea discussed in this context is the use of biological markers, such as specific immune signatures which may contribute to understanding the disease process itself. In this context, the present work was performed with the hypothesis that Cytomegalovirus (CMV) and dementia (primarily AD) influence the immune system. CMV infection has been implicated as a potential risk factor for AD (Table 1.1). It has a major impact on immune signatures which may be relevant to MCI and in determining which patients with MCI may progress to AD. Earlier, more accurate diagnoses would allow earlier therapeutic intervention which might increase the cognitively healthy time period of affected individuals.

1.1.2 Alzheimer's Disease (AD)

First reports on AD

Alzheimer's Disease is the most common form of dementia, it occurs predominately in elderly people above 65 years of age. According to the 2007 Facts and Figures report of the Alzheimer's Association of the United States, 96% of all AD patients are over 65 years of age. The worldwide prevalence of AD was estimated to be 18 million in 2008 [70], with a predicted increasing prevalence due to rising life expectancies and a reduction in deaths from infectious diseases associated with advances in modern medicine. Because of this, the study of dementia is becoming more relevant to societal health and the burden of disease. AD was first described by Aloysius Alzheimer in the beginning of the 20th century. Alzheimer observed memory decline, disorientation and

confusion in a 51 year-old patient in 1901 [70]. After autopsy in 1906, Alzheimer described the main characteristics of AD: an atrophied brain, dead and dying brain cells, plaques consisting of amyloid beta and neurofibrillary tangles (NFT) [5]. This led eventually to the "amyloid hypothesis".

Amyloid beta and tau

Despite controversial issues surrounding the cause and classification of AD, formation of amyloid plaques remains the main diagnostic and causative factor. It has been suggested by the group of La Ferla that amyloid beta (Aβ) may change from being a beneficial molecule with antimicrobial activity that is involved in protection against oxidative stress, cholesterol transport and is implicated in certain signalling pathways, to one of the major candidate molecules suggested to contribute to AD pathology (Fig. 1.2). The amyloid precursor protein (APP) is processed in cholesterol rich membrane regions, so-called lipid rafts. Secretases cleave APP into proteins between 36 and 43 amino acids long. The most important forms for AD pathology are suggested to be Aβ-40 and Aβ-42. Aβ can accumulate and aggregate, which according to the amyloid hypothesis initiates the onset of AD [117]. It is believed that Aβ exerts negative (for example neurotoxic) effects and has an immunomodulatory role. Synaptic dysfunctions within the brain and a systemic inflammation are some of the consequences of Aβ accumulation. With microscopy studies the La Ferla group showed co-localisation of ubiquitin and Aβ, implying that proteasomal functions are involved in the role that Aβ may play in AD pathology. One target for proteasomal degradation is phosphorylated tau, which can accumulate and form neurofibrillary tangles, as observed by Alzheimer within the brain of one of his patients. Tau is a microtubule-binding protein, which is involved in axonal transport, also of APP, and can so influence Aβ distribution and/or production. Another hypothesis is that Aβ activates tau kinases which lead to hyperphosphorylation of tau. The consequence is dissociation from microtubuli which then depolymerise, which results in axonal transport deficits and accumulation of hyperphosphorylated tau in AD patients. Additionally, consistent with a state of systemic inflammation, microglia and astrocytes are activated and are located near Aβ plaques, - as for example shown by La Ferla. These cells possess phagocytic activity and degrade Aβ plaques. In addition they produce high levels of pro-inflammatory cytokines, which can activate certain kinases, leading to tau hyperphosphorylation [13].

Amyloid beta as the cause of AD - a controversial hypothesis

Among several theories suggested to explain the cause of AD, the amyloid beta hypothesis is the most prominent. In 1991 Hardy and Allsop proposed a sequence of events characteristic of AD. Due to an imbalance between production and clearance of Aβ, the primary component of the plaques seen by Alzheimer, there is an accumulation of Aβ. This leads to formation of neurofibrillary tangles and subsequent to neuronal death [57]. This hypothesis is supported by the fact that the gene for the Aβ precursor protein (APP) is located on chromosome 21 and that individuals suffering from Down Syndrome that possess an extra copy of chromosome 21 frequently develop AD [89]. In addition, mutations in the APP gene could cause Aβ accumulation
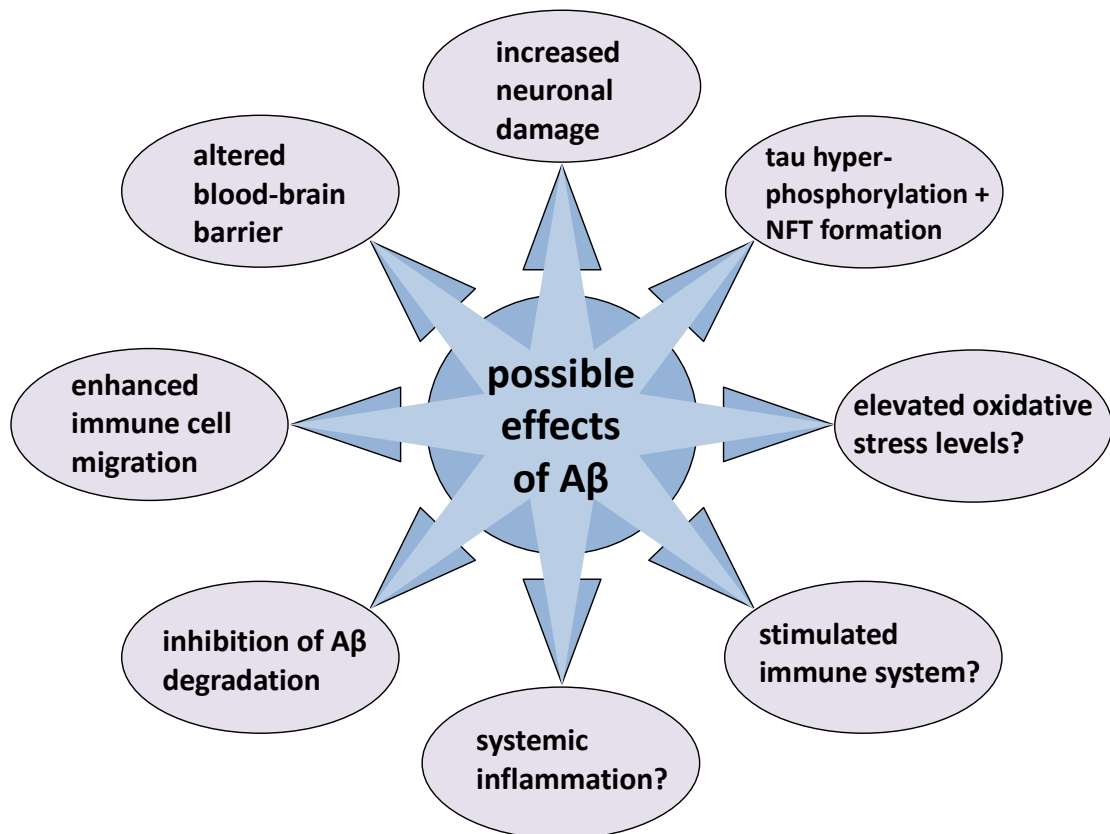
Figure 1.2: **Suggested pathophysiological effects of amyloid beta**
Contributions of Aβ to systemic inflammation and AD pathology. According to the amyloid hypothesis Aβ causes tau hyperphosphorylation and the formation of characteristic neurofibrillary tangles. Through inhibition of its own proteasomal degradation Aβ enhances this effect. In addition some studies showed Aβ stimulation of immune cells. They can secrete chemokines and cytokines and recruit peripheral cells which can enter the brain via a blood-brain barrier which is compromised, e.g. by Aβ. These peripheral cells can secrete cytokines and so contribute to the inflammatory milieu in AD.

outside the brain parenchyma and promote Aβ self-aggregation into fibrils [58]. In the limited number of cases of hereditary AD, mutations in the presenilin genes were detected. Presenilins are a family of transmembrane proteins that are found in the complex which processes APP to Aβ. Mutations in the *PS1* and *PS2* genes, which code for presenilins, were observed to result in enhanced Aβ production. Apolipoproteins play an important role in Aβ catabolism. The ApoE protein promotes proteolytic Aβ degradation. The gene for the E4 isoform ApoE-E4, with inefficient catabolic function, facilitates Aβ accumulation, and is a prominent genetic risk factor for AD. APP transgenic mice accumulate Aβ and show AD pathology. When these were crossed with ApoE-deficient mice, the offspring showed less Aβ accumulation in the brain [58].

It is also possible that mutations in the *tau* gene could be responsible for AD. As reviewed by Hardy & Selkoe, mutations in this gene cause frontotemporal dementia with parkinsonism including severe neurodegeneration. In contrast to AD, in this neurodegenerative disorder whereas aggregation of tau in neurofibrillary tangles in the brain occurs, no Aβ accumulation is present. The amyloid cascade model is not applicable in these cases. Therefore the authors concluded that tau aggregation in AD follows alterations in Aβ metabolism. In another study (by Braak and Braak) human brains were analysed postmortem. In younger people without amyloid plaques, age-dependent damage to neurofibrills and cells was already present [58]. Hardy & Selkoe postulate that it is not possible to predict whether the neurofibrillary alterations seen in nondemented elderly of this study would have resulted in AD. Rather they attribute these lesions to a *"process separate from AD"* pathology. To strengthen their hypothesis they cite studies performed with Down Syndrome patients at different ages which show amyloid plaques prior to the formation of neurofibrillary tangles and AD onset. In contrast, mice with mutations in the APP gene showed Aβ accumulation without neuronal loss. This could be explained - according to Hardy & Selkoe - by the absence of human tau and / or cytokines. It appears as though interplay of both tau and Aβ is essential for AD development, as studies using primary mouse cell cultures showed Aβ toxicity to be tau dependent. Additionally, mutations in the *APP* gene resulted in more rapid production of neurofibrillary tangles in *APP/tau* double transgenic mice [58].

There are other observations which are difficult to explain with the amyloid hypothesis. Given the hypothetical central role of Aβ, it would be expected that a correlation between cognitive decline and amyloid plaque formation would exist, but this has not been confirmed. Some healthy individuals show plaque formation, but no associated neuronal damage [97]. On the other hand, the amount of soluble Aβ does correlate with disease severity [58].

An overview of different hypotheses and the role of suggested key players is presented in Figure 1.3. This suggests that not all factors act independently, but instead within a complex network in which they may influence each other. An explanation of Aβ accumulation is genetic variability, while another hypothesis proposes that infections, especially with herpesviruses like HSV-1 (Herpes simplex virus 1) play a role [171]. HSV primarily infects infants, but the virus is latent in the peripheral nervous system for life. In some cases HSV can result in herpes simplex encephalitis - a severe brain disorder. In this disorder the same brain regions are affected as in AD [172]. However, among different viruses studied, HSV-1 was not found to be a risk factor for AD [60] - but in combination with the possession the *ApoE-E4* allele it was shown to be a risk factor [64]. One hypothesis is that carriers of this allele degrade Aβ less efficiently, so that much more Aβ is able to accumulate. It has therefore been suggested that HSV-1 inhibits degradation of Aβ

as well as tau [63]. Another herpesvirus, Cytomegalovirus (CMV), can be present in the brain of healthy elderly and AD patients [83]. In longitudinal studies, higher anti-CMV antibody levels were reported to correlate with more rapid cognitive decline, although the cause remains unclear [1, 146]. Further issues regarding the contribution of CMV to AD are discussed in Section 1.3. Yet another AD risk factor is oxidative stress, which has been observed to be elevated from early AD onwards. Serum oxidative load is higher and antioxidant capacity lower in AD patients compared to controls [70]. Brain lesions due to reactive oxygen species and reactive nitrogen species have been observed in AD patients [116]. In response to oxidative stress, heat shock proteins (HSPs) are over-expressed. For example, HSP70 was identified as a protective factor against intracellular Aβ accumulation. Its over-expression was shown to rescue neurons from Aβ-mediated toxicity [35, 91]. Therefore, it has been proposed that HSP70 acts to attenuate the cytotoxicity of Aβ by binding amyloidogenic peptides and restoring the balance between aggregation, folding and degradation [35]. Although the oxidative stress hypothesis is based on several findings in AD patients, including the observation that dietary intake of antioxidants reduces the risk of AD, clinical trials using antioxidant supplementation have shown only a marginal positive effect or no benefit at all [116]. This indicates that none of these hypotheses is by itself capable of explaining AD cause and pathology.

Diagnosis of AD

Given the above, it is not surprising that the diagnosis of AD cannot be made on the basis of a single test, but several measures are compiled into an overall picture. Some of these are measured with the use of questionnaires, such as the Folstein test (see Section 1.1.5), others with MRI images (as discussed for MCI above), positron emission tomography (PET) scans which show brain metabolism and Aβ deposition, analysis of blood or CSF (cerebrospinal fluid). To diagnose AD, other diseases with similar symptoms, such as delirium, HIV-infection, encephalitis and other forms of dementia must be excluded. Non-specific early AD symptoms are memory loss and language alterations, but an unequivocal diagnosis of pathological hallmarks, such as amyloid plaques and neurofibrillary tangles are often still only detectable at autopsy. At the cellular level, reduced numbers of nerve cells, dendrite branches and synaptic densities within the brain are characteristic of AD. According to Kidd, pathological deterioration is detectable years prior to diagnosis [70]. Therefore it is essential to find biomarkers that detect AD in early stages. Robust biomarkers would allow earlier therapeutic intervention. Of the possible biomarkers, those detectable in the peripheral blood would be most convenient for diagnosis. These may include plasma levels of tau and Aβ and the presence of specific immune signatures [42, 110]. As discussed in a following section, cells from the periphery may enter the brain of AD patients (Section 1.4). There they could contribute to Aβ phagocytosis and cytokine secretion by immune cells, which are also altered in AD patients [39], and potentiate disease.

Amyloid hypothesis

PS1 = component of the γ-secretase complex that cleaves APP

Missense mutations in *APP*, *PS1* or *PS2* genes

PS1 may be regulated by the chaperone network

HSV hypothesis

Other Hsp can regulate Aβ production and aggregation

PS1 forms aggresomes within the ER

APOE- ε4 carrier

increased risk

HSV infection

Chronic antigenic stress for the immune system?

Hsp drive APP to its destination

Impaired proteasomal activity

Amyloid beta accumulation

Hsp and APP mRNA copies are produced

Inhibition of degradation

Apoptosis of monocytes/macrophages of AD patients

Interaction with heat shock proteins (Hsp)

Extracellular chaperones, small Hsp

Plaques

Soluble Aβ

CXCR2 ↑ CCR5 ↑

Phosphorylation of heat shock factors

Stress response

Effects on proteases and kinases

T-cell mobility ↑

External factors

Altered neuronal, ionic homeostasis; oxidative injury

Tau hyperphosphorylation ⇨ NFT formation

activate T-cells

Intracellular Aβ

Neuropeptide transmitters

↓ Transport of APP carriers?

↑ Chaperoning function?

Axonal transport deficits

Phosphorylation of small Hsp

Neuronal damage, synaptic injury

cytotoxic effects

Tau

Hsp induces T-cell regulation of chronic inflammation.

IL-1 TNF

Inflammation + cell death

CMV

induced effector T-cells

Oxidative stress hypothesis

Negative influence + possible neuro-protective role of CCR5

Activation of microglia, astrocytes and other immune cells

Inflammation hypothesis

Phosphorylated tau is targeted for proteasomal degradation by chaperones ⇨ disruption of proteasomal activity or tau ubiquination leads to ↑ phosphorylated tau

Release of reactive O, cytokines and chemokines

Altered BBB

Infarctions in AD brains

↓ Inflammation via anti-inflammatory cytokines

Reduced cell migration

Beneficial roles of immune cells

Systemic inflammation

Recruitment of peripheral cells

Secretion of nerve growth factors by e.g. B-cells to promote regeneration
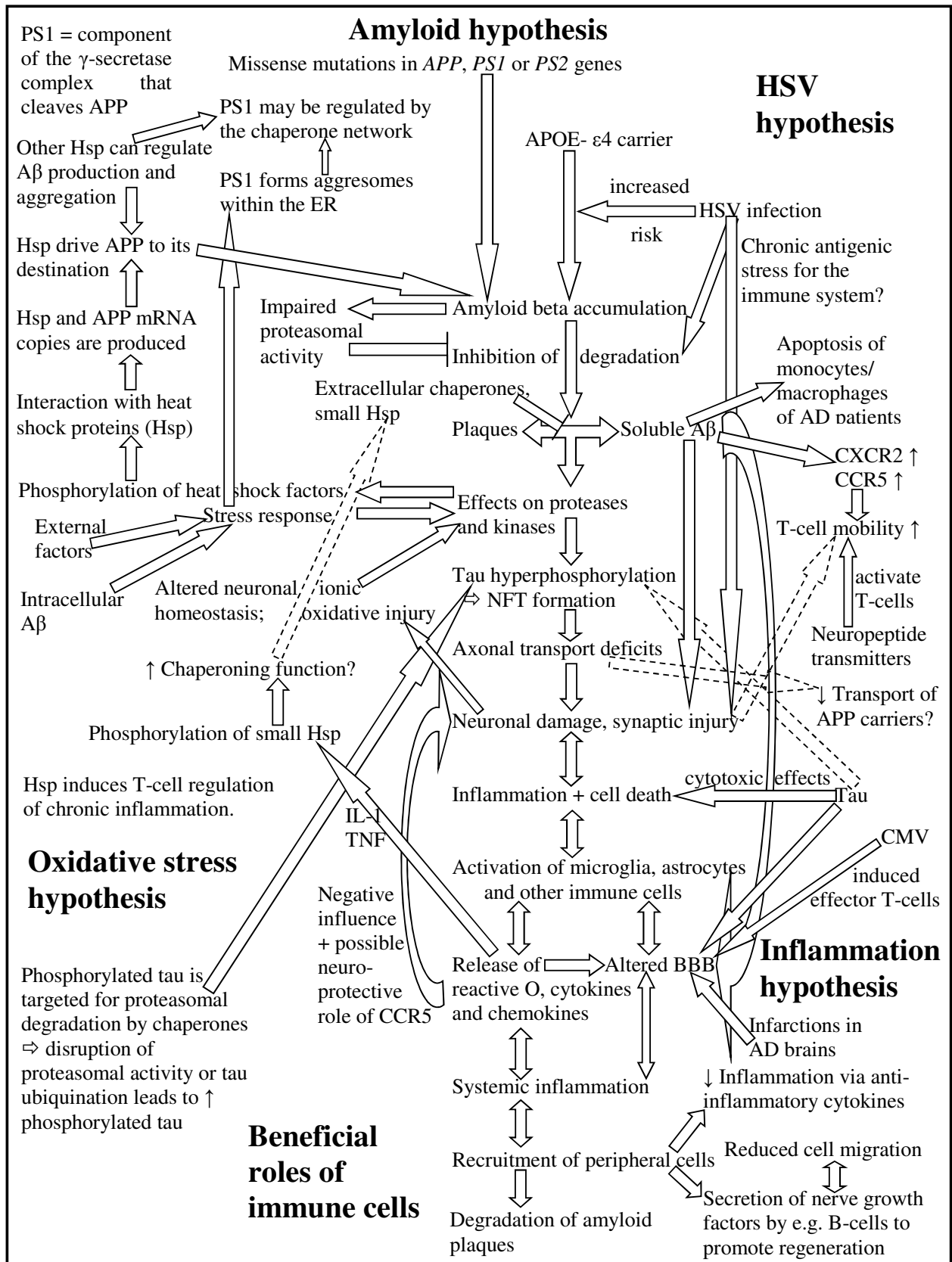
Degradation of amyloid plaques

8

Figure 1.3: **Proposed mechanisms of pathophysiology in AD**
Diagram displaying how the alternate AD hypothesis interact

Rapidly progressive AD (rpAD)

According to Goldberg, disease duration of slowly progressing AD is around 93 months, $\sim$24 months from the onset of clinical symptoms until definitive diagnosis, then $\sim$25 months until admission to a care facility, such as a nursing home, and a further $\sim$44 months patient survival time) [50]. When the disease is rapidly progressive, it is referred to as "rpAD". The term "rapidly" is defined as a decline of the MMSE value (value defining the rate of cognitive decline, see Section 1.1.5) of five points per year or by a survival time <4 years compared to up to ten years for "usual" AD patients. This type of rapid progression is not limited to AD, but can also be observed in other dementia. The symptoms of rpAD can mimic those of other neurological diseases, such as Creutzfeldt-Jakob disease [136], making diagnosis even more problematic.

1.1.3   Vascular dementia (VaD)

In Western countries vascular dementia (VaD) ranks as the second most common type of dementia after AD, while in Asia it may actually be the most prevalent form [11]. The incidence of VaD increases significantly above age 65. The NINDS-AIREN (National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherche et l'Enseignement en Neurosciences) gives the criteria for VaD as mentioned by Black [11]: *"a) cognitive decline in memory and two other domains sufficient to impair functional abilities (attributable to cognitive decline, not just physical disabilities); and b) evidence for CVD (cerebrovascular disease), indicated by focal (i.e. sensory-motor) signs and relevant imaging evidence of stroke."* Once these criteria are confirmed, time of onset determines whether diagnosis of VaD is probable, possible or definite [11]. For a definite diagnosis to be made, other brain diseases need to be excluded, especially other forms of dementia. One way to achieve this is through the use of neuroimaging techniques to study the structure, biochemistry, metabolic state and functional capacity of the brain. Tartaglia *et al.* postulate that *"All of the major neurodegenerative disorders have relatively specific imaging findings that can be identified."* and which support a diagnosis [150]. A three year follow-up study with 193 patients reviewed by Pantoni and Gorelick provides an example of the information that imaging can contribute to a diagnosis. In approximately 80 % of the cases, MRI combined with single photon emission CT provided useful information for diagnosis. Another study with 588 patients suggested that AD could be diagnosed by patients having solely medial temporal atrophy, whereas vascular dementia was diagnosed along with other information, such as patient history of stroke, white matter changes in addition to medial temporal atrophy [108]. A summary of methods to discriminate between these two forms of dementia is provided in the following section.

1.1.4   Differential diagnosis

Discrimination between different forms of dementia is a major challenge. Multiple criteria to achieve effective discrimination have been developed and evaluated in different schemes. NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke - Alzheimer's Disease and Related Disorders Association) and DSM (Diagnostic and Statistical

Manual of Mental Disorders) propose two sets of features for AD which achieve approximately 90% sensitivity of diagnosis. In the past, AD diagnosis largely relied on the exclusion of other diseases with similar symptoms. The major reason that accounted for these difficulties in effective diagnosis was insufficient knowledge of the disease, as the authors of the first NINCDS-ADRDA publication stress [95]. Currently, there is more emphasis placed on discovering new and utilising existing biomarkers. Changes in brain structure, for example volumetric loss of the hippocampus, and tau distribution are detected using MRI and metabolic differences are visualised by PET scans. In addition, CSF biomarkers, such as presence of $A\beta$ and tau, are potential candidate biomarkers. These advances may help to reduce the incidence of incorrect diagnosis. Therefore, Dubois *et al.* published revised NINCDS-ADRDA criteria for research, used for the patients studied in one series of studies presented in this thesis. The authors state that *"a more refined definition of AD is still needed to reliably identify the disease at its earliest stages"*. In addition, incorrect patient diagnosis led to unsuccessful (and expensive) trials with novel therapeutics with hundreds of participants. The basis for diagnosis is the progressive reduction in memory function, along with impaired recall and recognition. These symptoms should be accompanied by other factors, such as: 1. Volume loss of certain brain regions, as detected by MRI; 2. Altered concentrations of CSF markers (low levels of $A\beta$ and high levels of tau and as the authors state *"other well validated markers to be discovered in the future"*); 3. A particular pattern of functional neuroimaging measured with PET; 4. Specific genetic influences. Next, the authors list several exclusion criteria, for example the presence of other non-AD dementias. Nonetheless, these symptoms collectively can only lead to a diagnosis of probable AD. For a confirmed diagnosis, only post-mortem examination is sufficient. Another important consideration is that lumbar puncture to obtain CSF is not routinely performed in clinical patient assessment but only in certain cases. This reinforces the need for novel biomarkers [32].

In addition to these diagnostic criteria, others are included for VaD diagnosis. One of these is the Ischemic Scale - first introduced by Hachinski and subsequently modified by Rosen. Therein, several clinical symptoms are considered, such as nocturnal confusion, depression, evidence of atherosclerosis and history or presence of hypertension or strokes. Each of these features is assigned one or two points which are then tallied to give the ischemic score. These values are used to discriminate between AD, multi-infarct dementia (a kind of VaD) and a mixed form of both (mixed dementia) as summarized in Table 1.2. However, this scale has disadvantages. It does not consider the locations of the vascular lesions nor the type of cognitive syndrome. Therefore, the following alternative list of criteria was developed [167]:

- The DSM (Diagnostic and Statistical Manual of Mental Disorders) criteria (DSM-III, DSM-III-R, and DSM-IV)

- ICD-10 (International Statistical Classification of Diseases) criteria with six subtypes of VaD

- ADDTC (The State of California Alzheimer's Disease Diagnostic and Treatment Centers) criteria

- NINDS-AIREN (National Institute of Neurological Disorders and Stroke - Association Internationale pour la Recherche et l'Enseignement en Neurosciences) criteria

These classification strategies differ in several aspects and are variably strict. For example, the number of exclusion criteria differs, some diagnostic criteria exclude vascular brain lesions that, although typical for VaD, are still controversial. Each list also provides a different assessment of subtypes, in some sets of criteria mixed dementia is clearly defined, but in others it is not [167]. In another review, Wiederkehr *et al.* conclude from the results of clinical studies that there is a large degree of variability regarding sensitivities and specificities, incidence, and prevalence rates and a lack of discrimination between VaD and mixed dementia. They suggest several improvements, such as the use of the ADDTC criteria for clinical settings, while reserving the NINDS-AIREN criteria for research only [166]. Pohjasvaara *et al.* studied 107 patients comparing the different sets of criteria. The conclusion of this study was that comparability of the features is difficult. The authors suggested that new criteria should be tested in longitudinal studies [113]. Hence, there is need for improved methods of diagnosis.

Table 1.2: Dementia classification according to Ischemic Scales [167]

category of dementia	Hachinski Ischemic score	Ischemic score of Rosen
Alzheimer's Disease	≤ 4	≤ 2
mixed dementia	5-6	3
multi-infarct dementia	≥ 7	4-10

Some criteria applied for VaD are summated in the Ischemic Scale, which was first introduced by Hachinski and then modified by Rosen. Therein, several clinical symptoms are considered, e.g. nocturnal confusion, depression, evidence of atherosclerosis and history or presence of hypertension or strokes, etc. Each of these features is given one or two points which are then tailled as the ischemic score. These values are used to discriminate between AD, multi-infarct dementia (a kind of VaD) and a mixed form of both.

1.1.5 The Folstein Test and the MMSE value

In 1975, Folstein *et al.* introduced the mini-mental state examination (MMSE) consisting of a 30-point questionnaire designed to screen for dementia as well as to estimate the severity of cognitive impairment. In the span of approximately five to ten minutes, the MMSE tests various mental functions including arithmetic, memory and orientation. One advantage of this test is that it is quick to administer, which is important given that elderly people, particularly those suffering from a condition, cooperate well only for a short period of time [45]. The maximum score is 30 and the lower the value, the unhealthier a participant is. A method for estimating the rate of progression in AD was proposed by Doody *et al.* in 2001, after studying AD patients in a longitudinal study. A decrease of five points in the MMSE per year was considered as rapidly progressing AD. These patients developed clinically-detected symptoms significantly earlier than patients that had an initial score that was lower or equal, but decreased by two or fewer points per year. These patients were labelled as slow progressors, while average progressors showed a loss between two and 4.9 points per year [31].

1.1.6 Treatments for AD

Currently, there is no cure for AD. Existing therapies are only able to extend functioning by six to 18 months, if at all [70]. As of 2008, five drugs with approval from the U.S. Food and Drug Administration were available. Tacrine (brand name Cognex®), donepezil (Aricept®), galantamine (Razadyne®) and rivastigmine (Exelon®) are cholinesterase inhibitors. They are now rarely prescribed as they have shown minimal efficacy in improving cognitive function, while in the case of tacrine adverse side effects, such as liver toxicity, have been reported. These drugs are designed to prevent a decrease in the acetylcholine neurotransmitter at synaptic junctions. This decrease has been observed in the brains of AD patients [70]. Memantine (Namenda®) is an n-methyl-d-aspartate-receptor antagonist. When glutamate, another brain neurotransmitter, binds to this receptor calcium entry and nerve cell stimulation occurs - this is required for learning and the formation of memory. High levels of calcium due to overstimulation can lead to functional disruption and cell death. Pharmacological blocking of the n-methyl-d-aspartate receptors can reduce these negative effects. Memantin has been shown to result in improved cognitive functioning, behaviour and the performance of daily living activities in patients with moderate to severe AD [70]. As these drugs are only able to slow disease progression, if they even do that, other strategies such as Aβ-targeted immunotherapy have been considered. Wisniewiski and Konietzko describe six possible mechanisms of Aβ-directed immunomodulation. These approaches are not mutually exclusive and their effectiveness might differ depending on the stage of disease [170]. The six are as follows:

- Direct disassembly of plaques can be achieved by conformation-selective antibodies.

- Antibodies capable of activating microglial cells so that they dissolve plaques by Fc mediated phagocytosis.

- Microglia can also be activated via Fc-independent pathways, as suggested by experiments with mice and passive immunisation.

- Creation of a peripheral amyloid sink with the clearance of circulating amyloid may reduce amyloid burden in the brain.

- A humoral response resulting in IgM-mediated plaque-hydrolysis may be created with amyloid beta peptides as adjuvants.

- Antibodies may be capable of neutralising oligomer toxicity.

After promising studies in mice, a trial of the AN1792 vaccine was initiated. This vaccine consisted of preaggregated Aβ-42 in an adjuvant. In the phase I trial, 53% of the treated patients developed a humoral response against amyloid beta. The phase II trial was terminated when 5% of the patients showed signs of menigoencephalitis. Postmortem autopsies of selected patients revealed effective amyloid beta clearance after treatment with the vaccine, but this was not associated with a significant improvement in cognitive function or prolonged survival. Because

tau pathology remained in these patients, Wisniewski and Konietzko suggest that active vaccination needs to begin earlier - before AD-related pathology with neurofibrillary tangels develops [170, 154]. One possible explanation for the observed toxicity in the phase II trial is a strong Th1 response, which led to increased inflammation followed by amyloid beta-induced apoptosis of Th1 cells. This was modelled with the *in vitro* stimulation of PBMCs from the trial participants in which production of IL-2 and interferon gamma was observed. In order to prevent the induction of a Th1 response, it has been suggested that IgM be applied, which, due to its relatively large size compared to other immunoglobulins, may be less likely to enter the brain, where it can activate T-cells. This strategy relies on the creation of a peripheral $A\beta$ sink and is T-cell independent. Studies using passive immunisation of monoclonal amyloid beta antibodies were tested in clinical trials, but were not continued, because no clinical benefit was observed. Yet another approach sought to exploit the innate immune system by CpG oligodeoxynucleotides administration. CpG binds and activates Toll-like receptors on microglia via T- and B-cell independent pathways [170].

Considering the results of all trials performed to date, it must be concluded that none has resulted in a therapy able to cure AD. Instead, disease progression could at best be delayed only by several months [70]. The mechanism for this beneficial, if marginal effect has been suggested to be via a reduction of inflammation. Reale *et al.* demonstrated that treatment with acetylcholinesterase inhibitors was followed by reduced expression of pro-inflammatory cytokines such as $TNF\alpha$, IL-1β and IL-6 by peripheral blood cells of AD patients. These cytokines play a role in amyloid beta metabolism as they increase APP levels. One proposed role of acetylcholine is that of an immunomodulator which facilitates a Th2 response [120]. Other studies demonstrated that IL-4 can activate microglia and block the increase of pro-inflammatory cytokines and toxic nitric oxide *in vitro* [120]. Suggestions for future treatments include modulation of immune cell migration in order to reduce their brain entry. This process contributes to inflammation and supports the trafficking of cells with $A\beta$ phagocytic ability (Fig. 4.1).

1.2 Immune signatures of healthy individuals and AD patients

1.2.1 T-cell heterogeneity

T-cell differentiation following antigen exposure

Pathogens which enter the body encounter antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages or B-cells. Receptors, for example toll-like receptors (TLRs) located on the surface of APCs, recognise foreign structures such as lipopolysaccharide on bacterial cell walls, or nucleic acids. They may also bind and present self components, such as $A\beta$ under certain conditions. This recognition leads to the migration of APCs to the lymph nodes. After development in the thymus, T-cells circulate between the bloodstream and peripheral lymphoid organs as naïve T-cells. Cell adhesion molecules mediate the initial interaction between APCs and naïve T-cells in the lymph nodes. The T-cells then may become activated by interaction with antigen and cell surface and soluble mediators from the APC, and then migrate into infected tissues. Foreign antigens are presented by APCs on major histocompatibility complexes (MHCs).

The MHC molecules on APCs bind to the T-cell receptor and a co-receptor (either CD4 or CD8). This interaction initiates a signalling cascade which leads to T-cell activation. For this, additional interactions are necessary, involving the binding of co-receptors to their ligands on APCs (e.g. CD28 on T-cells to B7, CD27 to CD70 or CD40L to CD40). One function of these co-receptors is the maintenance of T-cell survival by inhibiting apoptosis pathways and stimulating secretion of the IL-2 growth factor. Once T-cells differentiate into effector cells, co-stimulation is not longer required. CD8+ T-cells can differentiate into cytotoxic lymphocytes with the ability to kill infected target cells. In contrast, CD4+ T-cells can develop different effector functions. CD4+ T-cells control infected macrophages, stimulate neutrophils to act against extracellular bacteria and fungi, promote eosinophil and mast cell responses towards parasitic infections and stimulate B-cells to produce antibodies. In addition, CD4+ T-cell subsets referred to as regulatory T-cells (Treg) can suppress T-cell responses (Section 1.2.3) thereby limiting autoimmune responses.

T-cell phenotype models

After activation, T-cells differentiate into memory and effector cells. These are characterised by the presence of several marker proteins including CD27 and CD28. Sallusto *et al.* proposed a T-cell phenotype model based on the chemokine receptor CCR7 or alternatively the homing receptor CD62L, together with the phosphatase CD45RA. In this model T-cells are divided into naïve cells (CCR7+CD45RA+), central memory (CM) (CCR7+CD45RA-), effector memory (EM) (CCR7-CD45RA-) and effector memory cells re-expressing CD45RA (TEMRA), which represent the more antigen experienced cells (CCR7-CD45RA+) (Fig. 1.4) [130]. Following priming by antigen, CCR7+ memory cells migrate into secondary lymphoid organs and stimulate other immune cells, such as B-cells and DCs. In contrast, CCR7- T-cells predominately mediate inflammation or defend against invasive pathogens in peripheral tissues, for example via the production of cytokines such as IL-4, IL-5 or IFNγ or by the release of perforin. In co-culture experiments CCR7+ memory T-cells were more potent activators of DCs than CCR7+ naïve T-cells, which stimulated them less effectively in terms of IL-12 production [130]. One explanation suggested by Romero *et al.* for this apparent division of labour is the different lifetimes of these cell types. CM and a subset of EM T-cells expressed high levels of CD127, a molecule necessary for the survival of memory T-cells. This is the likely reason why CM cells are more effective against systemic infections [126]. Furthermore, Romero *et al.* presented an expanded model suggesting that the progression of differentiation is naïve > CM > EM > TEMRA, with several stages further discriminated by their CD27 and CD28 expression. This sequence correlated with progressively reduced telomere lengths and higher granzyme B and perforin expression at each CD8+ T-cell differentiation stage.

Additional T-cell characterisation markers

Additional markers of T-cell phenotypes have been reviewed many times, e.g. by Appay *et al.* [6]. These authors defined naïve T-cells as CD45RA+CCR7+ CD27+CD28+ and suggested different T-cell differentiation phases following antigen contact where these markers are different to those proposed in other models. The authors describe the expression of several other mar-
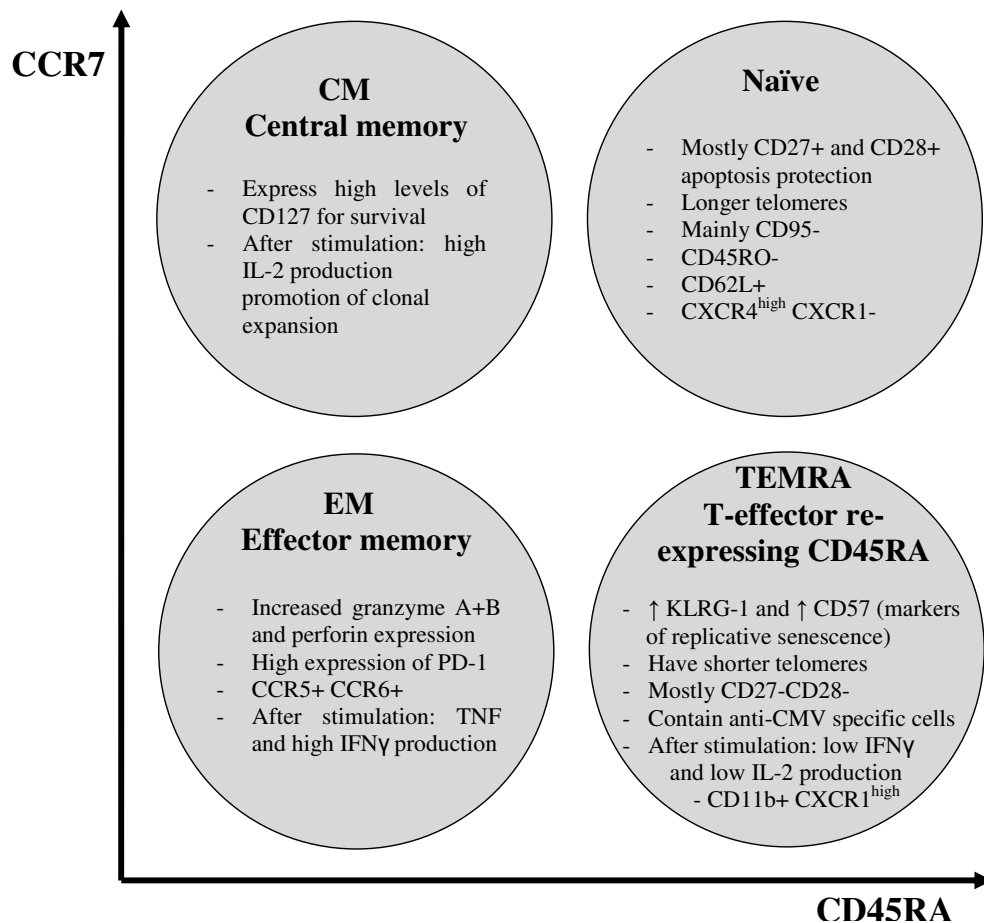
Figure 1.4: **T-cell differentiation model**
T-cells classified according to Sallusto *et al.* including naïve, central memory, effector memory and effector memory re-expressing CD45RA cells [130]. Characteristics such as surface marker expression, cytokine and chemokine receptor profile and telomere length of each phenotype are listed.

kers at each stage. While losing CD45RA expression, expression of the CD45RO isoform is increased. Another marker which is absent in naïve cells but increases during differentiation is CD57. This protein is considered by some to be a marker for replicative senescence and immune deficiency. CD4+ CD45RO+CD57+ cells possess a tendency for increased apoptosis while CD4+CD57+ cells do not support immunoglobulin production by B-cells [44]. For CD8+ T-cells, other markers such as the coinhibitory receptor KLRG1 (Killer cell lectin-like receptor subfamily G member 1) have been utilised. The expression of this protein increases in memory T-cells. Another marker used to define T-cell subsets is the Fas receptor CD95 (APO1/FASR), enhanced expression of which might be indicative of apoptosis. CD95 is expressed on memory cells and its expression, either alone or together with CD45RO, can be considered a sign for late-stage differentiation or immunosenescence [114]. Some marker proteins, such as the T-cell regulator PD-1, are absent in naïve cells, but are expressed during differentiation, and are down-regulated again in late-stage cells [6].

1.2.2 T-cell phenotypes in healthy elderly and AD patients

Age-related accumulation of late-differentiated T-cells

Ageing is associated with changes in the immune system, for example greater susceptibility to infectious diseases with increasing age. Due to age-related thymic involution, generation of naïve T-cells is decreased in the elderly. A more detailed description using the model previously described indicates a shift from naïve to late-differentiated TEMRA cells within the CD8+ T-cell subset, with less pronounced changes in CD4+ T-cells, where percentages of central memory, but not TEMRA cells are higher in the elderly. CD4+ T-cell differentiation with increasing age was not associated with shifts in naïve, CM, EM and TEMRA stages, but are more apparent when describing these phenotypes using additional markers such as CD27, CD28, CD57 and KLRG1 [71]. Although frequencies of the CD4+ T-cell sub-populations are changed with age, functionality in terms of proliferation and cytokine production assays have been reported not to differ [74]. An age-related decrease in naïve CD8+ T-cells and an increase in effector memory T-cells were also observed by Yan *et al.*, who also investigated the influence of gender. This commonly observed age-related increase in the frequency of CD3+CD45RA-CCR7- effector memory cells in peripheral blood leukocytes of healthy individuals occurred earlier in men than in women [175].) It has been suggested that although these observations are associated with age, they may be in fact be caused by chronic antigenic stress associated with viral infection [109].

Antigen-experienced T-cells accumulate more in AD-patients than in healthy elderly

Amyloid beta may be able to act as an immune stimulus in AD, as Monsonego and colleagues demonstrated. There may be an increased T-cell reactivity to Aβ-42 in the elderly and AD patients [103]. Tan *et al.* proposed that T-cell differentiation, as indicated by a switch from CD45RA to CD45RO expression, may be a marker for AD [149]. Togo *et al.* demonstrated that memory cells were present in the brains of AD patients. In their study, they also assessed

the expression of CD45 isoforms, and reported that CD45RA+ cells were rarely found in the brains of AD patients, whereas CD45RO, a marker for memory T-cells, was highly expressed on brain T-cells. In the hippocampus, the authors could detect a greater frequency of CD3+CD27- than CD3+CD27+ T-cells [151]. Further signs of T-cell differentiation in AD were reported by Pellicanó *et al.* who showed that stimulation of PBMCs from AD patients with Aβ-42 lead to an increased production of, amongst other cytokines, IFN-γ, but not IL-2 [110]. It has been reported that IL-2 expression by naïve cells is decreased as they differentiate [106]. Other studies report that *"shorter telomeres may indicate dementia status in older individuals with Down syndrome"* [67] and that *"telomere shortening in T cells correlates with Alzheimer's disease status"* [107]. Even if a longitudinal study was able to demonstrate that *"telomere length is not predictive of dementia or MCI conversion in the oldest old"* [177], the majority of published literature suggests that shorter telomere length is indicative of antigen experienced T-cells in AD patients, as telomere length is negatively correlated with T-cell differentiation [6]. A discrimination between AD-associated and CMV-associated effects on the immune system was not present in these publications. For this reason, some of the work described in this thesis aimed at discriminating between CMV+ and CMV- AD patients and healthy elderly.

1.2.3 Treg phenotypes and their association with ageing and dementia

Regulatory T-cells (Tregs) are important for immune tolerance. They control the immune system by suppressing activation, proliferation and effector functions of several immune cell types. Accordingly, they could be beneficial by reducing strong inflammatory responses in diseases such as AD. A hallmark of Tregs is their expression of the transcription factor forkhead box P3 (Foxp3) and high levels of the IL-2 receptor α-chain (CD25). Increased expression of these surface proteins is associated with greater suppressive function of Tregs and their reduced proliferative capacity. Slightly different from other T-cells, this shift from a resting to an activated effector state is accompanied by a loss of the naïve phenotype (CD45RA+) and elevated expression of CD28, CD95 and CD45RO [128]. A list of additional Treg surface marker proteins and their association with differentiation states are provided in Table 1.3. Tregs are typically produced in the thymus, but they can also derive from naïve CD4+ T-cells following stimulation of the TCR (T-cell receptor) in the presence of TGFβ, as *in vitro* experiments have demonstrated, although it has not yet been confirmed if these cells occur *in vivo* [128]. As with other T-cells, Treg phenotype changes are associated with age. Despite the presence of activated Tregs in fetal tissues, in cord blood, predominately naïve CD45RA+Foxp3low Tregs are found, while in adults and the elderly a greater frequency of effector Tregs is observed. Age associated thymic involution leads to lower production, but not a total absence of T-cells, including Tregs [128]. Naïve and differentiated Tregs are distributed differently in different tissues. The former express CXCR4 and are found mainly in the bone marrow, while CD45RO+ Tregs express skin homing chemokine receptors as CCR4. That the proportion of Tregs in the skin is increased with age might help to account for the reduced immune responses observed in the elderly [16].

In diseases such as AD, several factors may influence T-cell function and differentiation. Tregs can suppress other T-cells, but not necessarily completely. When T-cells are strongly ac-

tivated, they become resistant to Treg suppression [128]. Higher levels of T-cell activation and later differentiation phenotypes are found in CD4+ T-cells in AD patients [111]. During inflammation, Tregs secrete the pro-inflammatory cytokine IL-17. The suppressive capacity of IL-17+Foxp3+ Tregs is high when there is low TCR activation, but the greater the TCR signal, the lower the Treg efficacy [128]. One scenario of strong TCR-activation is an environment consisting of high levels of pro-inflammatory cytokines, such as in AD. Regarding the frequencies of Tregs and CD25+ activated T-cells, disparate observations have been reported. In a pilot study, CD25 expression was reported to be downregulated in T-cells of Canadian patients with mild AD [77]. In contrast, an opposite relationship was found in a Sicilian cohort of AD patients. In order to characterise CD4+ Tregs in more detail, Foxp3 and CD127 were included as Treg markers in the latter study. No differences between AD patients and controls were found with respect to CD25+CD127lowFoxp3+ CD4+ T-cells [111]. In order to assess functionality, Saresella *et al.* measured the intracellular and cell surface expression of programmed death receptor 1 (PD-1). CD4+CD25highFoxp3+ cells, especially those also PD-1-positive, were found in higher percentages in patients with MCI or severe AD, compared to healthy controls. On the other hand, PD-1-negative Tregs - those Tregs with the strongest suppressive capacity - were only increased in MCI patients. When T-cells were stimulated with Aβ in MCI patients, proliferation was reduced and Treg-mediated suppression was more efficient in comparison with AD patients and healthy controls. These investigators place their findings in the context of the systemic inflammation present in AD and suggest *"a possible beneficial role of these cells in MCI that is lost in patients with fullblown AD"* [132]. In another study, the same investigators demonstrated an impairment of the PD-L1/PD1 pathway in AD and MCI, which in turn influenced cytokine production and apoptosis of immune cells [133].

In addition to CD4+ Tregs, CD8+ regulatory T-cells are also present. These cells are characterised by the tissue specific homing receptor CD103. CD8+ Tregs specifically target activated T-cells by recognising peptides either presented by the TCR or derived from heat shock proteins. Among their functions is the ability to induce cell death, secrete immune suppressive cytokines and inhibit cell functions via cell-cell contact and signalling pathways [143]. Little is known about their presence and function in AD.

1.2.4 B-cell phenotypes

As with T-cells, B-cell phenotypes can be categorised according to the expression of certain surface proteins. One rudimentary model utilises CD27 and Immunoglobulin D (IgD). IgD is expressed in place of IgM on immature B-cells during development and its expression is associated with early stages of B-cell activation. One function of IgD is to stimulate basophils and mast cells in the defence of microbes, resulting in a pro-inflammatory environment [24]. Throughout differentiation stages the following progression of B cell phenotypes has been suggested: CD27-IgD+, CD27+IgD+, CD27+IgD- and finally CD27-IgD- may represent the most differentiated phenotype. B-cell functions are impaired in the elderly. They show a reduced response to novel antigens, but also have lower levels of memory cells (IgG+IgD-CD27-) which may be exhausted [21, 20]. A shift from early- to late-differentiated phenotypes is considered to be associated

Table 1.3: Characteristic markers of Treg differentiation according to Sakaguchi *et al.* [128]

Marker cell	Naïve T-cell	Activated T-cell	Memory T-cell	Activated converted Treg-like	Naïve Treg	Effector Treg	Terminal effector Treg
Foxp3	-	low	-	+	low	hi	hi
CD45RA	+	-	-	-	+	-	-
CD45RO	-	+	+	+	-	+	+
CD62L		+	-				
CD95	-	+		+	-		
CD127	+	-/low	+	-/+	-/low	low	-
CD25	-	hi	+	+	hi	hi	hi
HLA-DR	-	+			-	-	+
CD69		+					
CTLA4	-	+	+	+	-	+	+
ICOS		-/+		-/+		-/+	-/+
Ki67						+	+
IL-2		+					
IL-10				-/+			
IL-17				-/+			

All T-cell lineages are produced in the thymus as naïve cells and migrate to the peripheral tissues and organs. After activation they differentiate into conventional T-cells and Tregs. In addition naïve T-cells can differentiate into converted Treg-like cells with similar marker expression as conventional Tregs and possibly further to effector Tregs.

with B-cell senescence, as with T-cells. Another factor to be considered is the total number of B-cells. In addition to changes in T-cell numbers, CD19+ B-cells have been shown to be diminished in AD patients compared to age-matched controls as reviewed by Rezai-Zadeh *et al.* [123]. Thus, for these reasons and because disease-driven differences in the frequencies of T-cell sub-populations can be observed in AD patients, one aim of the work presented in this thesis was to investigate the influence of AD on B-cells.

1.2.5 Systemic inflammation in AD

As discussed at length above, the major pathological hallmark of AD is $A\beta$ accumulation forming plaques within the brain. Molecules of this type are in principle cleared by the innate immune system, but studies by Fiala *et al.* suggest that $A\beta$ phagocytosis by macrophages is deficient in AD patients. One proposed compensatory mechanism for this loss of function is further activation of macrophages and microglia and higher levels of pro-inflammatory cytokines. This inflammatory state may result in side effects such as tissue damage and neurodegeneration [38]. This inflammation is not limited to the brain, but occurs systemically, as shown by other investigators with genomic and proteomic analysis, as reviewed by Pellicanó *et al.* [110]. In addition to mono-

cytes and macrophages, other types of immune cells play an important role in AD. Rezai-Zadeh *et al.* provide an overview of this emerging field and suggest blood lymphocytes as noninvasive biomarkers and therapeutic targets [123]. The work presented in this thesis was undertaken to increase our understanding of the role of peripheral immune cells and its contribution to AD pathology in more detail by characterising immune cells in AD patients and comparing these to healthy individuals.

1.2.6 Plasma and serum cytokine levels in the context of inflammation in AD

Thus in AD an inflammatory state is typically present in the brain. This state may have both beneficial and detrimental consequences [13]. In the AD-affected brain, increased levels of several pro-inflammatory cytokines and chemokines (IL-1β, IL-6, TNF, TGF-β and IL-8) have been reported to occur in proximity damaged regions. One source of inflammation are microglia which are activated by fibrillar Aβ or by monocyte chemoattractant protein-1 (MCP-1) which is increased in association with senile plaques. These cytokines can decrease phagocytic activities of microglia which may be restored by anti-inflammatory drugs. In addition pro-inflammatory cytokines has been shown to accelerate tau pathology and NFT formation. For example, IL-1 or IL-6 can enhance kinase activity to hyperphosphorylate tau [13]. AD associated inflammation is not limited to the brain, but also occurs in the periphery. A number of studies have been performed to measure cytokine and chemokine levels in plasma and serum, several of which have been proposed as biomarkers for AD. In their critical review Lee *et al.* outline the hypothesis that peripheral molecules activate neurons, astrocytes and microglia to secrete pro-inflammatory cytokines and to enhance APP synthesis. In turn, Aβ can trigger further cytokine production and induce neurotoxicity. CSF is one source of information regarding inflammation states and Aβ levels, but given that it is not routinely collected, blood biomarkers are likely be of more of value. One of the main advantages of blood biomarkers is that blood collection is inexpensive, quick and minimally invasive. Brain proteins and metabolites that pass through the blood-brain barrier (BBB) into the plasma become *"markedly diluted into a biochemically complex medium"* [78]. Therefore, many molecules are present in concentrations so low that they are below current methods of detection. One example given in the review by Lee *et al.* is an IL-1β ELISA with a detection limit of 10 pg/ml. Many investigators nevertheless publish data using these assays, producing potentially controversial results. Multiplex systems with higher levels of sensitivity are becoming more widespread. These have the advantage of measuring several cytokines simultaneously. As indicated by Lee *et al.*, information regarding the level of multiple cytokines measured in a single assay is more likely to be trustworthy [78].

In AD, the levels of certain cytokines, such as IL-12, IL-16 and IL-18, differ according to the disease stage. Of note is that IL-18 induces Th1 cytokine production and IL-16 is a CD4+ T-cell growth factor. In patients with mild and moderate AD, elevated plasma levels of these cytokines have been observed, whereas lower levels were found in patients with severe AD. There are studies that indicate elevated TNF concentrations in the serum of AD patients. TNF, amongst others, is produced by activated microglia. Given that the BBB is altered in AD, these cytokines

might be able to enter the serum. In addition to these alterations, lower serum concentrations of neuroprotective factors have been observed in AD patients. The concentration of factors such as insulin-like growth factor is negatively correlated with TNF levels. TNF itself plays a role in neuroprotection and is implicated in apoptosis. Considering the body of literature, it is not possible to draw conclusions. The studies investigating other cytokines are also inconclusive. TGF-β is claimed to stimulate astrocytes to produce Aβ peptides, in addition to inducing Aβ clearance by microglia. Not only have discrepancies in the blood level of cytokines been reported, but also of those in the CSF [78].

1.3 CMV, dementia and the immune system

Cytomegalovirus (CMV) is a herpesvirus transmitted via bodily fluids which can remain latent within the body. The incidence of infection increases with age and varies across countries, but is strongly related to living standards. CMV acts as a stimulus to immune cells. As discussed above one possible stimulus causing AD-associated immune alterations is Aβ [110], which shares the feature of affecting the BBB with CMV - Aβ directly [73] and CMV via induction of T-cell effectors [155]. Both CMV-seropositive individuals and AD patients typically have higher percentages of late-differentiated CD4+ and CD8+ T-cells. To investigate this in more detail, both of these potential drivers of immunosenescence were analysed in the work presented in this thesis. In order to discriminate between CMV- and AD-related effects on the immune system, CMV status in healthy controls and AD patients was assessed. The hypothesis was that CMV and AD process act in concert to promote disease. Supporting this is the observation that high anti-CMV IgG titres are considered a risk factor for AD (Table 1.1 above). This is associated with an exhausted immune system which is more susceptible to other diseases. Several comorbidities in the elderly share inflammation as a feature. Considering inflammation as a driving force for disease development and progression, patients with pre-existing diseases such as those listed in Table 1.1 may be at risk for developing AD.

1.3.1 Association between CMV infection and T-cell differentiation

CMV represents a chronic stimulus for the immune system. As Pourgheysari *et al.* demonstrated, the percentage of CMV-specific CD4+ T-cells increases with age. The authors of this study describe a T-cell phenotype which they suggest is associated with immunosenescence. The authors observed higher CD57 expression concomitantly with decreased CD27 and CD28 expression in CMV+ people who were otherwise considered a healthy elderly cohort. This therefore suggested that CMV is capable of altering immune functions [115]. Further characterisation of these T-cells showed that in CMV+ subjects the frequencies of late-differentiated CD4+ cells (CD27-CD45RA- and CD27-CD45RA+) were higher than in CMV-seronegative people, in whom some investigators fail to find them at all. These CD27-CD45RA+ CD4+ T-cells produce granzyme B and perforin, two markers of T-cell-mediated cytotoxicity [82]. A correlation between the frequency of CD27-CD45RA+ T-cells and CMV infection status was demonstrated for the CD8+ T-cell subset [75]. Weinberger *et al.* discriminated between age- and CMV-related differences

in the immune system analysing T-cell phenotypes in a healthy elderly population. They reported lower frequencies of naïve (CD45RA+CD28+) and memory (CD45RA-CD28+) T-cells, while higher levels of effector (CD28-) CD4+ and CD8+ T-cells in CMV+ compared to CMV-elderly individuals were observed. Within the CMV-negative cohort, these authors observed negative correlations for the CD8+ but not the CD4+ T-cell subset, while correlations within CMV+ elderly individuals were detected in both CD4+ and CD8 T-cells. From these experiments the authors concluded that *"in the elderly different T-cell subsets compete for space within the CD8+, but not the CD4+ T-cell population"* and that differences in CD4+ T-cells in CMV+ elderly are virus induced, as they differed from the age-related alterations observed in the CMV-cohort [161]. Higher frequencies of late-differentiated CD4+ and CD8+ T-cells were seen in CMV-seronegative, but not HSV-infected, donors pointing out the special role of CMV [30].

1.3.2 Herpesviruses and AD

Age is the most significant risk factor for AD. The risk of developing AD increases with age, while CMV seropositivity is also observed to increase with age, but obviously not all CMV+ elderly individuals suffer from AD. In elderly individuals, the majority are CMV-seropositive. Amyloid plaques and neurofibrillary tangles occur not only in AD patients, but also healthy elderly individuals; but hence there still might be some connection. Some authors have postulated that the neurofibrillary tangles are an ageing marker. These tangles may be influenced by Aβ which in turns acts to accelerate the ageing process. Is CMV another modulator of ageing and AD? Are CMV+ individuals more susceptible to AD? Given the fact that CMV affects the immune system, it may contribute to inflammation that is characteristic in AD patients, potentially increasing the risk of developing AD. Given that CMV specific T-cells are present in CMV-seropositive individuals, the question is then posed; are there Aβ specific T-cells present in AD patients as well?

One of the small number of genes that has been associated with AD risk is the *ApoE (Apolipoprotein E)* gene. The ApoE protein plays an important role in lipoprotein catabolism. ApoE promotes Aβ degradation. Within the ApoE isoforms the E4 isoform is less efficient than the E3 and E2 isoforms and as a consequence is associated with greater Aβ deposition. The *E4* allele of the *AopE* gene is therefore associated with a higher risk of AD development [68, 42]. This allele of the *ApoE* gene correlates with the expression of the apoptosis-related T-cell marker CD95 [87]. Lin *et al.* have shown that when viewed in combination, the *AopE-E4* allele and the presence of herpes simplex virus 1 in the brain may be a major risk factor for developing AD. Other herpes viruses are capable of entering the brain and potentially contributing to disease. For example HSV2 increases the risk of developing a form of AD and HHV6 correlates in a small number of cases with mental retardation. Whether HHV6 is the cause or the consequence of the disease has so far not been determined [83]. In AD, the area of the brain that is most compromised by the disease is the frontotemporal region. This area is also the region that is commonly invaded by herpesviruses. Also CMV can be present in the brains of healthy and AD-affected elderly [83]. In another study, Lin *et al.* indicated that CMV, but not HSV1 or HHV6 is more prevalent in VaD patients than healthy controls [84]. In longitudinal studies, higher levels of antibodies

against CMV correlated with more rapid cognitive decline [1, 146]. But in the NONA longitudinal study performed in an elderly Swedish population, CMV seropositivity did not correlate with cognitive impairment, nor did it correlate with the inflammatory marker IL-6 (detected in plasma), although both factors separately predicted 2- 4- and 6-year mortality [168, 109].

1.4   Brain infiltration by peripheral immune cells along a chemokine gradient in AD

Immune cells must first develop in distinct tissues before they are able to execute their function. Precursor T-cells are produced in the bone marrow. Following maturation in the thymus they migrate to the periphery. At sites of development, injury or inflammation small proteins (8–14 kDa), referred to as chemoattractant cytokines, or chemokines, are secreted. Certain cell types are attracted to these proteins, and they modulate cell migration along concentration gradients. Chemokines may also play roles in cell activation, proliferation, apoptosis and cytokine secretion. They interact with G protein-coupled seven-transmembrane domain receptors. Microglia, astrocytes, PBMCs and monocytes have been reported to show increased production of chemokines after stimulation with Aβ *in vitro* [40, 119]. In one study, microglia were obtained from the brains of AD patients. Aβ levels were observed to be associated with levels of CCL2, CCL3 and CXCL8 chemokines *in vitro*. Regarding the role of chemokine receptors in AD, Lee *et al.* propose a suppressive role for Aβ by CCR5. These authors showed that mice lacking CCR5 developed Aβ deposits, increased expression of CCR2, more activated astrocytes, higher levels of β-secretase and impaired memory [78]. Another study demonstrated that Aβ accumulated earlier in CCR2-deficient AD mice than in control mice. The following mechanism was invoked to explain these results; *"Ccr2-dependent microglial accumulation plays a protective role in the early stages of Alzheimer's disease by promoting Abeta clearance"* [34]. The brain of AD patients is infiltrated by peripheral immune cells through an altered BBB. Several factors such as Aβ, tau and others affect the permeability of the BBB (see list below). With regard to the systemic inflammation observed in AD, it has been reported that brain cells such as astrocytes and microglia become activated and secrete cytokines and chemokines. When these cytokines and chemokines are secreted into the bloodstream they form a gradient which leads to the attraction of peripheral leukocytes via chemotaxis [40].

Factors which affect the BBB in AD

- The expression of adhesion molecules on endothelial cells was shown to be enhanced in inflammatory diseases, such as AD. [3, 4, 162]

- The endothelial low-density lipoprotein receptor-related protein 1 (LRP-1) and P-glycoprotein are responsible for the export of small molecules, whereas Aβ influx transport occurs via the receptor for advanced glycation end products. The expression of these markers correlated with age-related accumulation of brain Aβ. [28, 140, 141]
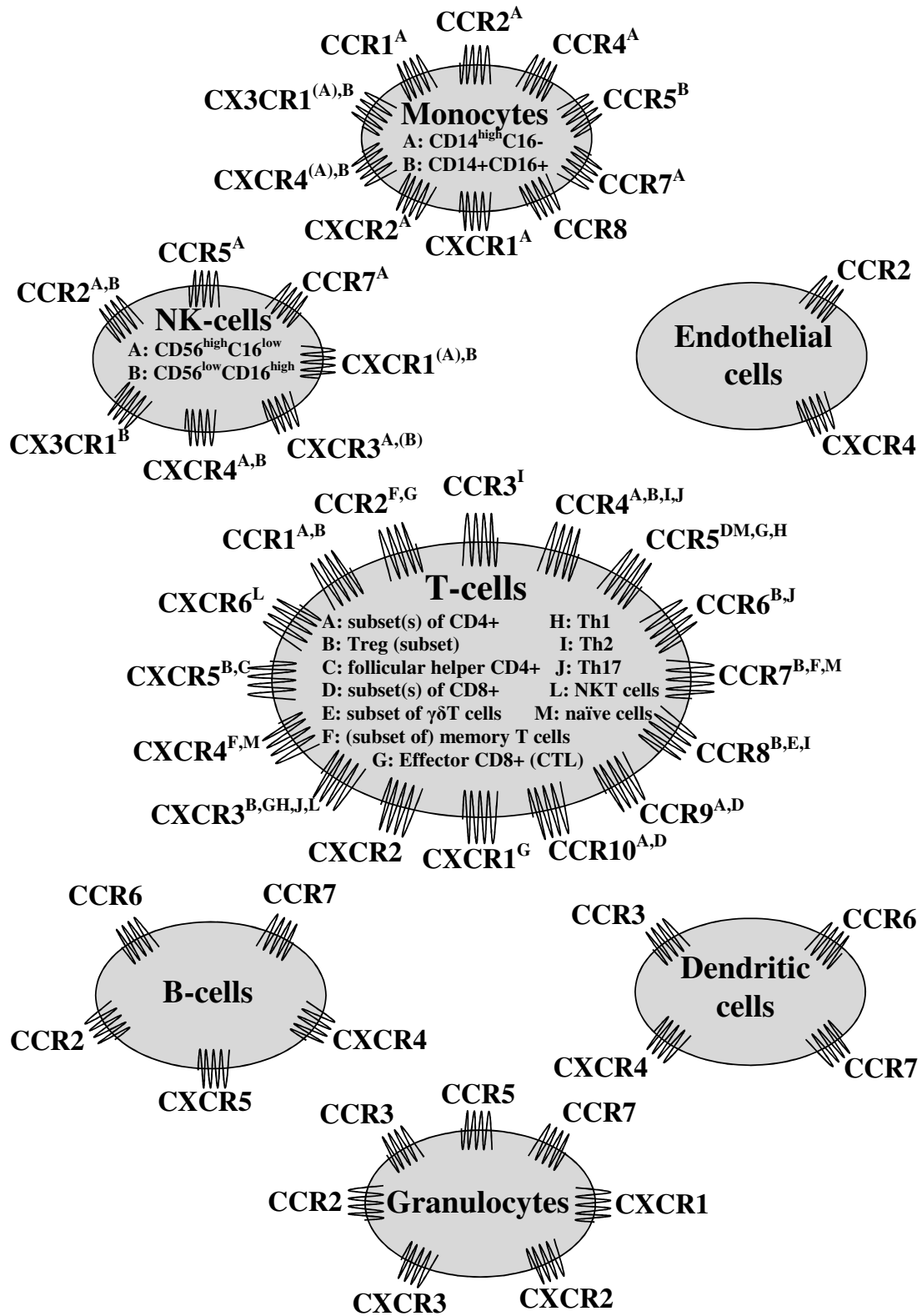
Figure 1.5: **CCR expression on different immune cell subsets**
Chemokine receptors expressed on different subsets of leukocytes and endothelial cells as currently
reported [130, 155, 129, 19, 23, 51, 52, 56, 62, 139].

- $A\beta$ led to higher expression of MIP-1α (macrophage inflammatory protein-1α) on the peripheral T-cells of AD patients. This induced the expression of CCR5 on brain endothelial cells. MIP-1α and CCR5 interactions facilitated brain infiltration of T-cells. [81, 93]

- $A\beta$ upregulated CXCR2 expression in rats, which resulted in enhanced T-cell mobility through the BBB. Inhibition of the CXCR2 ligand IL-8 in human brain microvascular endothelial cells by an anti-TNF antibody led to reduced T-cell migration. [85]

- Infarctions that are regularly found in the brains of AD patients damage the BBB. They led to a *"complication of hypoxic and ischemic episodes"*. Additionally, anatomical and functional alterations of cerebral microvessels and endothelial cells were reported. [40]

- $A\beta$ increased microvascular permeability. [73]

- In rats, basolateral treatment of the BBB with truncated tau led to a very strong polarity-dependent effect. A significant decrease of transendothelial electrical resistance and increase of endothelial permeability resulted. In addition Kovac *et al.* reported cytotoxic effects on astrocyte-microglia. In addition to tau, TNF and the chemokine MCP-1 also play a role. [73]

- *"CMV-induced effector T-cells cause endothelial cell damage."* [155]

- *"Cytomegalovirus directly enhances MHC class I and intercellular adhesion molecule-1 (ICAM-1) expression on cultured proximal tubular epithelial cells."* ICAM also plays an essential role in B-cell migration through the BBB. Although not definitively demonstrated, CMV may affect the BBB. [156, 4]

- Damage to the tight junctions in the BBB has been shown to be caused by proteases and gelatinases from neutrophils recruited by MMP-3. The activation of MMP-3 is induced by the production of IL-17 - a cytokine secreted during inflammatory states. [157, 158]

- CSF and plasma levels of the inflammatory mediator soluble CD40L (sCD40L) are elevated in cognitively impaired, HIV infected individuals and its receptor, CD40, is expressed on brain endothelial cells. The HIV transactivator of transcription (Tat) can induce activation of platelets which secrete sCD40L. CD40-CD40L interactions led to increased inflammation and production of adhesion molecules in endothelial cells. In addition levels of reactive oxygen species were enhanced, which promoted chemokine secretion. sCD40L decreases ATP levels - required for maintenance of tight junction permeability in the BBB. In mice, Tat has also been shown to be associated with increases in inflammatory monocyte subsets with adherent potential to BBB cells. These results were supported by migration experiments using an *in vitro* BBB model. [27, 118]

1.5 Rationale of the present study

The brain of AD patients is infiltrated by immune cells [151]. T-cells and other immune cell types are able to migrate into the brain of AD patients possibly due to alterations in the BBB associated with AD pathophysiology. Under normal physiological conditions, an intact BBB regulates the transport of many molecules and cell types present in the blood. In typical inflammatory diseases, permeability of the BBB increases and lymphocytes migrate into the CNS and inflammation-associated molecules reach the periphery. Because not only the brain but also the periphery is affected in AD, the inflammation is considered to be systemic. Peripheral immune cells can contribute to brain inflammation, thus changes in the periphery may have a significant impact on the brain. The root cause of the systemic inflammation observed in AD is not yet fully understood. Persistent CMV infection has a large impact on the immune system. Therefore this study separately considered impacts that both CMV and AD may have on the immune system. It was important to determine if CMV+ individuals are under additional antigenic load and thus have more stressed immune systems, thereby potentially making them more susceptible to dementia. To investigate this, immune signatures were compared in a cohort of CMV- and CMV+ healthy individuals and additionally CMV- and CMV+ AD patients.

In order to investigate the systemic immunopathology of AD, this study examined several leukocyte subsets, including T-cell populations, B-cells and monocytes found in PBMCs isolated from the blood of AD patients and healthy age-matched controls. This study was performed in three phases. In phase one, the differentiation stages of leukocyte populations were investigated by analysing the expression of surface marker proteins. For this analysis, the CMV status of the individual was considered, since it has a significant impact on the immune system. In phase two, the activation state of the aforementioned leukocyte subsets was examined by measuring the expression of HLA-DR, CD25, CD69 along with other markers. In phase three, the cytokine secretion of T cells in response to Aβ and/or PMA-Ionomycin was measured using functional assays. Not only cytokines but also chemokines play an important role in inflammation and leukocyte migration. Therefore the expression of several chemokine receptors (CCR2, CCR4, CCR5 and CCR6) on leukocytes was also studied.

The aim of this study was to identify immune signatures that are characteristic of AD, and which therefore might qualify as biomarkers or as novel therapeutic targets. It has been speculated that the specific interactions between various immune cells types may be beneficial during the early stages of AD, but harmful in advanced stages of disease. This idea is supported in several experimental settings [41, 34, 153, 176]. In addition, CMV infection should be considered in the context of the immunopathology of AD as it influences brain infiltration of T-cells [155]. The results of the present study suggest new therapeutic targets. These are based on biomarkers that are more highly expressed by peripheral blood cells from AD patients than from controls. Town *et al.* reviewed the role of T-cells in AD and highlighted the beneficial role that Th2 T-cells may play in murine AD models. In AD patients, one reason why T-cells fail to exert similar beneficial effects to limit disease progression may be due to the high NO production surrounding amyloid plaques, which may result in T-cell apoptosis [154]. Therefore, promoting

chemotaxis to result in increased brain infiltration by anti-inflammatory peripheral T-cells may help curb disease progression. On the other hand, decreasing migration of inflammatory T-cells with antibodies directed against certain chemokines or their receptors such as CCR6 could reduce the inflammation present in AD. But before therapeutic advances along these lines can be made, it is important to first investigate the chemokine receptor expression in AD patients. It is also of importance to assess the frequencies of leukocyte subsets and differentiation stages in AD patients compared with healthy controls. For these reasons, this is study aimed to investigate the immune profiles associated with different disease stages in AD and other forms of dementia.

Chapter 2

Materials and Methods

2.1   Reagents

2.1.1   Buffers and reagents

Table 2.1: List of buffers and reagents

abbreviation	reagent	company	catalogue number
Aβ1-42	[Asp22]-beta-Amyloid (1-42); E22D beta-Amyloid (1-42)	Mobitec, AnaSpec	65217-05AS
Ampuwa	Ampuwa	Fresenius Kabi	0571041/00
	Compbeads Negative Control (FBS)	BD	51-90-9001291
	Compbeads Anti-Mouse	BD	51-90-9001229
	Compbeads Anti-Rat	BD	51-90-9001189
	Compbeads Anti-Rat and Anti-Hamster	BD	51-90-9000949
cytofix cytoperm	Cytofix cytoperm	BD	554714
CS&T	Cytometer setup & tracking beads	BD	642412
DMSO	Dimethylsulfoxide, research grade	Serva	20385
EDTA	Ethylenediaminetetraacetic acid	Serva	11278
EDTA	Ethylenediaminetetraacetic acid	Sigma	E-1644
FBS	Fetal bovine Serum	Sigma	F0804
FicoLite	FicoLite-H (human)	Linaris	GTF1511YK
fix/ perm	Foxp3 fix /perm buffer set	BioLegend	421403
gamunex	Gamunex (human IgG)	Talecris	
Golgi Plug	Golgi Plug (Brefeldin A)	BD	555029
HBSS	Ca2+Mg2+ free Hank's BSS	PAA	H15-009
ionomycin	Ionomycin Calcium Salt	Calbiochem	407952
mouse serum	Mouse serum	Milipore	S25-10ML
PBS	Phosphate buffered saline	PAA	H15-002
PMA	Phorbol 12-myristate 13-acetate	Sigma	P-8139
RPMI	RPMI1640 + GlutamaxTM-I + 25 mM HEPES	Gibco (Invitrogen)	72400-054
NaN$_3$	Sodiumazide	Sigma	S-8032
XVIVO-15	XVIVO-15	BioWhittaker/ Lonza/ Cambrex	EO4-418Q

2.1.2 Antibodies

Figure 2.1: **Antibodies used**
Antibody panels employed. For each antibody the conjugated fluorochrome and the company from which it was purchased is given.

2.1.3 Kits

anti-CMV IgG kit: Genesis Diagnostics CMVIgG ELISA kit GD84
cytokine kit: BD Cytometric Bead Array (CBA) human Th1/Th2 cytokine kit

2.1.4 Instruments

Plate reader for ELISA measurements: Dynex Technologies MRX Revelation Microplate reader
Flow cytometer: 14 channel three laser BD LSRII (violet, blue and red lasers). The configuration according to Table 2.1 columns 1 and 2 was used.
Incubator for cells: Hera Cell 150, Heraeus

2.2 Sample collection and processing

PBMCs are typically isolated from fresh blood before undergoing experimental analysis, but some samples in this study were only available in the form of frozen blood. This therefore required the development of a protocol to allow for this. The final product of this protocol has been validated and published in Journal of Immunoassay & Immunochemistry [2]. This method has several advantages. Blood is more likely to better represent the *in vivo* conditions compared to isolated PBMCs. For the laboratory performing the blood collection, no special equipment is required as in the case for PBMCs that require isolation. While long term storage of isolated PBMCs is not possible at -80 °C, it is possible to obtain viable cells when whole blood is stored at this temperature, liquid nitrogen storage is not necessary. This is particularly advantageous in case of field studies. The following section provides an overview of this protocol. Isolated PBMCs were obtained from one source. These samples were processed following subsequently described standard protocols.

2.2.1 Collaborators who provided samples

- PBMCs from healthy young and elderly and patients with mild or moderate AD: Prof. Dr. Calogero Caruso and Dr. Guiseppina Colonna-Romano at the Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy

- Frozen whole blood from healthy elderly patients suffering from VaD, AD or mixed dementia: Prof. Dr. Roberto Paganelli, Università degli Studi G. d'Annunzio Chieti e Pescara, Dipartimento di Medicina e Scienze dell'Invecchiamento, Chieti, Italy

- PBMCs from healthy elderly and AD patients at different stages: Prof. Dr. Tamas Fulop of the Immunology Program, Geriatric Division, Faculty of Medicine, Research Center on

Aging, University of Sherbrooke, Sherbrooke, Quebec, Canada

- Frozen whole blood from AD and rpAD patients: Dr. Christian Schmidt and Prof. Dr. Inga Zerr, Clinical Dementia Center, Dept. of Neurology, University Medical School, Georg August University Göttingen, Germany

- Fresh whole blood from healthy participants of the Berlin Ageing Study BASE-II: Prof. Dr. Elisabeth Steinhagen-Thiessen, Charité–Universitätsmedizin Berlin, Germany

- Buffy coats from healthy blood donors: Prof. Dr. Dorothee Wernet, Zentrum für Klinische Transfusionsmedizin, Tübingen, Germany

2.2.2 PBMC isolation, freezing and storage

According to a standard laboratory protocol, fresh whole blood was diluted 1:2 with HBSS (phosphate buffer) and carefully layered over Ficoll-Hypaque. After centrifugation for 30 minutes (810 g, no brake, room temperature) a gradient containing separated PBMCs was formed. Mononuclear cells (PBMCs) were removed from the interphase and washed three times with HBSS, before undergoing centrifugation for ten minutes (300 g, room temperature). After the third washing step, cell numbers were determined using a Neubauer Chamber and the required volumes of freezing solutions determined. Centrifuged cells are resuspended carefully in 40% (v/v) FBS dissolved in RPMI at room temperature. Following this, half the volume of 20% (v/v) DMSO in RPMI was added and the solution was carefully mixed. After 5 min the remaining volume of DMSO was added. The samples were then aliquoted in pre-prepared vials at room temperature. The vials were then immediately stored at -80 °C overnight. Vials were transferred to liquid nitrogen storage the following day as they require temperatures below -80 °C for optimal long term storage. Samples were stored on dry ice during shipping.

2.2.3 Frozen whole blood storage

For the storage of whole blood, ten percent (v/v) DMSO was added before being stored at -80°C. In the case of the samples obtained from Dr. Schmidt and Prof. Zerr in Göttingen, samples were kept at room temperature for 24 h before the addition of DMSO and subsequent storage at -80°C.

2.2.4 Preparation of samples for flow cytometric analysis

Thawing of frozen blood samples

Frozen whole blood was thawed in a 37 °C waterbath until the majority of ice clumps had disappeared. The samples were then transferred to a 50 ml centrifuge tube, diluted 1:25 with washing

buffer (PBS, 2 mM EDTA, 2% (v/v) FBS) in order to reduce the toxic effects of DMSO and centrifuged for 5 min at 300 g.

Thawing of PBMCs

Vials were taken from liquid nitrogen storage and placed directly into a +37 °C waterbath. Vials were gently shaken until all of the larger ice clumps had disappeared. The samples were then immediately transferred to a fresh tube with an equal volume of 4 °C RPMI. After gently shaking for 5 min another equal volume of RPMI was added. The tubes were shaken gently before being centrifuged (5 min, 300 g). The cell pellet was resuspended in medium / buffer and the number of cells determined with the use of a Neubauer chamber. Cells were divided according to experimental requirements and centrifuged. Following centrifugation, the supernatant was discarded and the appropriate volume medium / buffer added. The experiments performed with the PBMCs from Canada are listed in Table 2.2.

2.2.5 Experimental design

Different experiments were performed for each sample cohort. T-cell phenotypes were analysed for all sample cohorts, while Treg, leukocyte subsets and functional assays were analysed in selected cohorts. For these experiments, it is important to consider that some samples consisted of whole frozen blood, while others were separated PBMCs. An overview of the studies performed can be seen in Table 2.2. The samples obtained from Dr. Schmidt and Prof. Zerr at Göttingen were taken at 2 time points. These samples were shipped and analysed separately. To ensure consistency of results, PBMCs from one control donor were stained with the same panel of antibodies for each experiment and compared. Blood samples of healthy elderly individuals from Berlin were additionally included as controls.

Table 2.2: Overview of the sample cohorts and the analysis performed for each[1]

panel	Chieti	Göttingen 1[2]	Göttingen 2[2]	Berlin[3]	Palermo	Canada1[4]	Canada2[4]
T-cells	2 ml	2 ml	2 ml	1 ml	PBMC[5]	PBMC	PBMC
Tregs/CCR	2 ml	-	1 ml	1 ml	PBMC[5]	-	PBMC
Leukocytes	2 ml	2 ml	2 ml	1 ml	-	-	-
Aβ uptake	2 x 2 ml	2 x 2 ml	-	-	-	-	-
Cytokines	-	-	-	-	PBMC	-	PBMC

ml indicates the volume of blood used for each experiment. Depending on availability, separated PBMCs were used for the samples from Palermo and Canada. Pilot tests indicate that the volume of blood used does not affect experimental results [2].

[1] The details of each panel are listed in Table 2.1.

[2] Göttingen 1 includes the first batch of 20 samples, Göttingen 2 the second with 30 samples, including follow-up samples.

[3] The Berlin controls were processed in parallel to Göttingen 2 ones. Out of 20, ten controls per panel were used.

[4] From Canada 2 independent cohorts were recruited.

[5] T-cell and Treg analysis was performed together with one panel.

2.2.6 Staining cell surface proteins and intracellular Foxp3 with fluorochrome-conjugated antibodies for flow cytometric analysis

Cells were first equilibrated in PFEA staining buffer (PBS, 2% (v/v) FBS, 2 mM EDTA and 0.01% (w/v) Na azide). Experiments were performed on ice (PBMCs) or room temperature (blood). After centrifugation for 5 min at 300 g, the cell pellet was resuspended in 50 μl of 1% Gamunex $^{\circledR}$ solution (100 μl of 10% Gamunex into 900 μl of PFEA) in order to block Fc receptors and promiscuously binding proteins. Optionally, 1 μg EMA for the discrimination between living and dead cells was included. After a 10 min incubation (plus an additional 10 min under light when EMA was used) 1 ml PFEA was added in order to wash the Gamunex solution from the cells. The following step was optionally used in the case indirect stainings with non-conjugated antibodies. Cells were stained for 20 min with primary antibody, washed with 1 ml PFEA, stained for 20 min with secondary antibody and washed again with one ml PFEA. The final step involved blocking with 50 μl 10% serum derived from the animal that the primary antibody was raised in (mouse in the case of this study). Cells were washed with 1 ml PFEA and centrifuged (300 g, 5 min) before the mixture of conjugated antibodies was applied to the samples. Samples were protected from light once the fluorochrome-labelled antibodies were applied. The total volume of each antibody incubation solution was adjusted to 50 μl.

Parallel to the above procedure, compensation controls were made for each antibody in order to to allow the flow cytometer to adjust for spectral overlap of the fluorochromes used in each experiment. For each antibody separately, an equal volume as used in experiments was incubated with 10 μl mouse or rat compensation beads, depending on the animal that the antibodies were raised in. Each sample also contains unstained beads. When discrimination between living and dead cells was required (for example with EMA or Vivid staining), no compensation was performed as only the living cells (Vivid or EMA negative) were gated and used for analysis. Samples and controls were incubated for 20 min (30 min with gentle agitation for blood samples) in the dark. During this time the flow cytometer was switched on in order to prewarm the lasers. Following the incubation, samples were washed with one ml PFEA and centrifuged for 5 min at 300 g. Cells were resuspended in 100-200 μl (200-300 μl for blood samples) of PFEA and measured with the flow cytometer.

In the case of analysing Foxp3 expression, it was possible to detect this intracellularly expressed protein following antibody incubation for cell surface proteins. To achieve this, 0.5 ml 1x Foxp3 Fix/Perm solution was applied to samples, vortexed and incubated for 20 min at room temperature in the dark. Following washing with 1 ml PFEA cells were centrifuged (300 g, 5 min) and washed with one ml of 1x Foxp3 Perm buffer for 15 min in the dark. After centrifugation (300 g, 5 min) cells were resuspended in 100 μl of 1x Foxp3 perm buffer along with a Foxp3 antibody. Additional antibodies recognising other intracellularly expressed proteins were included depending on the experiment. The antibodies were incubated for 30 min at room temperature in the dark. Samples were then washed with 1 ml PFEA, centrifuged (300 g, 5 min), and resuspended in 100-200 μl PFEA (200-300 μl for blood samples) before being measured on a flow cytometer.

2.2.7 Intracellular cytokine staining

PBMCs were thawed according to the procedure described in Section 2.2.4 and a maximum of $1x10^6$ cells were suspended in X-VIVO-15 cell culture medium. For each experiment, the same PBMC donor from the local blood bank was used as a control. The PBMC samples from each donor were divided into three conditions: untreated, PMA (50 ng/ml) and Ionomycin (750 ng/ml) treated, and amyloid beta (1 μg/ml) treated. For each condition, 1 μl Golgi Plug was included. After 17 h of incubation (37 °C, 5% CO2, 95% humidity) cells were collected by washing the wells of the culture plates with PFEA. After centrifugation (300 g, 5 min) cells are incubated with 50 μl 1% Gamunex to block non-specific antibody binding sites and with a live-dead discrimination marker (1 μg EMA under strong light). Following this, cells were washed with PFEA and fixed with 250 μl BD cytofix/cytoperm for 15 min. After washing with 1 ml perm/wash, cells were incubated for 15 min with perm/wash. Following this, 50 μl of the antibody mastermix diluted in perm/wash was added to the centrifuged pellet and incubated for 30 min. After the final washing step with PFEA, 100-200 μl of PFEA was added and samples measured with the flow cytometer. Compensation controls were prepared as described in Section 2.2.6 and measured before samples were analysed.

2.2.8 Testing PBMC uptake of amyloid beta

Two times 2 ml blood from each donor were thawed and washed as described in Section 2.2.4. Following, two 1 ml aliquots of the cell samples (suspended in XVIVO-15 medium) were plated on a 24 well plate. To 1 well 10 μl of 1:10 diluted of fluorochrome-labelled Aβ was added (0.1 mg + 50 μl buffer -> 10 μl + 190 μl PBS -> 1 μg/10 μl -> 10 μl for 1 ml to achieve 1 μg/ml). In parallel, $1x10^6$ PBMCs (suspended in 1 ml XVIVO-15) from a local blood bank donor was used as internal control. In addition, 1 sample of cells incubated with Aβ was used for compensation. Incubation was performed for 16 h (37 °C, 5% CO2). Following the incubation period, cells were harvested by washing the wells thoroughly with PFEA in order to detach adherent cells, particularly the monocytes. After 10 min of blocking with Gamunex at room temperature samples were washed with 1 ml PFEA buffer and centrifuged for 5 min at 300 g. Then cells were stained with fluorochrome-conjugated antibodies for 30 min at RT (or on ice in case of the blood bank donor). Finally, the cells were washed with 1 ml PFEA and resuspended in 200-300 μl PFEA before being measured with the flow cytometer.

2.3 Testing CMV serostatus

The CMV serostatus of the donors from Canada was established by collaborators at Sherbrooke University in Canada. The Chieti and Göttingen rpAD-study plasma samples were analysed in-house with an anti-IgG-CMV ELISA kit (Genesis Diagnostics CMV IgG ELISA kit GD84), according to the manufacturer's instructions. In case of the rpAD study, plasma was obtained by centrifugation frozen-thawed blood for 10 min at 1000 g. Given that elderly patients commonly possess high anti-CMV IgG titres, the Chieti samples were prediluted 1:10 in PBS. According

To the level of anti-CMV IgG titre, samples were categorized in CMV accordingly; < 3 U/ml < low < 5 U/ml < high.

2.4 Flow cytometry

The analysis of flow cytometry data was performed with either FlowJo version 7.2.5 or FACS-DIVA 6.1. The FlowJo gating strategy for the analysis of T-cells from the Chieti samples is shown in Figure 2.3, and an example of the leukocyte analysis from a sample from Göttingen in Figure 2.2. For each sample the cell numbers were not controlled and therefore the number of cells recorded by the flow cytometer varied. This was because of the difficulty in determining cell numbers with microscopy for whole blood samples due to the interference of debris and erythrocytes. Gated subsets of cells from donors where the parent population contained less than 50 events were excluded from the analysis. For example, if a donor contained 1,000 CD3+ events, the frequency of CD4+ and CD8+ cells was examined, but CD4+ subsets were only assessed if at least 50 CD4+ events were accumulated.

For the assessment of Aβ uptake by leukocytes, the mean fluorescence of HiLyte Fluor^{TM} 488 conjugated to Aβ was measured. To calculate the degree of uptake, the fold difference in fluorescence was calculated by dividing the mean fluorescence of the Aβ-treated samples by the mean fluorescence of the untreated samples. The mean fluorescence values were taken from CD45+ leukocytes after gating for leukocyte subsets

2.5 Statistics

All statistical analysis were performed with Graphpad Prism 4.02 (2004) or JMP 8.0 (SAS Institute, 2008). Non-parametric Mann-Whitney U test was used for the comparison of independent groups.

2.6 Samples

Parameters for patients and controls analysed in these studies are summarized in the following tables. All patients included in this analysis as AD patients required a diagnosis of probable AD consistent with the DSM-IV as set out by the American Psychiatric Association in 1994. In the case of the Göttingen patients, the Dubois criteria for research [32] were also considered. For the Canadian donors the criteria of the National Institute of Neurological and Communicative disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) were included, while for the Chieti study participants the Hachinski score was used to discriminate between AD, mixed dementia and vascular dementia. A CT or MRI scan was required to corroborate the AD diagnosis. Major Stroke or massive vascular lesions on imaging were exclusion criteria. All patients gave their written informed consent. All healthy controls and AD patients underwent the Folstein Mini-Mental State Examination test. All other dementias, neurological and psychiatric disorders were excluded.
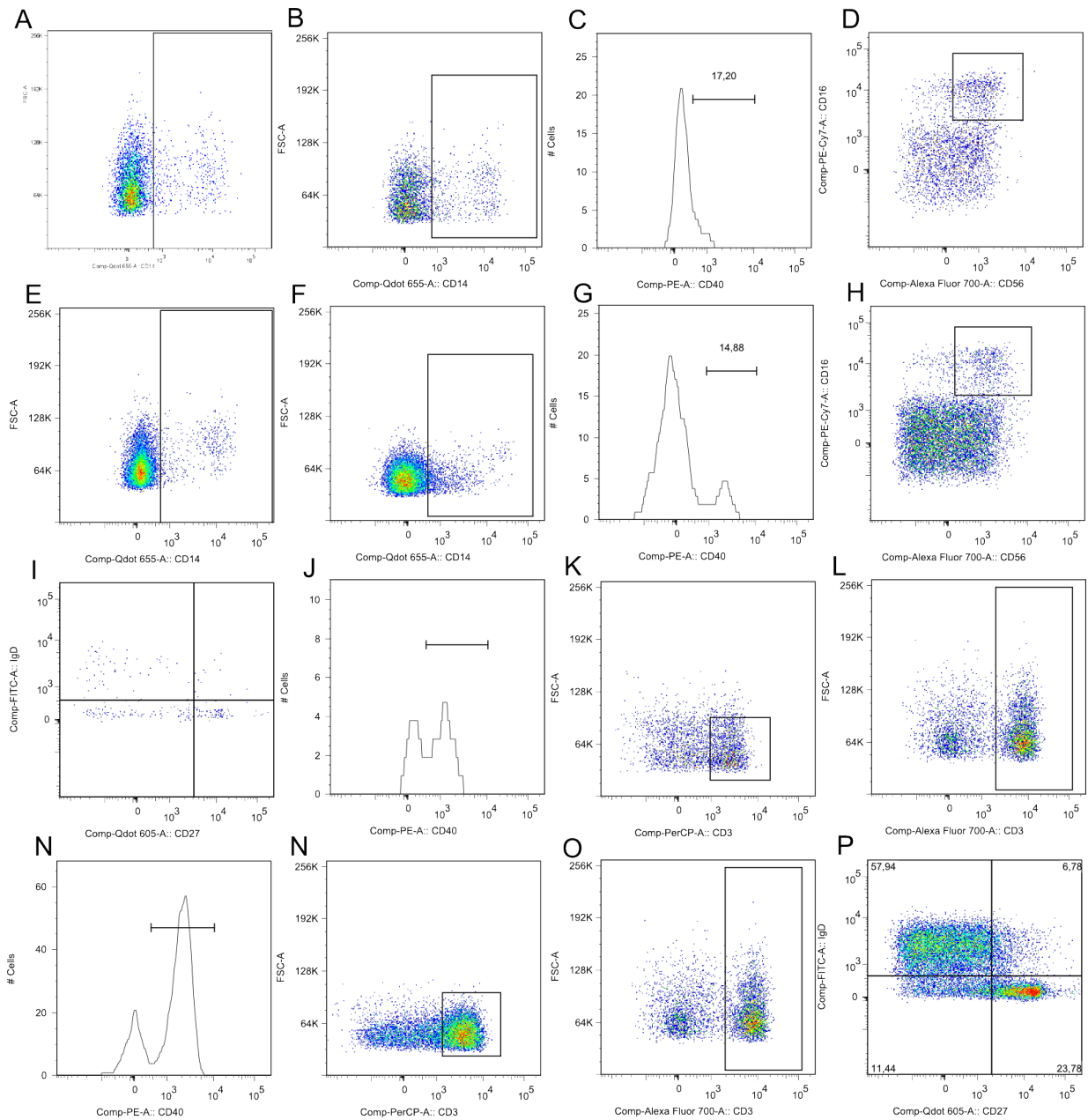
Figure 2.2: **Gating strategies for the leukocyte panel**
FlowJo images of the leukocyte analysis of the rpAD study. Representative examples were chosen from one AD patient (first and third row) and one healthy control. A and E show the gating strategy for monocytes (CD14+). B and F show parallel results of the CCR-panel analysis. In C and G the CD40 expression on monocytes can be seen. In D and H CD16 was plotted against CD56 for a NK-cell identification. I and P - with IgD plotted against CD27 expression - are examples of B-cell phenotypes. J and M show CD40 expression of CD19+ cells. K and L and N and O show the selection of CD3+ cells for the leukocyte and the CCR analysis.
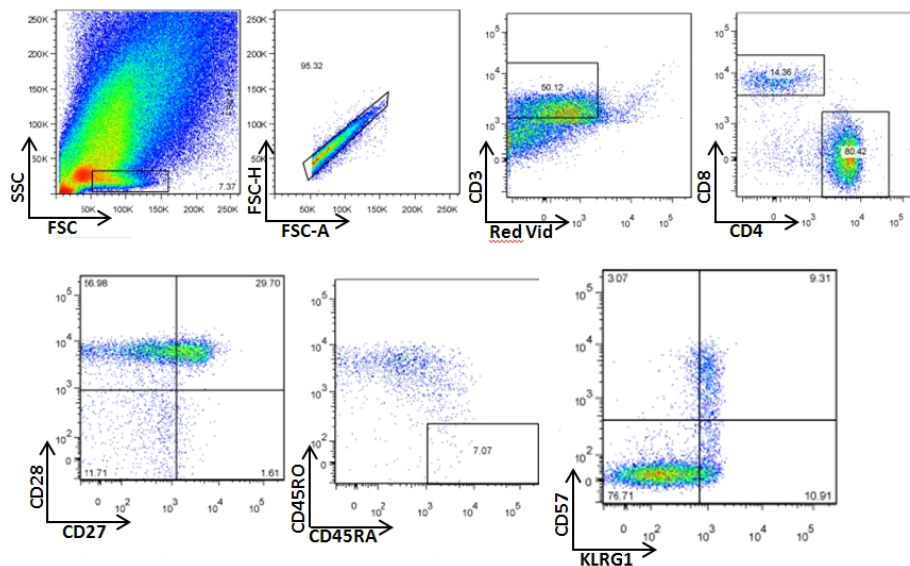
Figure 2.3: **Example of the gating strategy used for T-cell subsets with FlowJo software**
Populations and their subsets were selected in the following order: lymphocytes according to size and granularity -> singlets -> living (Red Vid-) CD3+ -> CD4+ (and CD8+) -> differentiation states, analysed with several markers in parallel, here shown for CD4+ T-cells. CD27 and CD28 characterise early- and late-differentiated cells. Naïve cells were considered to be CD27+CD28+ as well as CD45RA+CD45RO-. In the last image CD57 and KLRG1 expression is displayed.

Table 2.3: Clinical parameters of 2nd cohort of Canadian AD patients and healthy elderly individuals (Table 2.2)

	Healthy	mild AD	moderate AD	severe AD
no. of donors	9	10	10	10
with CRP > than 3	0	4	2	3
CMV status	$4-/5+$	$2-/8+$	$2-/8+$	$5-/5+$
MMSE	30	$24-29$	$16-26$	< 17
mean cortisol	513 ± 172	927 ± 1430	441 ± 164	466 ± 123
total cholesterol	5.55 ± 0.85	4.80 ± 1.12	4.84 ± 1.30	4.54 ± 1.24
mean triglycerides	1.10 ± 0.50	1.73 ± 1.18	1.65 ± 0.72	1.22 ± 0.45
mean HDL	1.83 ± 0.60	1.16 ± 0.31	1.20 ± 0.22	1.29 ± 0.37
mean LDL	3.23 ± 0.46	2.85 ± 0.85	2.89 ± 1.13	2.69 ± 1.17
mean GB	5.3 ± 1.1	6.6 ± 1.7	6.7 ± 1.9	5.8 ± 1.8
mean hemoglobin	138 ± 11	130 ± 16	120 ± 10	128 ± 10

CRP: C-reactive protein, HDL: high density lipoprotein cholesterol, LDL: low density lipoprotein cholesterol.

Table 2.4: Clinical data of the Chieti study participants

	Healthy	*AD*	*VaD*	*mixed dementia*
no. of donors	17	11	6	6
gender	$7F/10M$	$6F/5M$	$4F/2M$	$3F/3M$
mean age	82 ± 6	81 ± 3	85 ± 5	87 ± 4
MMSE	28.3 ± 1.2	8.8 ± 5.9	8.4 ± 6.0	6.8 ± 4.8
Hachinski ischemic score	0	$2 - 4$	$5 - 8$	$4 - 9$
mean CC	216 ± 159	166 ± 148	209 ± 165	168 ± 106
mean wbc	7.35 ± 3.45	8.62 ± 3.59	8.72 ± 1.95	9.01 ± 2.02
mean rbc	3.90 ± 0.65	4.44 ± 0.41	4.32 ± 0.33	4.07 ± 0.56
mean cholesterol	157 ± 24	158 ± 26	174 ± 20	158 ± 23
mean triglycerids	139 ± 68	102 ± 34	113 ± 26	100 ± 19
mean ves	42 ± 31	39 ± 25	33 ± 25	60 ± 3
mean hb	11.6 ± 1.6	12.5 ± 1.2	11.9 ± 0.5	12.0 ± 1.8
mean total protein	6.1 ± 0.7	6.8 ± 0.8	6.3 ± 0.8	6.4 ± 0.8
tc atrophy[1]	1/17	11/11	0/6	6/6
tc vas	3/17	3/11	6/6	6/6
CMV serostatus	16/17+	11/11+	6/6+	6/6+

wbc = white blood cells, rbc = red blood cells, hb = hemoglobin, tc = testicular

Table 2.5: Clinical parameters of healthy controls and AD patients from Göttingen

	Healthy	*MCI*	*AD*	*rpAD*	*other*[1]
no. of participants	20	3	23	7	11
thereof follow-up	0	0	5	0	1
mean age	68 ± 3	83 ± 5	69 ± 9	69 ± 5	67 ± 11
gender		$1F/2M$	$12F/11M$	$5F/2M$	$3F/8M$
CMV range		26-27	0-27	4-27	15-29
mean MMSE		26 ± 1	19 ± 8	17 ± 8	24 ± 4
mean Aβ level[2]	*n.d.*[4]	390	673 ± 462	336 ± 104	464 ± 230
mean tau level[2]	*n.d.*[4]	144	425 ± 473	585 ± 352	231 ± 188
anti-CMV IgG titre[3]					
negative	8	0	6	3	7
low positive	3	1	7	1	1
high positive	8	2	9	3	3

[1] VaD, mixed dementia, Parkinson's disease, Pseudodementia, possible AD
[2] values only for 20 participants available
[3] The donors were grouped according their anti-CMV IgG titre: negative < 3 U/ml < low < 5 U/ml < high; 1 AD and 1 healthy excluded
[4] n.d. = not determined

Chapter 3

Results

AD is a difficult to diagnose disease that affects people worldwide. For this study, it was hypothesised that identifying immune biomarkers associated with AD may; 1) Assist in the diagnosis of AD 2) Allow the identification of people at risk of progressing to AD prior to manifestation of clinical symptoms. This study therefore assessed the immune profiles of patients with different disease severity from different countries (Italy, Germany and Canada) with the goal of identifying robust biomarkers found in all AD patients. Given that AD is associated with systemic inflammation and a compromised blood-brain barrier (which may also be associated with pre-AD states), blood leukocytes were investigated because they may be potential markers for CNS events in situ. The primary objective was to determine the phenotype of peripheral immune cells. The secondary goal was to investigate the functionality of these cells. Because Aβ is a key player in AD pathology with well-established neurodegenerative effects, the capacity of leukocytes to take up Aβ was investigated as this may be a mechanism of Aβ clearance in the brain. In addition, PBMCs were stimulated with Aβ peptides to assess the presence of Aβ-reactive T-cells. Due to sample volume limitations, not all of these experiments could be performed for every cohort. The analysis performed for each sample is given in Table 2.2.

3.1   Characterisation of T-cell phenotypes in patients with mild and moderate AD

3.1.1   Mild AD is associated with dramatic shifts in circulating CD4 but not CD8 T-cell subsets relative to age-matched controls

For the initial study, a well characterised cohort of mild AD patients from Canada were analysed according to the Sallusto model for T-cell differentiation outlined in the introduction (Fig. 1.4). The results of this analysis indicated that lower percentages of CD4+CCR7+CD45RA+ T-cells were observed in the elderly compared with young individuals, while the frequency of these cells was even lower in age-matched patients with mild AD. Parallel to this, the percentages of late-differentiated CD4+CCR7-CD45RA+ T-cells were observed to be higher in the PBMCs of AD patients compared to the controls (Fig. 3.1). These differences were limited to the CD4+ subset -

differences between patients and controls were not observed in CD8+ cells. This was most likely because although CD8+ naïve cells may be affected in AD patients given that the age-matched controls already displayed very low frequencies of naïve T-cells it was unlikely that additional effects exerted by AD could have been observed (Fig. 3.2). This is more clearly seen in the following analyses. In addition to this CCR7/CD45RA model, the expression of costimulatory molecule CD28 was also analysed. Anti CD28-Alexa700 mean fluorescence values of CD4+ and CD8+ T-cells were significantly lower in AD patients compared to controls. In addition, percentages of CD28+ T-cells were lower in AD patients as well. These differences between AD patients and age-matched controls were observed within the total CD4+ and CD8+ population as well as in differentiation stages. The strongest effect on CD28 expression was observed in the late-differentiated TEMRA (CCR7-CD45RA+) cells [77].

This pilot investigation also indicated that lower percentages of CD4+ CD25high T-cells (a phenotype consistent with cells that are acutely activated) are present in AD patients, by comparison with healthy controls. This analysis once again indicated that a shift from naïve to late-differentiated T-cells is associated with ageing and disease. In this case, the percentages of CD8+CD25+ T-cells was also lower in AD patients compared to controls [77].

3.1.2 Frequency of naïve, late-differentiated and regulatory T-cells in the peripheral blood of patients with mild and moderate AD

In a second study, a larger cohort of patients was analysed for the frequency of T-cell differentiation stages in the peripheral blood. For this analysis, Sicilian patients with mild or moderate AD were tested, in addition to the previously analysed Canadian cohort with mild AD. Initially, the expression of CD27 and CD28 was investigated. As described in Section 1.2.1, early-differentiated cells are double positive for these markers, while late-differentiated cells are considered to be double negative. To clearly identify the naïve cells, CD45RA and CD45RO were chosen as additional markers. Naïve cells were identified as having the phenotype CD27+CD28+CD45RA+ CD45RO- and late differentiated memory cells as CD27-CD28-CD45RA+CD45RO+. The frequency of naïve cells was observed to be lower in AD patients compared to controls while the frequency of late differentiated T-cells was higher in this group. No differences between patients with mild AD and those with moderate AD were detected, suggesting that these phenotypes would not be useful as markers of progression. Henceforth these patients were considered as a single group (Fig. 3.3). These results confirm previous observations that differences in the frequency of T-cell differentiation stages are limited to the CD4+ subset [111]. This contrasts with a comparison between young and elderly individuals in which the predominant differences in T-cell frequencies occur in the CD8+ subset T-cell populations.

To more thoroughly investigate these observations additional markers of T-cell differentiation were studied. These results indicated that the percentages of CD57+ and KLRG1+ CD4+ and CD8+ cells were higher in the elderly compared to the young. For CD57, no differences between healthy elderly and age-matched AD patients were observed. In the case of KLRG1, there was a trend towards higher frequencies of CD8+KLRG1+ T-cells in AD patients, which was statisti-
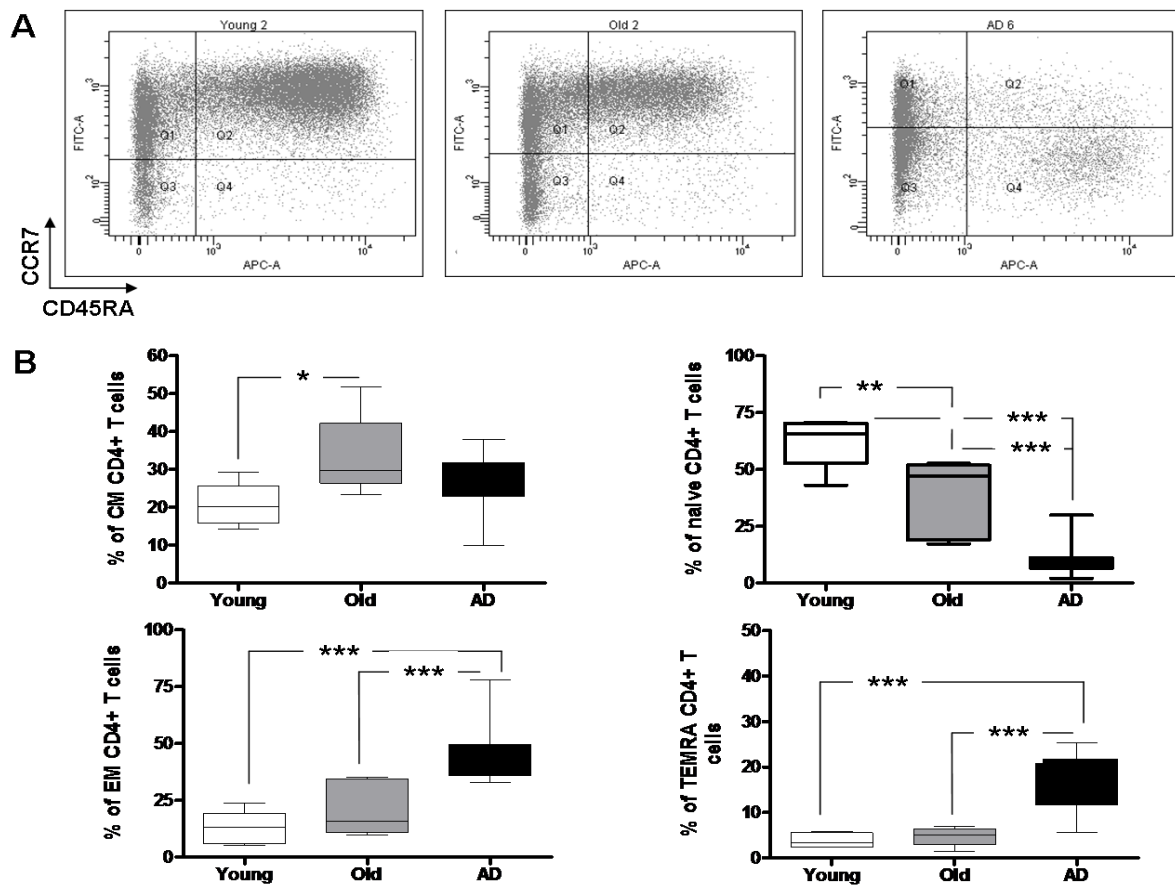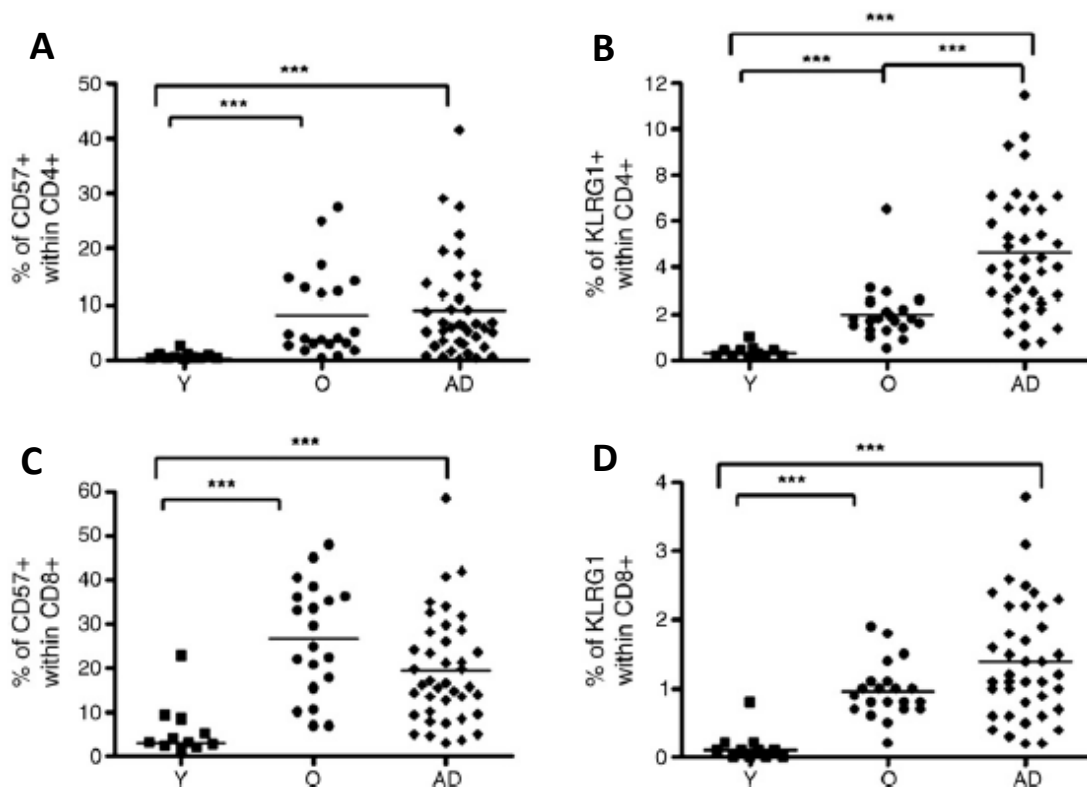
Figure 3.1: **Frequency of CD4+ T-cell phenotypes in AD patients and healthy controls**
PBMCs were stained for CD3, CD4, CCR7, and CD45RA to distinguish naïve cells (CD45RA+CCR7+),
CM (CD45RA-CCR7+), EM (CD45RA-CCR/-), and TEMRA (CD45RA+CCR7-) cells. A)
Representative plots of the CD4+ subset distribution of one young (left panel), one old (middle panel),
and one AD patient (right panel). B) Distribution of naïve (top right), CM (top left), EM (bottom left),
and TEMRA (bottom right) CD4+ T-cells. Significant differences are indicated by * $p < 0.05$, ** $p <$
0.01 and *** $p < 0.001$.
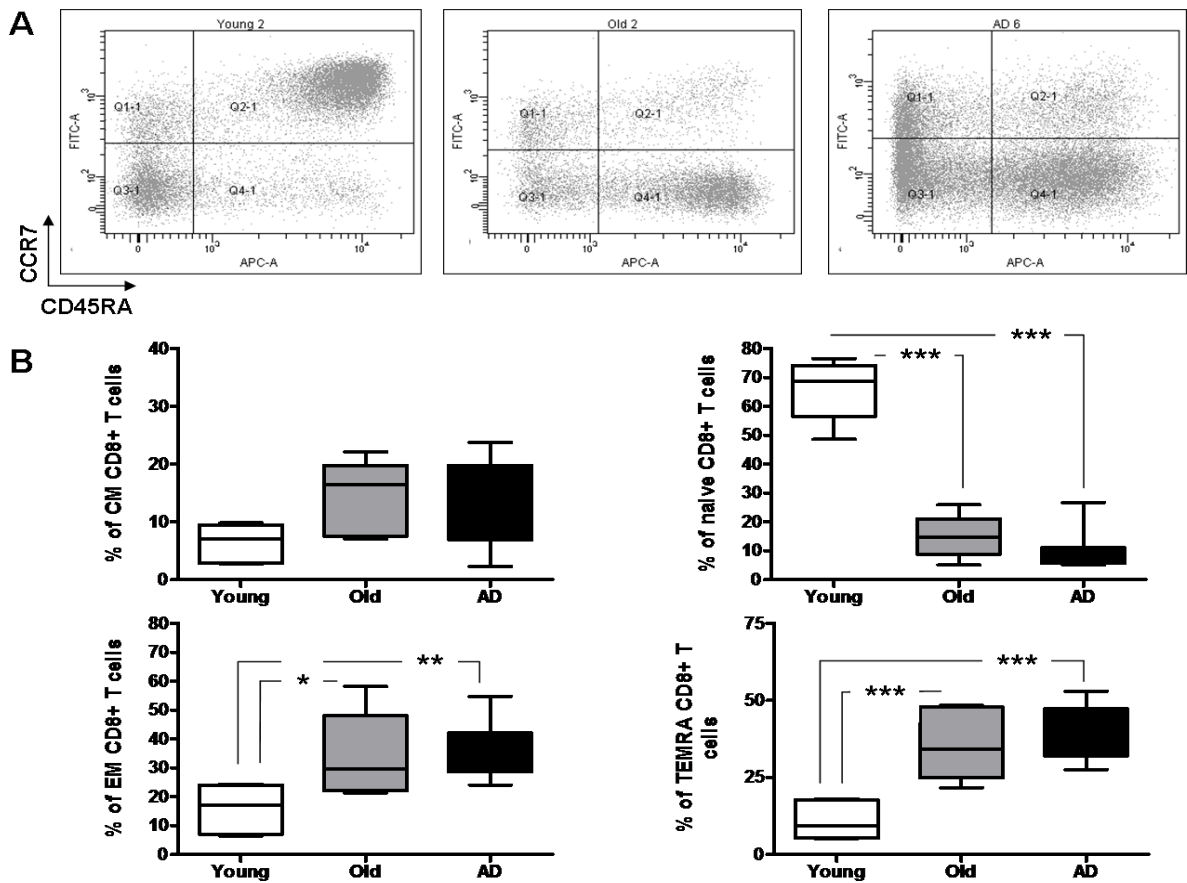
Figure 3.2: **Frequency of CD8+ T-cell phenotypes in AD patients and healthy controls**
PBMCs were stained for CD3, CD8, CCR7, and CD45RA for the identification of T cell differentiation stages as described in Figure 3.1. A) Representative plots of the CD8+ differentiation stages for the identification of T cell differentiation stages of one young (left panel), one old (middle panel), and one AD patient (right panel). B) Frequencies of naïve (top right), CM (top left), EM (bottom left), and TEMRA (bottom right) CD8+ T cell differentiation stages. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 3.3: **CD4+ naïve and late-differentiated subsets in AD patients and controls.**
PBMCs were stained with anti CD3, CD4, CD28, CD27, CD45RA, CD45RO antibodies to identify naïve and late-differentiated cells. (3A) Representative dot plots of naïve (top) and late-differentiated (bottom) CD4+ T cell distribution in a young control (left panels), in an old control (middle panels) and in an AD patient (right panels). (3B) Percentages of CD28+CD27+CD45RA+CD45RO- naïve cells (left) and of CD28-CD27-CD45RA+CD45RO+ late-differentiated cells (right) of 11 young controls (black squares), 21 healthy old (black circles) and 40 AD patients (black diamonds). Bars represent means. Differences between control subjects and AD have been assessed by Mann-Whitney nonparametric U testing with Bonferroni correction. Significant differences are indicated by * p < 0.005, *** p < 0.0005

Figure 3.4: **"senescence" marker expression on CD4+ and CD8+ T-cells**
PBMC were stained with anti CD3, CD4, CD8, KLRG1 and CD57 antibodies. Percentages of
CD4+CD57+ (upper left) and of CD8+CD57+ (bottom left) cells in 11 young controls (black squares),
21 healthy old (black circles) and 40 AD patients (black diamonds) are shown. Frequency of
CD4+KLRG1+(upper right) and of CD8+KLRG1+ (bottom right) cells of young (black squares), healthy
old (black circles) and AD patients (black diamonds). Bars represent means. Differences between control
subjects and AD have been evaluated by Mann-Whitney nonparametric U testing with Bonferroni
correction. Significant differences are indicated by *** $p < 0.0005$.

cally significant in the CD4+ population (Fig. 3.4).

As in the study presented in Section 3.1.1, this part of the study also investigated CD25+ T-cells. In contrast to the previous study, the percentages of CD4+CD25+ T-cells were higher in elderly compared to young controls, but the frequency of these cells was even higher in AD patients. To see whether these activated cells were likely to be regulatory T-cells, the expression of CD127 and Foxp3 was analysed. The results of this analysis showed that age but not health status (AD compared with healthy controls) was associated with higher percentages of Tregs (CD25+Foxp3+CD127low cells), as presented in Figure 3.5.
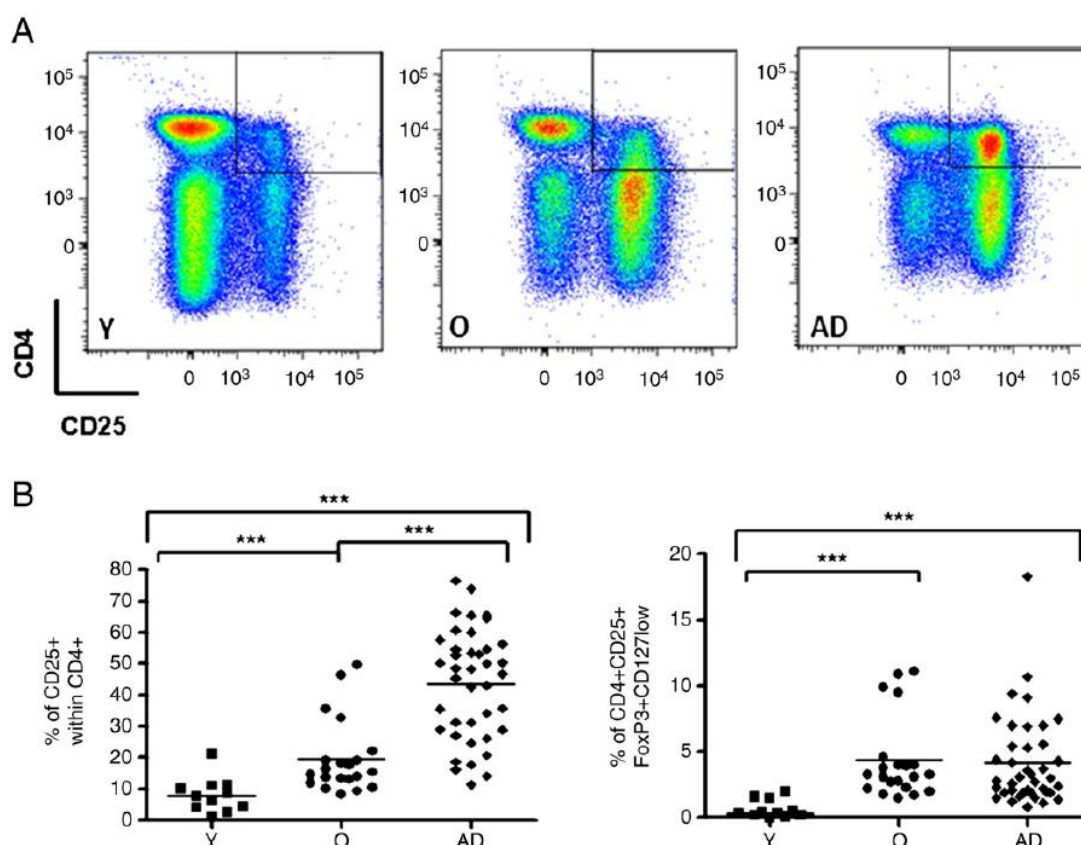
Figure 3.5: **Percentage of activated and Treg cells within CD4+cells of AD patients and controls.**

Representative dot plots of CD4+CD25+cells in a young control (left panel), an old control (middle panel) and an AD patient (right panel) (A). Median of CD4+CD25+cells in PBMC of 11 young controls (black squares), healthy old (black circles) and AD patients (black diamonds) are shown (B left). Percentages of CD4+CD25+Foxp3+CD127low, putative Tregs, in young (black squares), healthy old (black circles) and AD patients (black diamonds) (B right). Bars represent medians. Differences between control subjects and AD have been evaluated by Mann-Whitney nonparametric U testing with Bonferroni correction. Significant differences are indicated by *** $p < 0.0005$.

3.2 Immune signatures of Canadian patients with mild, moderate or severe AD

This study was designed to distinguish between stages of disease severity among AD patients using PBMCs obtained from the Sherbrooke population. Subjects were categorised as from mild, moderate or severe stage of disease and were compared with healthy age-matched controls. The CMV infection status of the donors were as follows: control group five CMV+ and three CMV-; mild and moderate groups contained eight CMV+ and two CMV-; while the severe group contained five CMV+ and five CMV-. In this population, therefore, the impact of CMV on immune signatures could be assessed.

3.2.1 T-cells

For the analysis of T-cells, they were divided into subsets. Out of the total living CD3+ cells, the percentages of CD4+, CD8+ and CD4-CD8- cells were evaluated. These results indicated a large range of values with no significant differences between groups (Fig. 3.6). TCR$\gamma\delta$+ cells were analysed within the CD3+ population and in the CD4-CD8- subset. This showed a non-significant trend of higher percentages of TCR$\gamma\delta$+ cells in patients with mild and moderate compared with severe AD (Fig. 3.7). The severe group showed a non-significantly lower frequency of this cell type, similar to those of the healthy controls. Frequencies of $\gamma\delta$T-cells within the CD4-CD8- population were not different between the groups.

Following this, the phenotype of the CD4+ cells was characterised in this cohort. As in the study of AD samples from Palermo [111], these samples were analysed with respect to CD27 and CD28 expression (Fig. 3.8 A-D shows the gating strategy employed). From the CD27 and CD28 double-positive and double-negative fractions, the CD45RA and CD45RO expression frequency was investigated. The outcome of this analysis indicated that patients with severe AD display a similar CD4+ T cell phenotype to healthy controls. In contrast, patients with mild or moderate AD showed slightly lower percentages of naïve cells (characterised superficially as CD27+CD28+ or more thoroughly as CD27+CD28+CD45RAhighCD45RO-) and higher percentages of late-differentiated cells (CD27-CD28- and CD27-CD28-CD45RO-) compared to patients with severe AD and healthy controls (Fig. 3.8).

Similarly, the percentages of KLRG1-CD57- CD4+ T-cells decrease with disease severity, although the patients suffering from severe AD showed similar median percentages as the healthy controls (Fig. 3.9). CD57 and KLRG1 expression levels were higher in patients with mild and moderate AD than in healthy controls, but this was the case only for a small number of patients with severe AD. These results might be attributable to differences in CMV infection rates. Eight out of ten patients with mild and moderate AD were CMV+, while only five of ten patients with severe AD were CMV+. Figure 3.10B displays the percentages of CD57+ CD4+ T-cells. The patients with severe AD were grouped according to CMV infection status. Observing these two groups separately indicates that CMV+ patients showed a slightly higher percentage of CD57+ CD4+ T-cells. This suggests that CMV seropositivity may influence the expression of these pro-

teins, but that patients with severe AD show similar expression frequencies to those observed in healthy controls. Taking CMV status into consideration may reveal another perspective for these data, but the number of patients in each group was too low to be able to draw conclusions.

In addition to the aforementioned analyses, the expression frequency of PD-1 (a marker for immune exhaustion) was analysed on CD4+ cells (Fig. 3.11). No significant differences between patients and controls were observed.
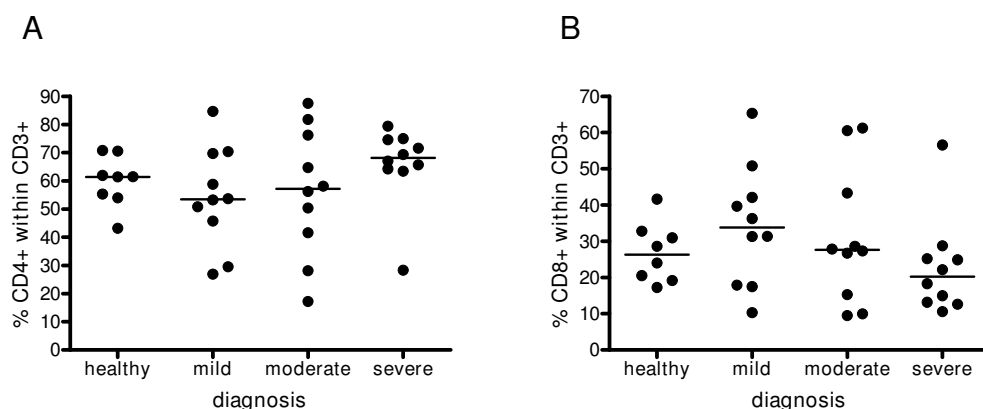
Figure 3.6: **CD4+ and CD8+ T-cell subset frequencies in dementia patients and healthy controls**
Frequencies of CD4+ (A) and CD8+ (B) T-cells of healthy elderly controls (n = eight) and AD patients (n =30). The AD patients were divided into three disease stages: mild (ten), moderate (ten) and severe (ten). Bars represent median values.

3.2.2 Tregs

For Treg analysis, Foxp3+ T-cells were gated from the CD4+ T-cells. The median is consistent across groups and statistical analysis showed no significant differences. Despite this, the AD patients show a greater range (11% spread) compared with the healthy controls that show a four percent spread in Treg frequencies. These results also suggest that there may be two groups of AD patients - those with a greater and those with a lower frequency of Tregs. This may have been due to disease severity or CMV status, but statistical analysis indicated that they were not associated with the frequency of Tregs. The regulatory T-cells can be characterised in more detail with the inclusion of CD45RA+ and CD45RO+ markers [16]. Miyara *et al.* divided CD4+Foxp3+ T-cells into three functionally and phenotypically distinct subpopulations in the following way: (1) CD45RA+FoxP3low (resting Tregs), (2) CD45RA-Foxp3high (activated Tregs) and (3) CD45RA-Foxp3low (cytokine secreting non-Tregs) [98]. In the present study Foxp3+CD45RO+ and Foxp3lowCD45RA+ cells showed a similar but more pronounced trend as Foxp3+ cells. Slightly higher values for AD patients were observed compared with healthy controls, but no significant difference were observed. No differences were observed within AD patient subgroups either. The expression frequency of CD45RA- in Foxp3-low and -high expressing cell fractions showed no statistical differences or trends (data not shown).

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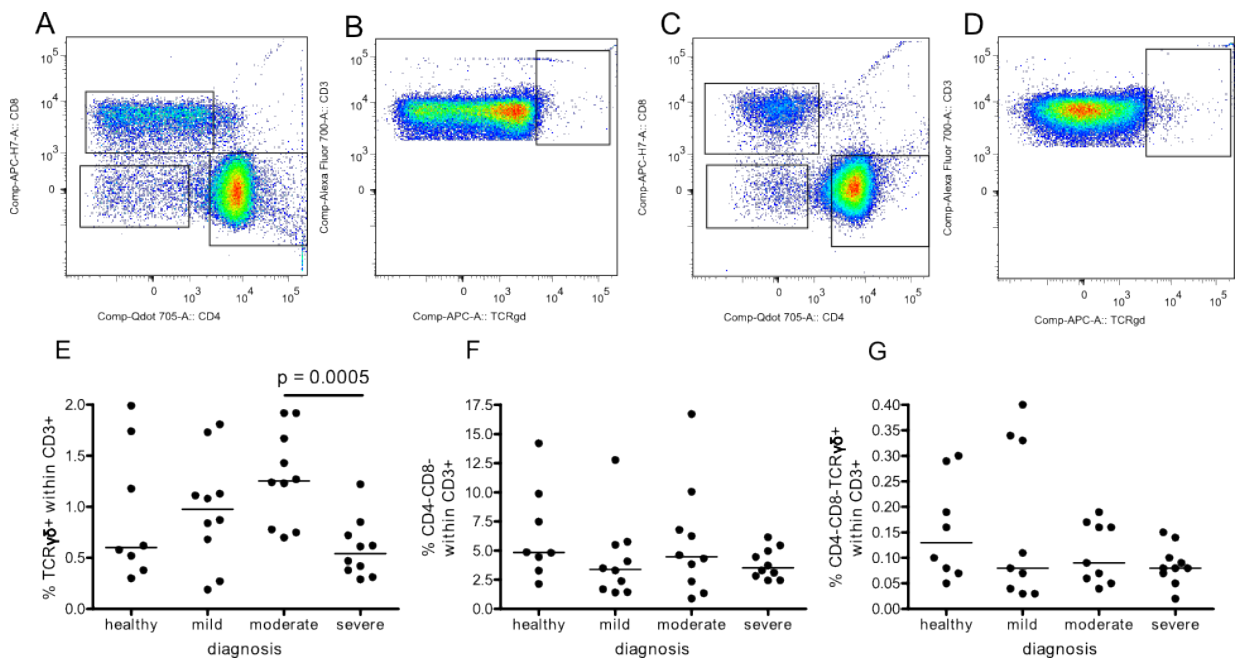
Figure 3.7: **Frequency of $\gamma\delta$T-cells** in dementia patients and healthy controls
Percentages of CD4-CD8- (E), TCR$\gamma\delta$+ (F) and CD4-CD8- TCR$\gamma\delta$+ (G) T-cells within the total CD3+ population, in healthy elderly controls and AD patients with different stages of disease. Bars represent median values. Examples of gating analysis for a healthy control is shown in A and B and for moderate AD in C and D. The statistical analysis of TCR$\gamma\delta$+ events gated in panels B and D is shown in panel E and the frequencies of CD4-CD8- events gated in panels A and C are displayed in panel F.
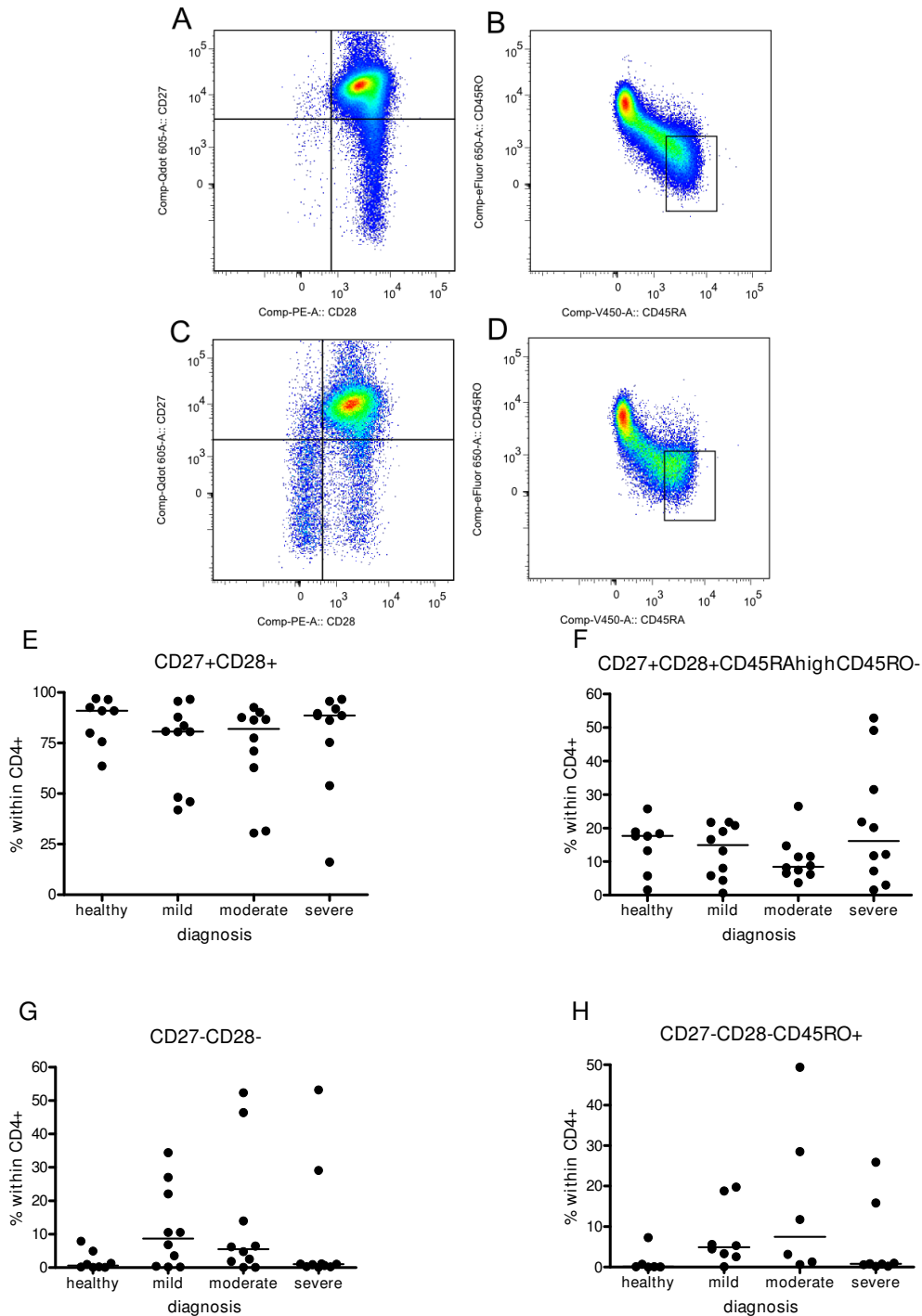
Figure 3.8: **CD4+ T-cell gating strategy and the resulting T-cell phenotype analysis**
Representative images from a healthy elderly control (A and B) and a patient with mild AD (C and D)
are given. This gating strategy was employed to identify differentiation stages for CD4+ T-cells. To
achieve this, CD27 was first plotted against CD28 before analysing the double positive CD45RA cells
against CD45RO-expressing cells. The resulting percentages of CD4+CD27+CD28+ are shown in panel
E. These cells were further characterised as CD45RAhighCD45RO- (F). G and H show
late-differentiated CD4+ T-cells (defined as CD27-CD28- and CD27-CD28-CD45RO+)
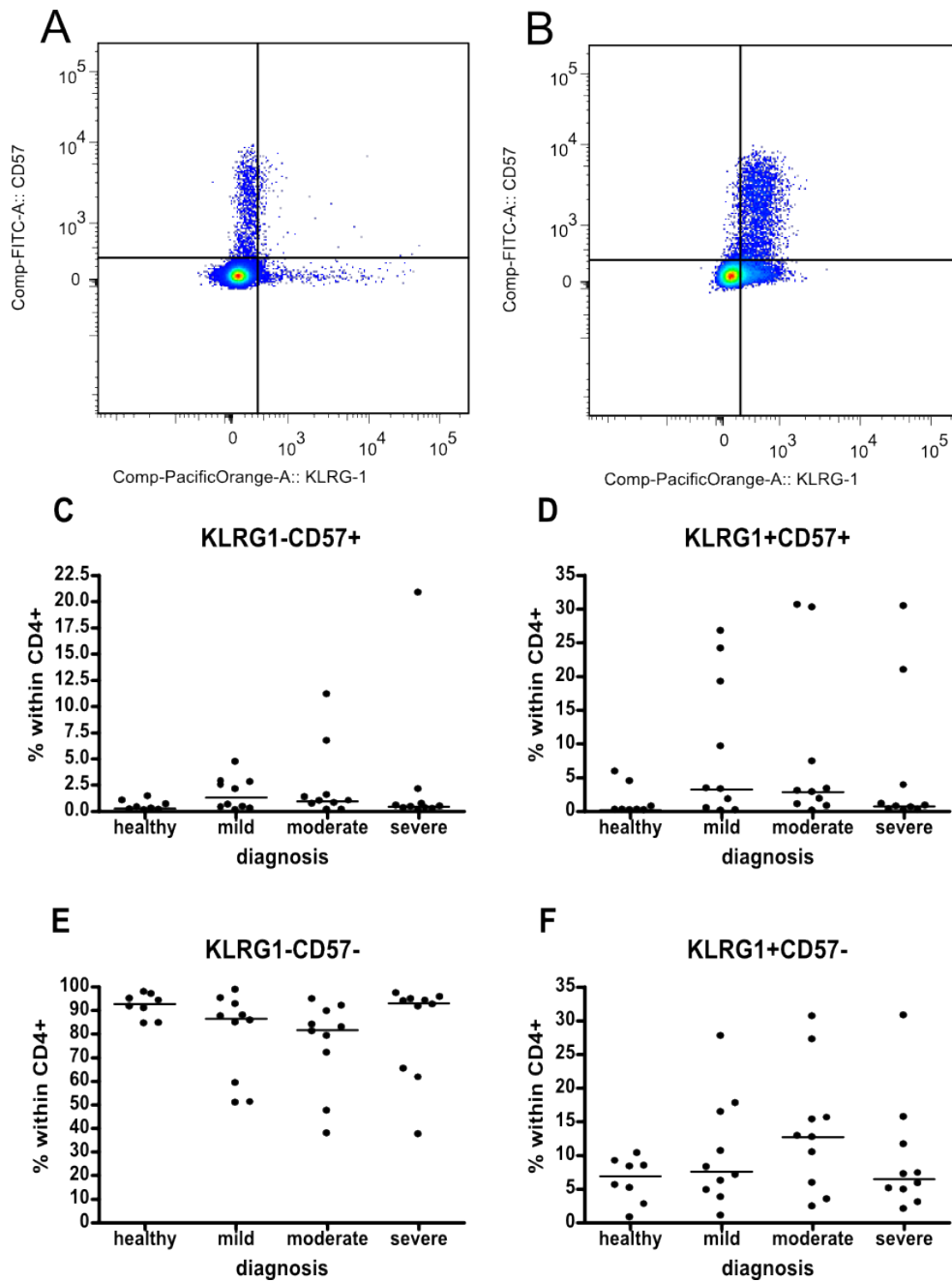
Figure 3.9: **Frequency of CD4+ KLRG1- and CD57-expressing cells**
A and B show typical CD57 and KLRG1 gating analyses for a healthy control and an AD patient (moderate AD). The percentage of CD4+ cells in the four different populations are grouped in panels C, D, E and F.
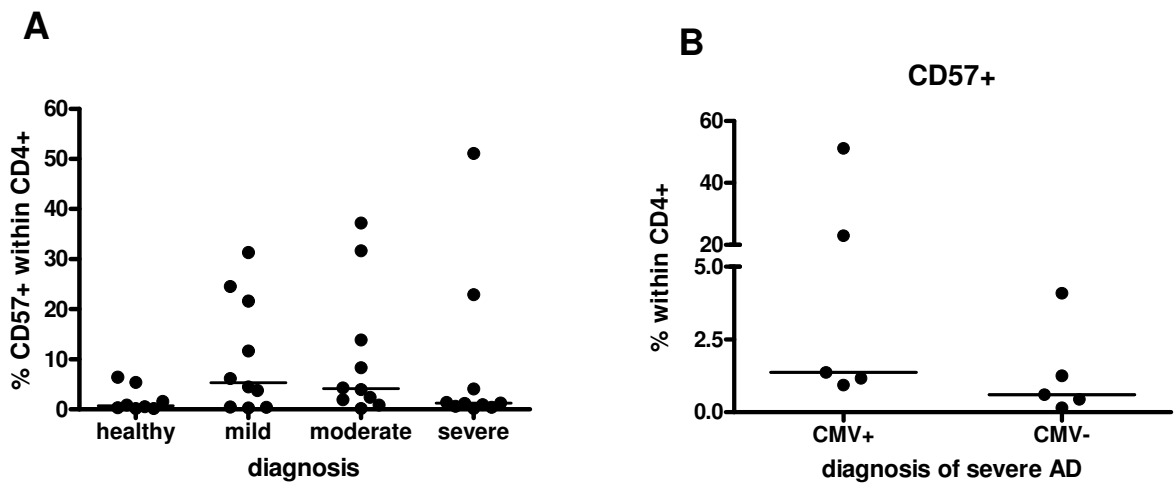
Figure 3.10: **CD57 expression on CD4+ cells in CMV-seronegative and CMV-seropositive AD patients with severe disease**

Frequency of CD57+ CD4+ T-cells in dementia patients and healthy controls (A). Panel B shows the frequency of CD4+ CD57+ T-cells according to CMV status in dementia patients.
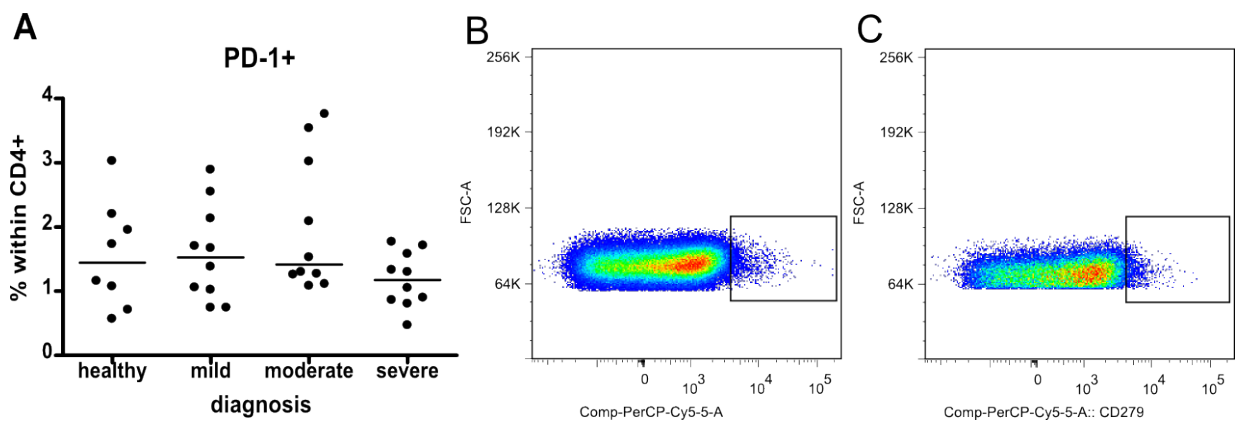
Figure 3.11: **Frequency of CD4+ PD-1+ cells in dementia patients and healthy controls**

Expression frequency of PD-1 on CD4+ T-cells (A). The analysis gates are shown for a healthy donor (B) and a patient with moderate AD (C).
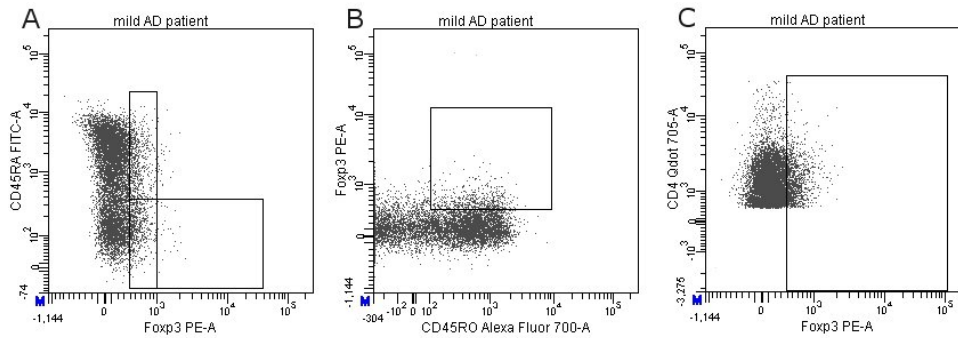
Figure 3.12: **Treg gating strategy**
An example of the gating strategy is given for a donor with mild AD. To isolate these cells the lymphocytes were gated first, followed by the singlets (using FSC-W against FSC-A), before identifying the living cells (EMA-). CD3+ T-cells were then selected, from which CD4+ and CD8+ T-cells were selected (images not shown). These images show different CD4+T-cell subsets according to Foxp3 expression. Panel A has been gated for CD45RA against Foxp3, while (B) shows Foxp3 against CD45RO. Panel (C) shows the gating of CD4+Foxp3+ cells. The classification of Tregs into resting Tregs (CD45RA+Foxp3low), cytokine secreting non Tregs (CD45RA-Foxp3low) and activated Tregs (CD45RA+Foxp3high) was developed by Miyara *et al.* [98]. The frequencies of these populations in the study participants are shown in Figure 3.13 and figure 3.14.
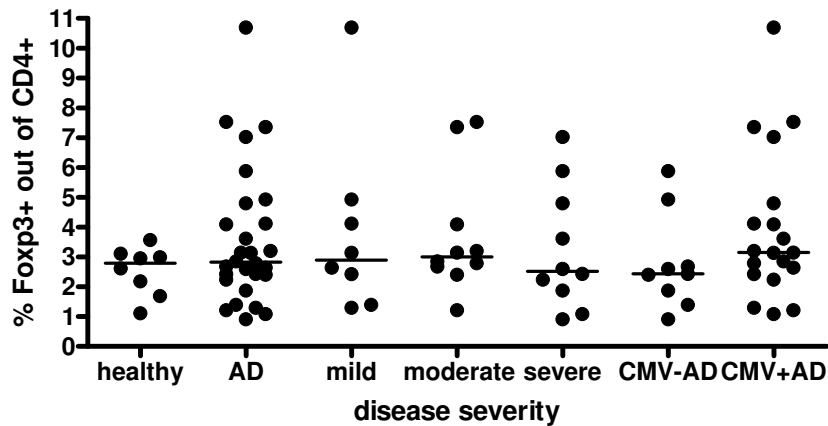
Figure 3.13: **Frequency of Tregs in AD patients and healthy controls**
Frequency of Foxp3+ T-cells within the CD4 T-cell compartment. Frequencies are shown for healthy elderly (n = eight) and AD patients (n = 28). The AD patients were divided into three disease stages: mild (n = eight), moderate (n = ten) and severe (n = ten) or into CMV- (n = nine) and CMV+ (n = 19). Bars represent median values.
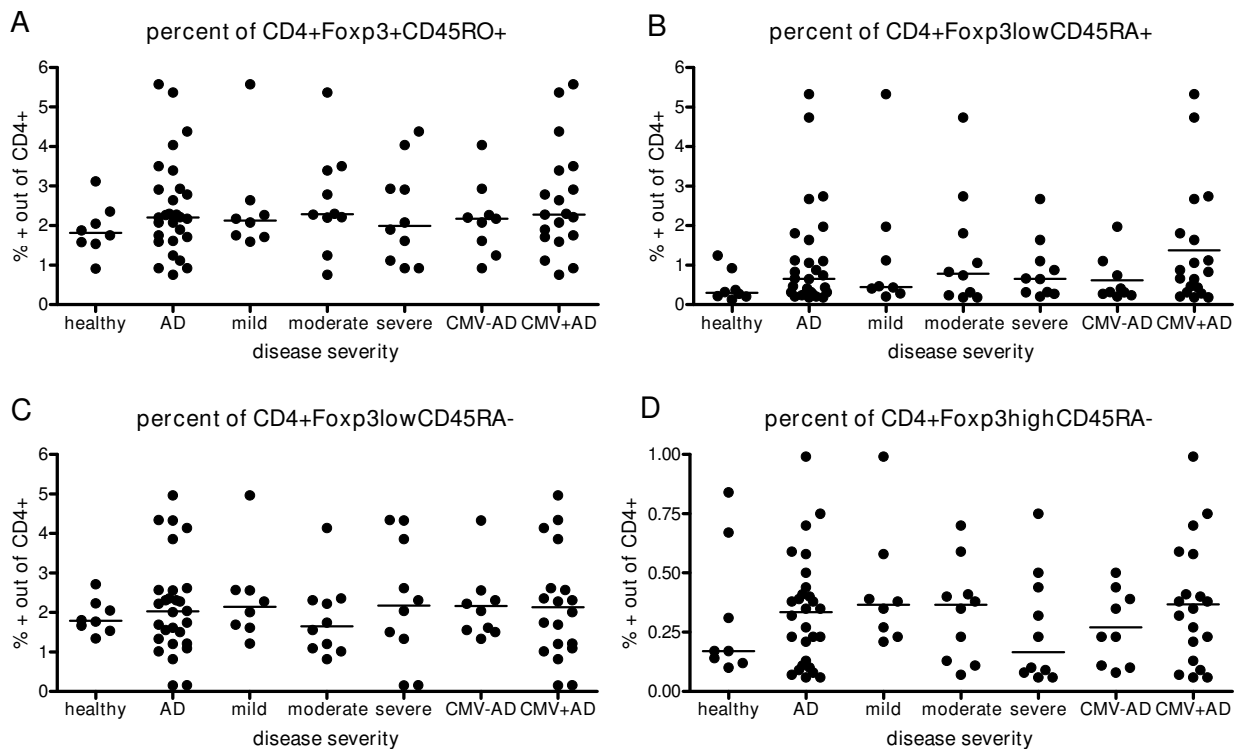
Figure 3.14: **Frequency of different Treg phenotypes in AD patients and healthy controls**
Frequency of Foxp3+ T-cell subsets within the CD4 T-cell compartment of healthy elderly (n = eight) and AD patients (n = 28). The AD patients were then divided either in three disease stage mild (n = eight), moderate (n = ten), and severe (n = ten) or in CMV- (n = nine) and CMV+ (n = 19). Bars represent median values. In panel A the percentages of Foxp3+CD45RO+ cells are given, panel B shows Foxp3lowCD45RA cells+, panel C Foxp3lowCD45RA- cells and panel D Foxp3highCD45RA- cells.

3.2.3 Chemokine receptor expression on the leukocytes of AD patients and healthy controls

Chemokine receptor expression is a factor that may be altered in AD given that immune cells are known to migrate along chemokine gradients through a defective blood-brain barrier. To investigate this, the expression frequency of CCR4, CCR5 and CCR6 on PMBCs was analyzed in dementia patients and healthy controls (Fig. 3.15). Trends across patient groups and controls in the expression frequency of these cells within the CD45+ population (total leukocytes) were observable. These trends were more pronounced when T-cells were investigated alone. Within the CD4+ T-cells, the median CCR4 expression was not observed to be different between healthy controls and AD patients, although a larger range is apparent for the AD patients. For the AD patients, the percentage frequency ranges up to almost 40 %, whereas in the healthy elderly the maximum frequency is 15 %. Dividing the AD patients according to mild, moderate and severe disease stages, or into CMV- and CMV+ status did not result in statistically significant differences. Similarly to CCR4, the results for CCR5 show similar median values across groups, but some AD patients have relatively high frequencies. The results for CCR6 differ slightly. Although no trends were present, the healthy controls showed slightly higher frequencies of CCR6 compared with those of CCR4 and CCR5, while ten AD patients for CCR4 and nine for CCR5 showed higher frequencies compared to the highest control subject, this was true only for one patient in the case of CCR6. The healthy controls show slightly higher frequencies of CCR6 compared with those of CCR4 and CCR5 and so no trend compared to the AD patients is visible. The distribution across AD patient sub-groups is also different. Mild AD patients show median expression frequencies lower than those of healthy controls, while severe AD patients show higher frequencies compared with controls. CMV infection status appeared to have no influence (Fig. 3.15).

3.2.4 Induced cytokine production profiles of CD4+ T-cells in AD patients and healthy controls

Several previous studies have reported different cytokine levels in the plasma and CSF in AD patients compared to healthy controls [78]. In the present study, the cytokines produced by CD4+ and CD8+ T-cells were analysed. Combinations of cytokines characteristic of certain T-cell subsets (Th1, Th2 and Th17) were considered. This analysis was not performed with all samples, due to insufficient material. Exclusion criteria were for CD4+ cell events observed with flow cytometry to be less than 500 (zero samples), and less than 100 events for CD8+ cells (two samples). Due to reduced sample numbers, all AD patients were considered as a single group and not according to disease severity. Stimulation of PBMCs with PMA and Ionomycin resulted in significantly increased cytokine production compared to unstimulated cells, but without differences between AD patients and healthy elderly controls (data not shown). This indicates that the capacity of PBMCs from AD patients and healthy controls is comparable in terms of producing the cytokines IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF.

One of the hallmark features of AD is amyloid beta accumulation in the brain, and it has been hypothesised that this contributes to the inflammatory conditions typically observed in AD patients.
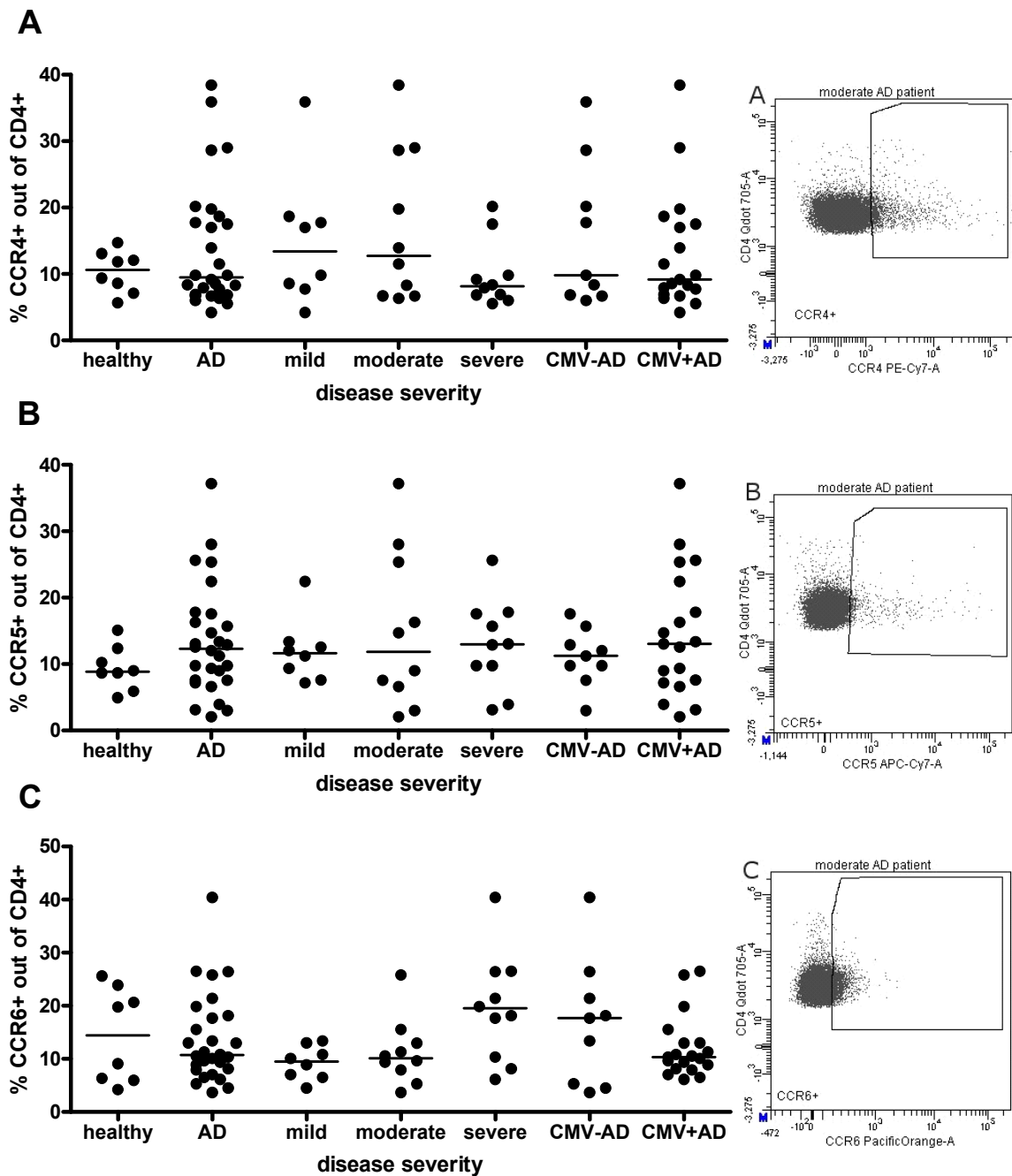
Figure 3.15: **CD4+ T-cell CCR expression in dementia patients and healthy controls**
Figure indicates the frequency of chemokine receptor expressing CD4+ T-cells as follows: CCR4 in A,
CCR5 in B and CCR6 in C. The median value is indicated by the bar. On the right hand side an example
of the gating strategy for the chemokine receptors CCR4 (A), CCR5 (B) and CCR6 (C) in CD4+ T-cells
from a patient with moderate AD is shown. The pre-gating was performed in the same manner as for the
regulatory cells because these markers were tested together in the same panel. Gating was performed in
the following manner: lymphocytes -> singlets (FSC-W vs. FSC-A) -> living (EMA-) CD3+ cells->
CD4+ from CD4 vs. CD8 (images not shown).
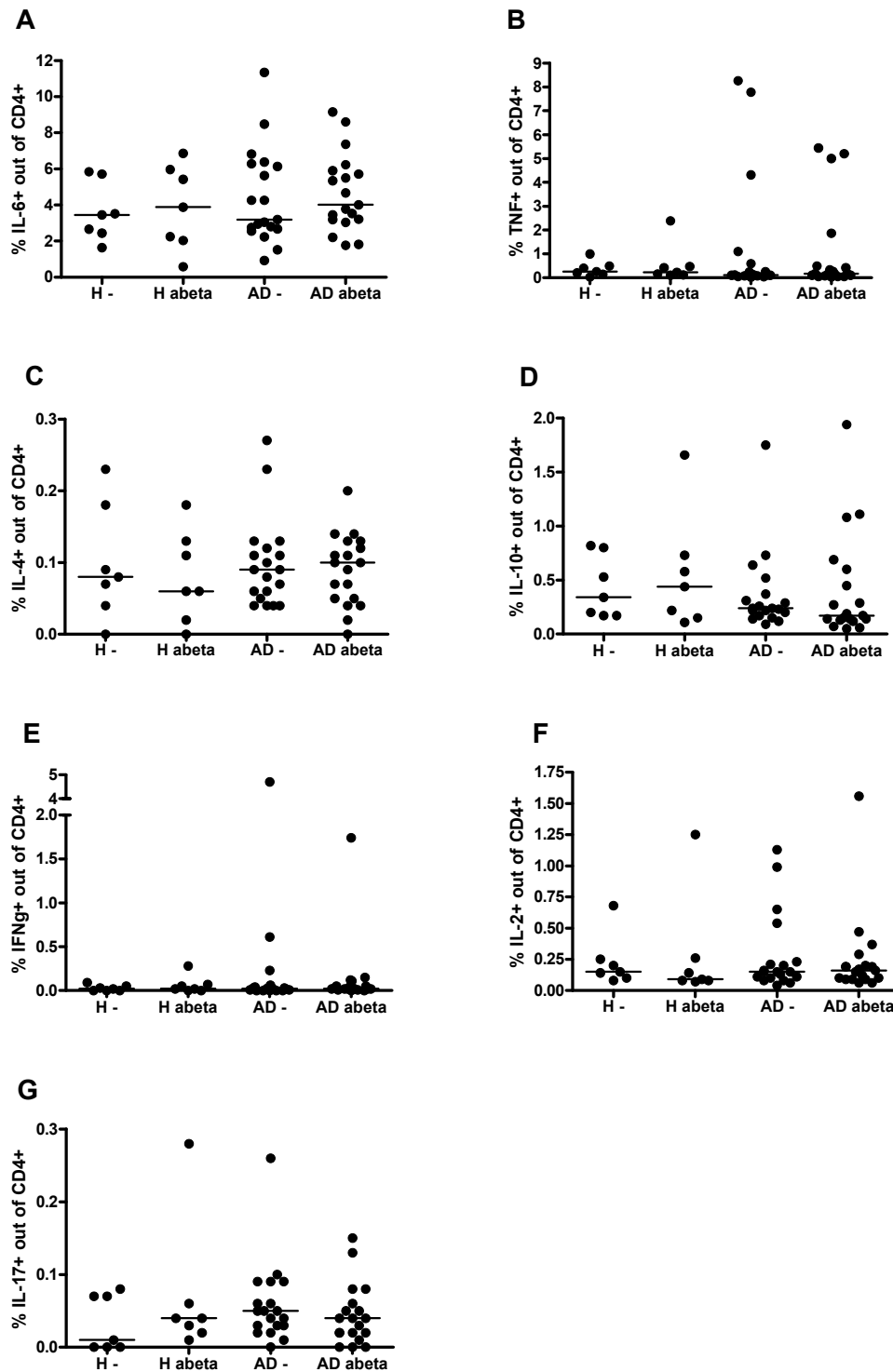
Figure 3.16: **Cytokine profile of CD4+ T-cells**

Cytokine production of CD4+ T-cells after incubation with Golgi plug alone (-) or with amyloid beta (abeta) shown for seven healthy elderly (H) and 19 AD patients (AD) (4 mild, 4 moderate, 7 severe AD). The cytokines analyzed were: IL-2 (A), IL-4 (B), IL-6 (C), IL-10 (D), IL-17 (E), IFN-γ (F) and TNF (G). The median value is represented by the bar in each column.

Another hypothesis is that amyloid beta reactive T-cells may be able to manifest an immune response that controls inflammation in AD. We therefore investigated the influence of amyloid beta on PBMC cytokine production. The results indicated that only in a small number of samples did stimulation with amyloid beta result in cytokine production, whether the PBMCs were from an AD patient or a healthy donor. In the cases where cytokine production was observed, there were no differences with respect to pro- or anti-inflammatory cytokines. The unstimulated samples showed a very low level of cytokine production for both AD and controls, although the AD patients appeared to show a slightly higher basal production of IL-6 and TNF cytokines (Fig. 3.16). The results were similar for CD8+ T-cells - no clear differences between AD patients and healthy controls were observed (data not shown).

3.3 Immune profile of Italian patients with AD, VaD or mixed dementia

The next series of experiments again included AD patients, but also those with vascular dementia to investigate whether immune parameters could contribute to differential diagnosis. Frozen blood samples from forty individuals with accompanying clinical data were analyzed. This cohort consisted of the following groups: healthy controls, AD patients, vascular dementia (VaD) and a mixed form of dementia (mixed dementia). These groups were formed according to their Hachinski ischemic score. The results of this analysis were plotted against the MMSE value for each patient in order to assess how the immune profile may be related to the severity of dementia. Patient frozen whole blood samples were used to perform four separate experimental investigations: (1) T-cell subsets; (2) Tregs and chemokine receptor expression; (3) other leukocytes and (4) amyloid beta uptake.

3.3.1 T-cells and their phenotype

T-cell phenotypes were analysed according to the classification model developed in this laboratory by Pellicanó *et al.* [111]. The results of this analysis demonstrated that the percentage of CD4+, CD8+ and TCR$\gamma\delta$+ cells was not different between the groups (Fig. 3.17). The percentage of cells with a CD27 CD28 double-positive phenotype has been reported to be lower in the elderly than in the young, particularly for CD8+ cells, while the CD4+ compartment is less affected. In contrast, the results of this study indicated that in AD patients, naïve T-cells in both the CD4 and CD8 compartments occur in lower percentages [77, 111]. This study is the first to report that the percentage of CD27+CD28+ cells is also significantly lower in VaD compared with healthy controls (p = 0.019), and in mixed dementia compared with healthy controls (p = 0.039). Reciprocally, CD27-CD28- (late-differentiated) T-cells occur at marginally higher frequencies in VaD patients than in healthy controls, although this was not statistically significant. This difference was also observed in AD, but to a lesser extent (Fig. 3.18). In order to characterise naïve and memory cells in more detail, more markers were analysed. For the purposes of this study, naïve cells were characterised as CD27+CD28+CD45RA+CD45RO-. This population

of cells was observed to be significantly lower in frequency in VaD (p = 0.0389) and tendentially in AD (p = 0.2041) and mixed dementia (p = 0.0542) than in healthy controls (Fig. 3.19A). In the dementia patients, the frequency of naïve cells was associated with MMSE values; lower MMSE values were associated with reduced frequencies of naïve cells (p = 0.0150) (Fig. 3.19F). Given this observation, the percentage of late-differentiated cells (CD27-CD28-CD45RA+CD45RO+ or CD27-CD28-CD45RA-CD45RO+ or CD27-CD28-CD45RO+CD57+KLRG1+) was expected to be increased in these patients, but such a relationship was not observed (Fig. 3.19B-E). Even more unexpected was the observation of a marginal reduction of these populations in mixed dementia. Another characteristic of naïve cells is the lack of CD57 and KLRG1 expression. In AD and VaD but not mixed dementia patients, the percentage of CD57-KLRG1- CD4+ T-cells was slightly lower, while CD57+KLRG1+ cells were observed to be slightly higher in frequency (Fig. 3.20). Concerning other T-cell groups, no differences were seen. CD8 and $\gamma\delta$T-cell percentages did not vary. In the CD8 population, the percentage of CD27+CD28+ CD8+ cells remained the same for all groups tested (Fig. 3.21).
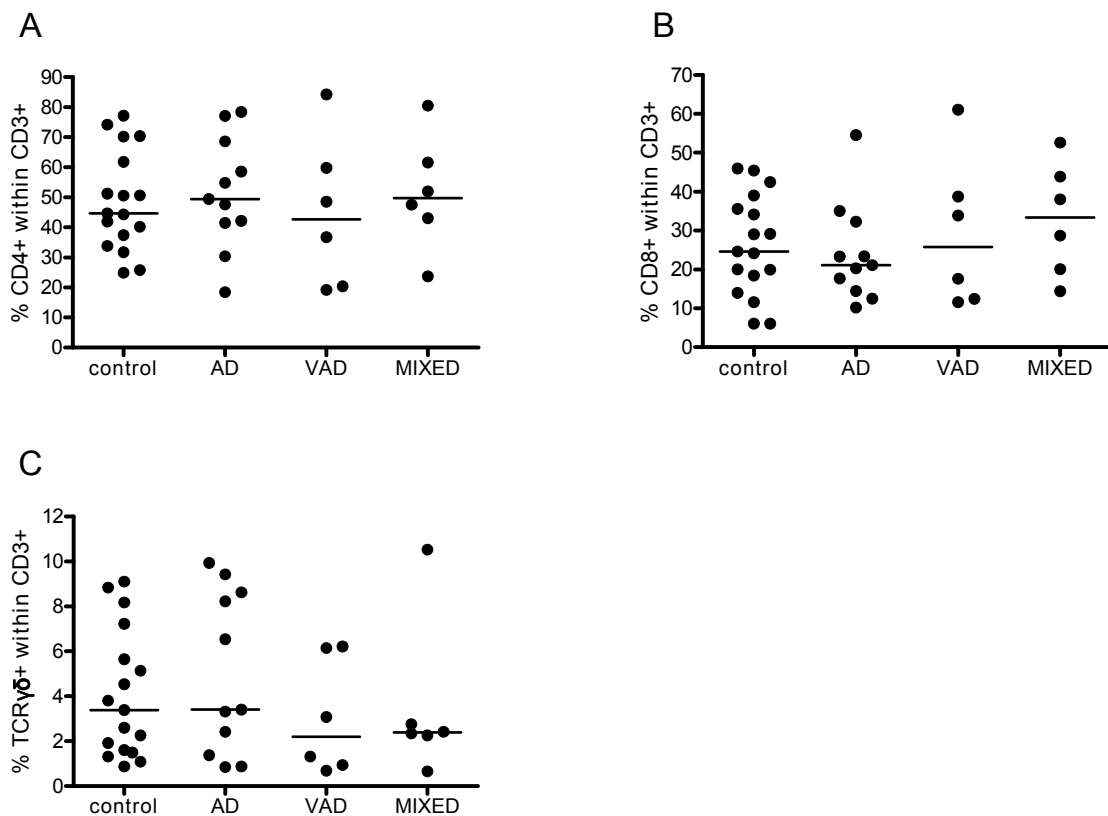
Figure 3.17: **Frequency of CD4+, CD8+ and TCR$\gamma\delta$+ T-cell subsets**
Percentages of CD4+ (A) CD8+ (B) and TCR$\gamma\delta$+ (C) cells within the CD3+ T-cells of controls, AD, VaD and mixed dementia patients. Bars represent the median values.
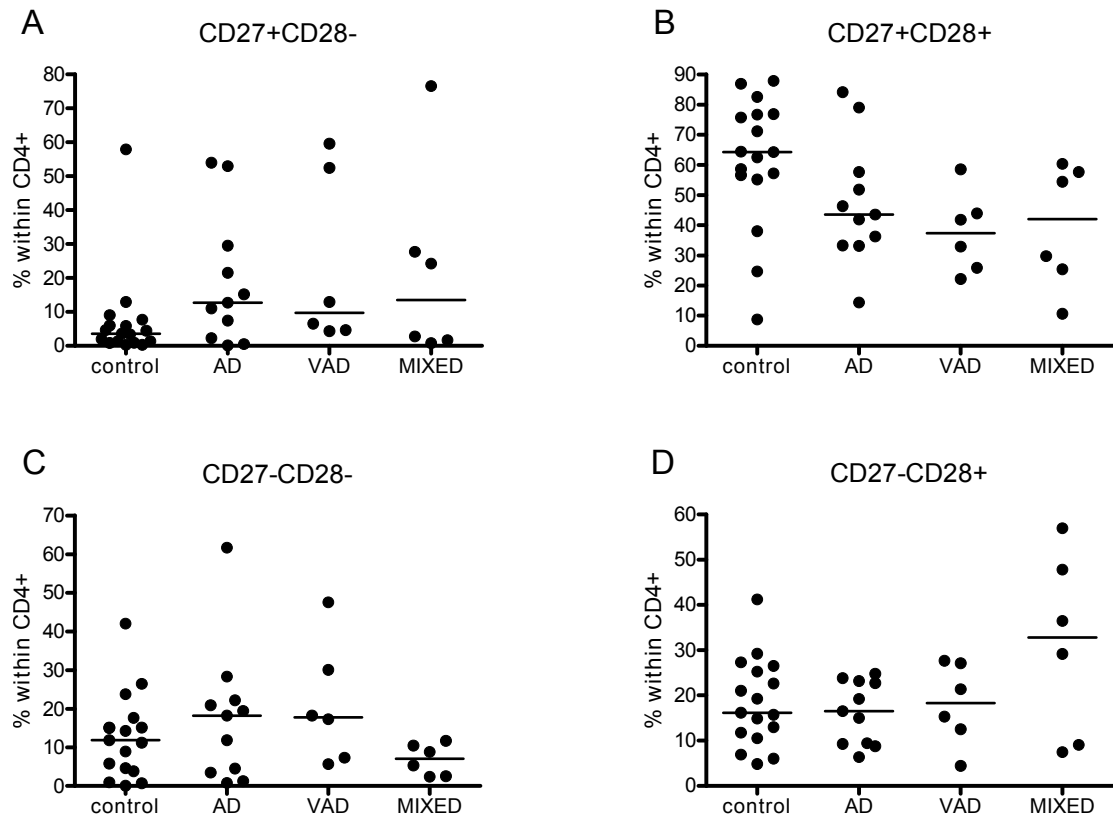
Figure 3.18: **CD4+ T-cell differentiation stages**
Identification of early to late-differentiated CD4+ T-cells according to the expression of CD27 and CD28. Panel A shows the percentages of CD27-CD28+ cells, (B) CD27+CD28+, (C) CD27-CD28- and (D) CD27+CD28- cells. The following significance levels were reached with Mann-Whitney test: control vs. VaD in (B) (p = 0.0190) and (D) (0.0461); control vs. mixed dementia in (B) (0.0389). The bars represent the median.
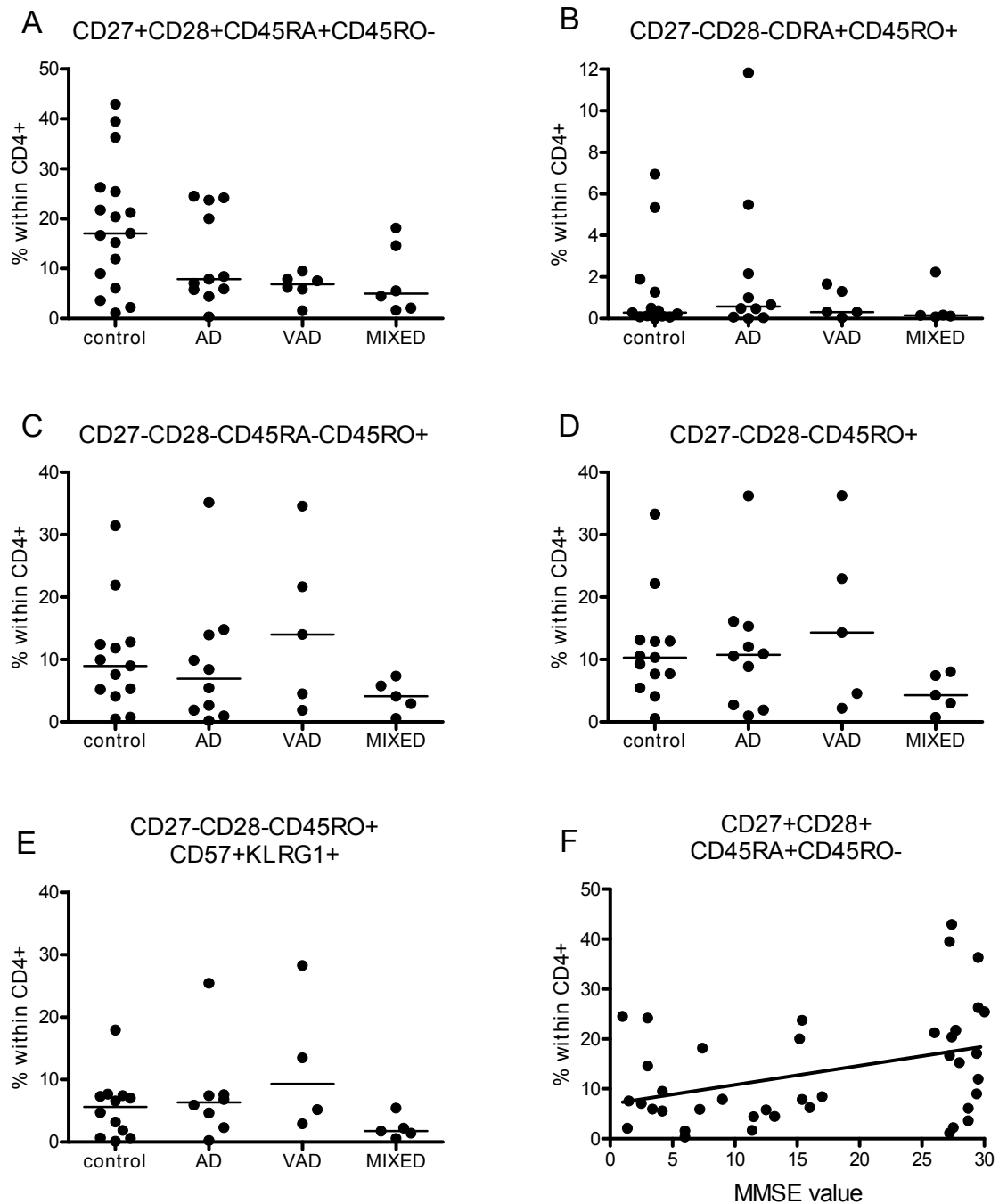
Figure 3.19: **CD4+ T-cell phenotyping with additional markers**
Detailed characterisation of early- and late-differentiated CD4+ T-cells with the inclusion of CD45RA, CD45RO, KLRG1 and CD57. Naïve cells (CD27+CD28+CD45RA+CD45RO-) are presented in panel A and were plotted against the MMSE value of all patients (panel F). Late-differentiated cells with different marker combinations are given in B, C, D and E. After application of the Mann Whitney test, $p = 0.0389$ when comparing VaD with the control group, while $p = 0.0542$ between mixed dementia and the control group in A. Bars indicate medians.

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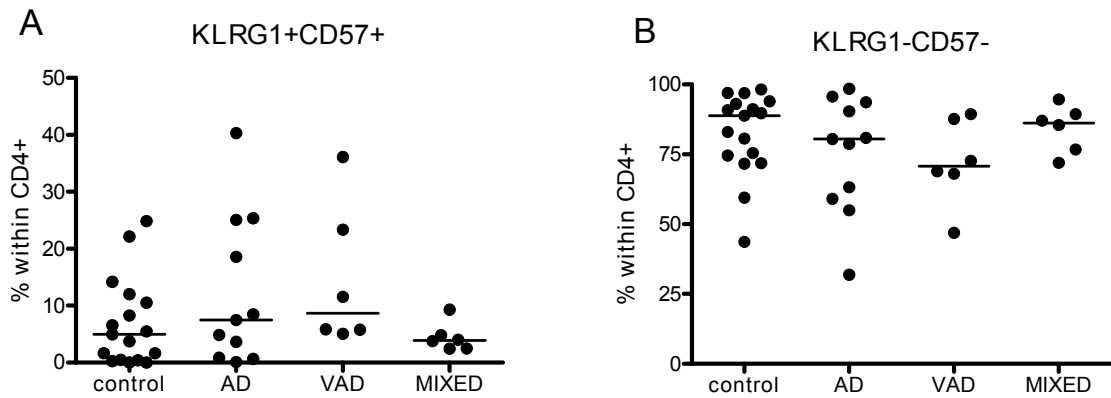
Figure 3.20: **KLRG1 and CD57 expression on CD4+ T-cells in dementia patients and healthy controls**

Frequency of KLRG1 and CD57 expression combinations in dementia patients and healthy controls. The KLRG1+CD57+ phenotype is characteristically expressed by senescent cells. This population of cells can be seen in panel A and compared with the frequency of cells negative for these markers in the same patients (B). Statistical analysis (Mann-Whitney test) revealed p = 0.0152 when comparing VaD with mixed dementia in panel A.
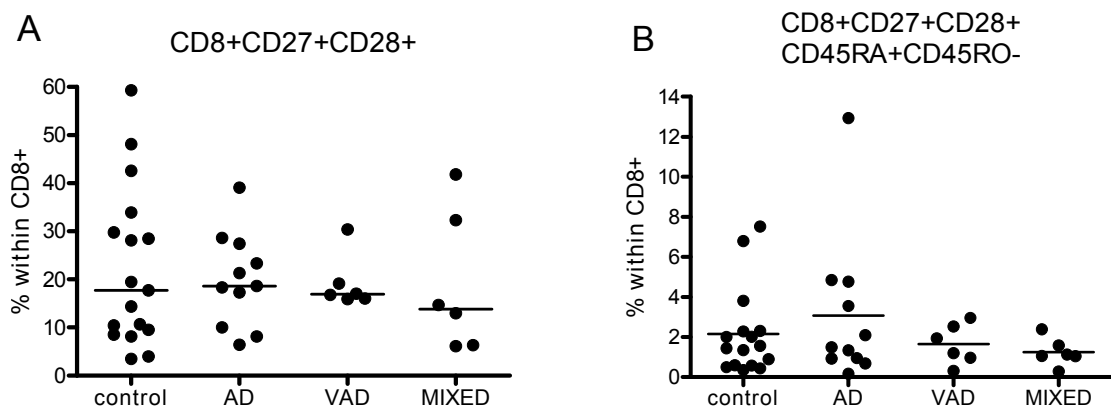
Figure 3.21: **Frequency of naïve CD8+ cells in dementia patients and healthy controls**

CD8+ early-differentiated T-cells characterised by CD27+CD28+ (A) or CD27+CD28+CD45RA+CD45RO- (B) in healthy controls and patients with dementia (AD, VaD, mixed dementia). Bars represent median values.

3.3.2 Regulatory T-cells and chemokine receptors

Particular attention was paid to Tregs and chemokine receptors in this study. With the antibody panel employed, the aim was to detect activated cells. We established the percentage of CD25+ cells as an acute activation marker for T-cells, but found no difference between healthy controls and dementia patients (Fig. 3.22A). Following this, CD4+ Foxp3+ cells were evaluated, but again no differences between patient groups were found (Fig. 3.22B). CD8+ regulatory cells, characterised by the expression of CD103, also showed no differences across patient and control groups (Fig. 3.22C).
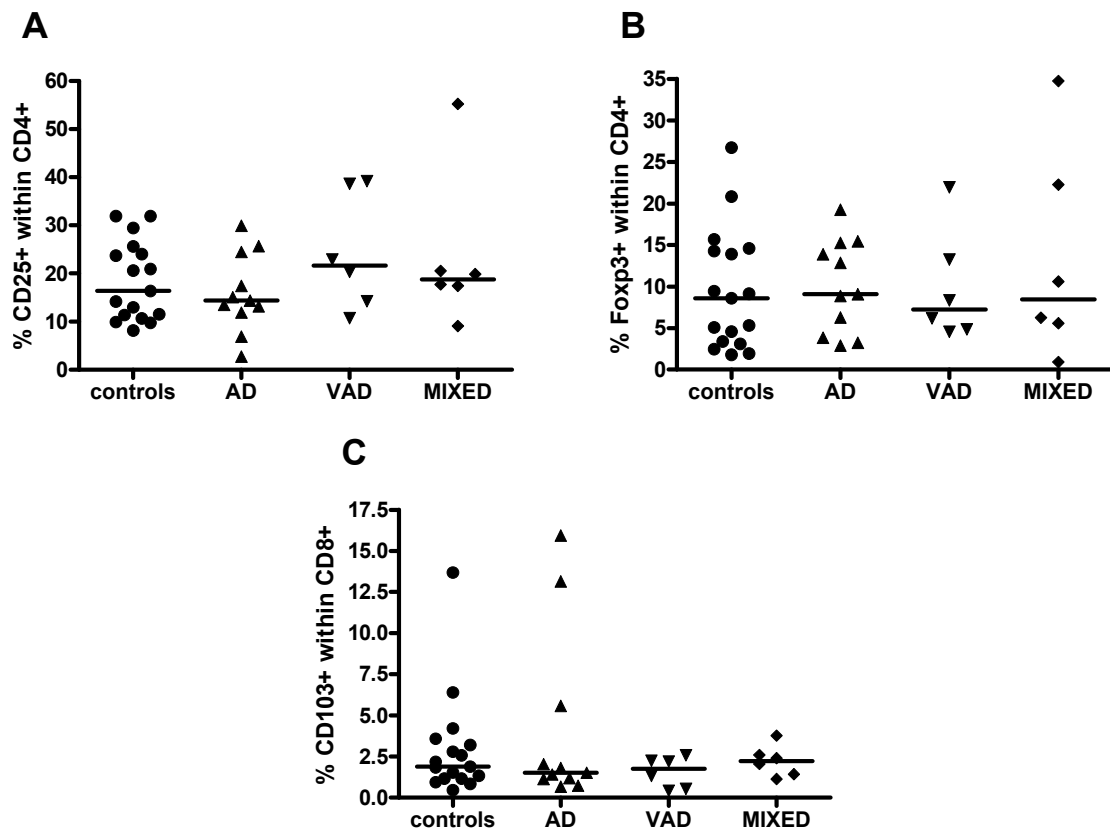
Figure 3.22: **Frequency of activated and regulatory T-cells in dementia patients and healthy controls**

Expression of activation marker CD25 (A) and Foxp3 (B) on CD4+ T-cells and homing receptor CD103 on CD8+ T-cells (C) of healthy controls and patients with AD, VaD and mixed dementia. Bars represent median values.

The frequency of cells expressing chemokine receptors showed no significant differences, but only slight trends in the case of CD4+ cells, with the trend becoming more pronounced for CD4+CD25+ cells. CCR4 (Fig. 3.23) and CCR6 (Fig. 3.24) expression was only slightly enhanced on CD4+ T-cells. One difference did reach statistical significance, namely, CCR6 expression between mixed dementia and healthy controls ($p = 0.0328$ for CD4+ T-cells and $p = 0.0157$ for CD4+CD25+ T-cells). Plotting the MMSE value against the frequency of CCR ex-

pressing cells shows a weak correlation between CCR expression and disease severity according to the MMSE (p = 0.1954 for CCR6+, p = 0.0310 for CD25+CCR6+). Additionally, PD-1 expression frequency (this protein is not a chemokine receptor, but a marker of exhaustion) was assessed, but was not observed to be different across patient groups and healthy controls (Fig. 3.25). In the CD8+ T-cell subset, no significant differences were observed, either for CCR4+ or for CCR6+ cells in healthy controls and patients from the three groups (Fig. 3.26).
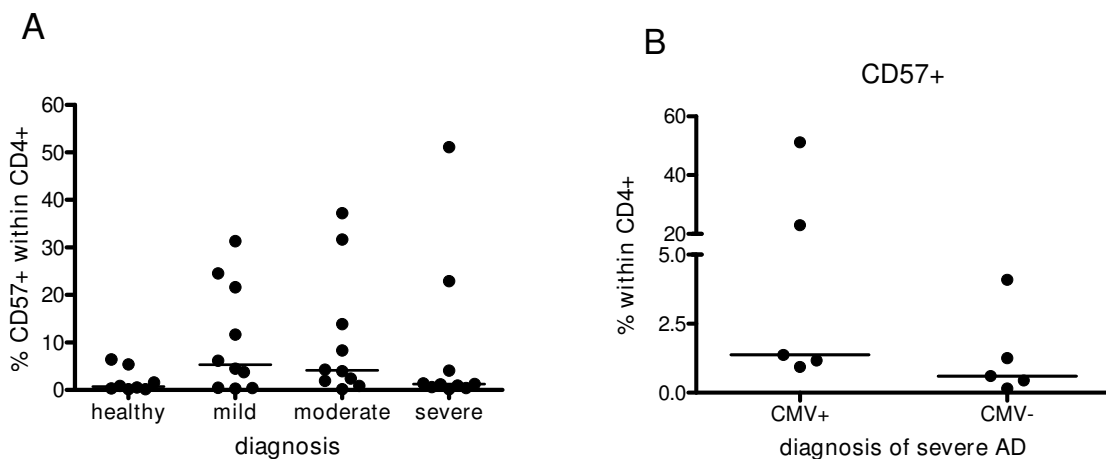
Figure 3.23: **CCR4 expression**
Percentages of CCR4+ (A) and CD25+CCR4+ (C) CD4+ T-cells plotted against the MMSE values or against diagnosis (healthy, AD, VaD, mixed dementia) (B, D). In the second column all patients (AD, VaD and mixed dementia) are grouped together. The medians are shown as bars.

3.3.3 Leukocytes

For this study, an antibody panel was employed to compare the frequency of leukocyte sub-populations in dementia patients and healthy controls in more detail. Within the CD45+ population (a protein expressed by all leukocytes) the frequency of different leukocyte sub-classes was calculated. No differences were observed between patients and healthy controls in the frequency of NK-cells (CD16+CD56+CD3-), NKT-like cells (CD16+CD56+CD3+), T-cells (CD3+), monocytes (CD14+) or B-cells (CD19+), (Fig. 3.27). As expected, in the elderly and particularly AD patients, the percentage of B-cells was low. Because of this, B-cell subsets could not be analysed due to insufficient cell numbers. The frequencies of monocytes covered a large range and showed similar median values for all groups (Fig. 3.27). These results were observed whether the activation marker CD69 or the scavenger receptor CD36 (which might play a role in amyloid beta uptake) were included or not (Fig. 3.28).

3.3.4 Amyloid beta uptake

$A\beta$ is one of the hallmark features of AD. The amyloid beta hypothesis proposes different roles that this protein may have in AD pathology (see Section 1.1.2). $A\beta$ promotes tau hyperphos-
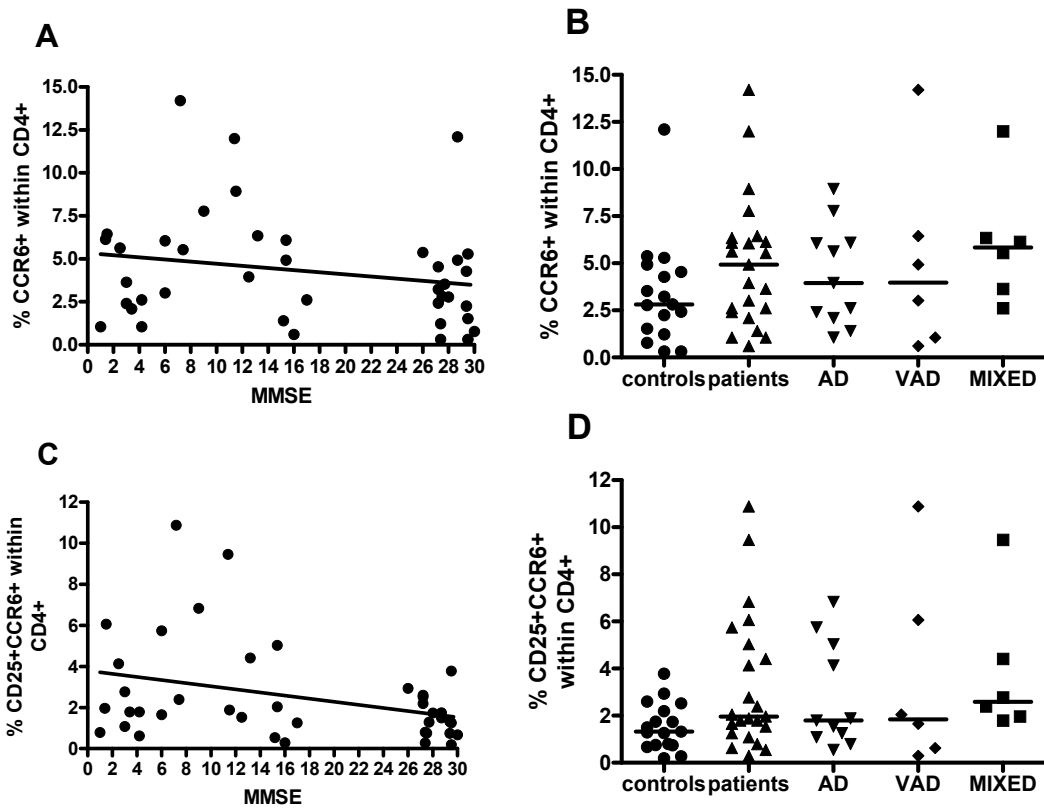
Figure 3.24: **Frequency of CCR6 expressing CD4+ T-cells in dementia and healthy controls**
The percentages of total CCR6+CD4+ (A) and CD25+CCR6+CD4+ (C) cells were plotted against the
corresponding patient MMSE values(p = 0.1954 for (A) and p = 0.0310 for (C)). In (B) the total CCR6+
percentages for healthy elderly and the different diseases are given (AD, VaD, mixed dementia) and in
(D) CD25+CCR6+ cells. The only significant differences were in the case of healthy controls compared
with mixed dementia (p = 0.0328 for panel B and p = 0.0157 for D). These differences were assessed
with Mann-Whitney test and statistically significant differences were no longer significant after
Bonferroni correction.
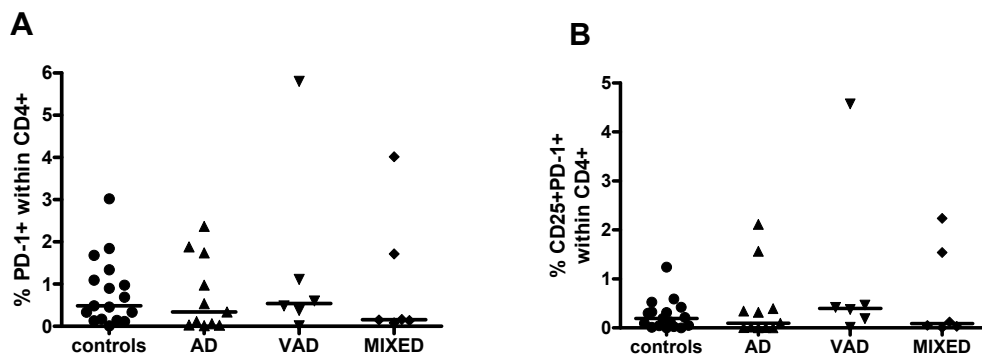
Figure 3.25: **PD-1 expression frequency on T-cells of dementia patients and healthy controls**
Frequencies of PD-1+ (A) and CD25+PD-1+ (B) CD4+ T-cells of healthy and patients (AD, VaD, mixed
dementia). No significant differences between the groups were observed with Mann-Whitney tests.
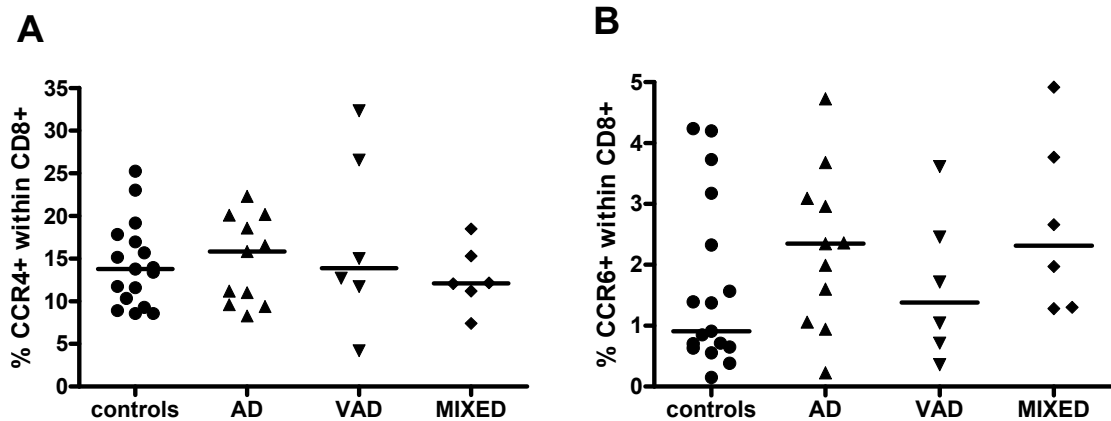
Figure 3.26: **Frequency of CD8+ T-cells expressing CCR4 and CCR6 in dementia and healthy controls**
CCR4 (A) and CCR6 (B) expression on CD8+ T-cells of healthy elderly and patients (AD, VaD, mixed dementia)

phorylation which leads to microtubule depolymerisation, impairing axonal transport. $A\beta$ also regulates protein degradation by the proteasome and may act as an antigen for certain immune cells. A method of reducing the negative effects of $A\beta$ is through promoting its phagocytosis by brain macrophages. Giving the function of these macrophages may be less effective in AD, the recruitment of monocytes from the periphery into the brain might assist in reducing the pathology caused by $A\beta$. Because of those roles, the capacity of blood leukocytes to take up $A\beta$ was investigated in this study.

After incubating frozen thawed blood for 17 h with amyloid beta, the degree uptake of $A\beta$ was determined. Mean fluorescence intensity (MFI) of HiLyte Fluor dye conjugated to amyloid beta was used as a measure of $A\beta$ uptake by PBMCs. To assess the degree of uptake, HiLyte fluor MFI of PBMCs incubated with HiLyte-conjugated $A\beta$ was divided by the MFI of PBMCs that were incubated in parallel without $A\beta$ in order to express the fold increase in fluorescence. This was performed for total leukocytes as well as monocytes (CD14+ cells within the CD45+ population) (Fig. 3.29). No significant differences between patients (AD, VaD and mixed dementia were considered as a single group) and controls were observed, but in contrast to the controls which all showed similar low levels of fluorescence, a small number of dementia patients showed relatively high fluorescence values. These findings suggest that leukocytes of some individual dementia patients may be more effective in taking up $A\beta$ than healthy individuals.

3.3.5 Cytokine profile

As cytokines can serve as biomarkers for inflammation, six different cytokines were measured in the plasma of dementia patients and healthy controls with a BD CBA multiplex kit. In almost all cases, the level of detected cytokine was below the sensitivity range of the multiplex kit used to measure it. Results are therefore not shown.
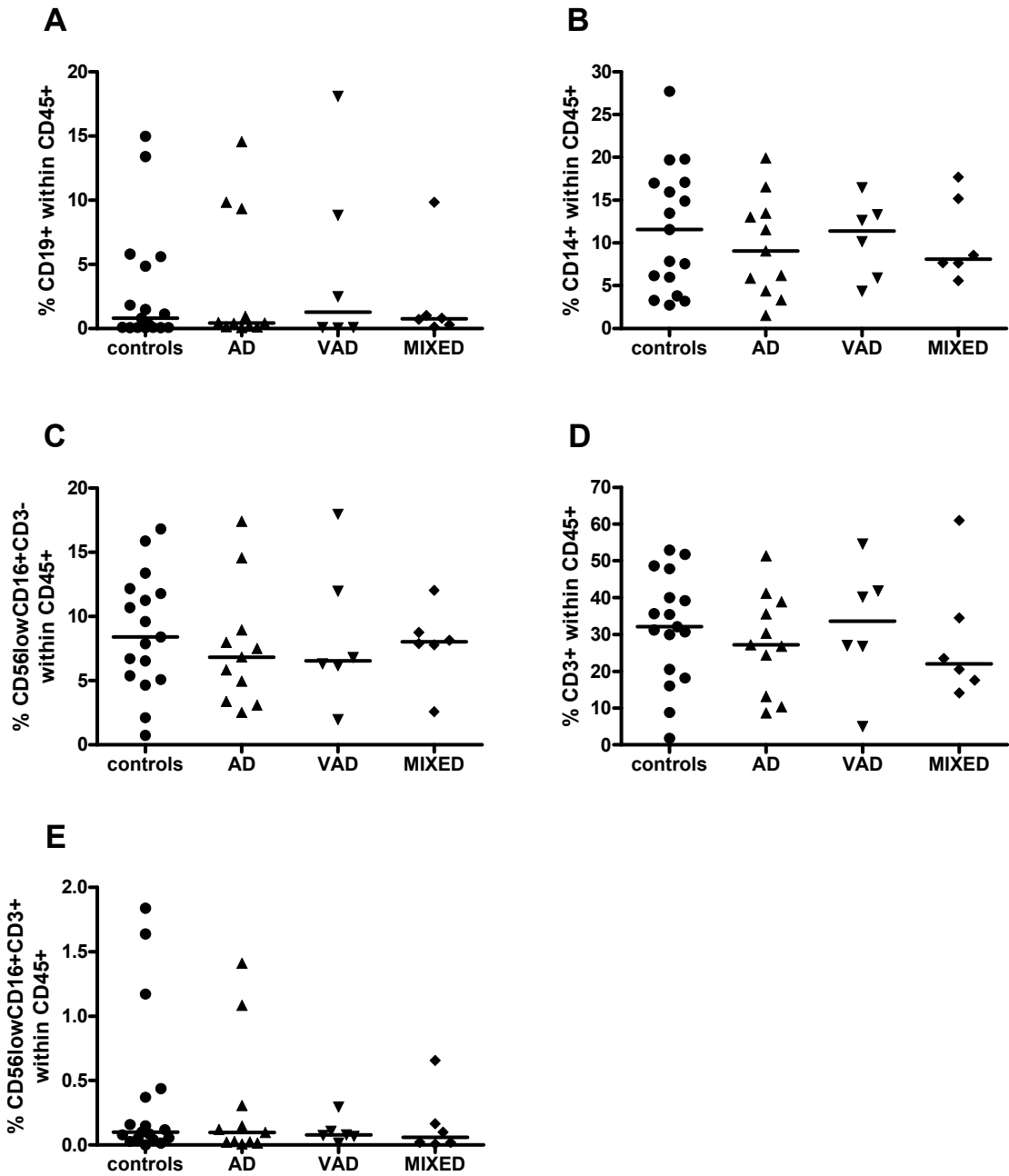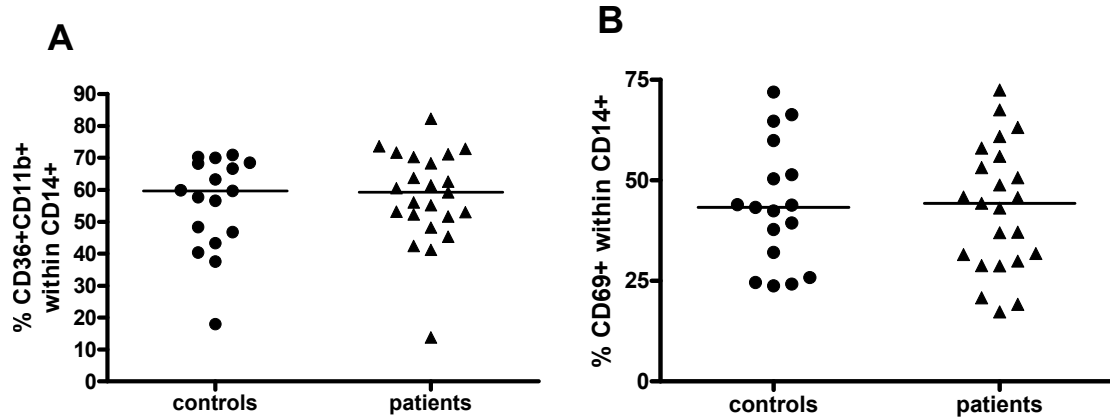
Figure 3.27: **Leukocyte subset frequency in dementia patients and healthy controls**
Percentages of B-cells (CD19+, A), monocytes (CD14+, B), NK-cells (CD56lowCD16+CD3-, C),
T-cells (CD3+, D) and NKT-like cells (CD56lowCD16+CD3+, E) within the total leukocyte population
(CD45+). Bars represent median values.

Figure 3.28: **Frequency of monocyte phenotypes in dementia patients and healthy controls**
Percentages of CD36+CD11b+ (A) and CD69+ cells (B) within the CD14+ leukocytes of healthy
controls and patients (AD, VaD and mixed dementia were grouped together). Bars show the
corresponding median values. Mann-Whitney test showed no significant differences between groups.
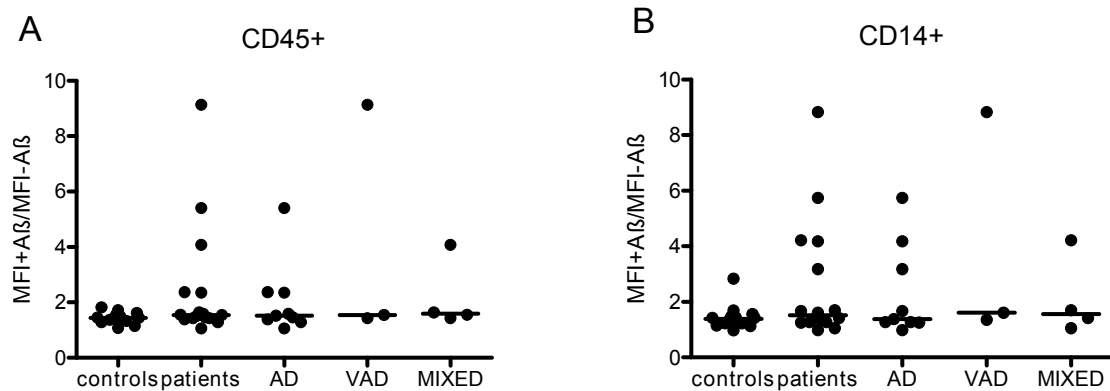
Figure 3.29: **Amyloid beta uptake by leukocytes in dementia patients and healthy controls**
Aβ uptake by the total leukocyte population (CD45+) and monocytes (CD14+). The fold fluorescence
increase of samples incubated with fluorochrome-labelled Aβ compared with samples not incubated with
Aβ is given on the Y-axis. Comparing controls with dementia patients (AD, VaD, mixed dementia)
showed there to be no significant difference between groups.

3.3.6 CMV serostatus

Plasma titre of anti-CMV IgG was measured with a CMV ELISA kit in order to determine the CMV infection status of dementia patients and healthy controls. This was performed because of the likelihood that CMV infection influences the immune signatures of interest in this study. The results indicated that 39 of the 40 subjects were CMV-seropositive. Therefore, it was not possible to distinguish CMV-associated influences on immune signatures. Preliminary analysis indicated that the anti-CMV IgG titres was also not associated with differences in immune signatures.

3.4 Comparison of rpAD, MCI, AD and healthy immune status

The final cohort of patients investigated was a more diverse group including rapidly progressive AD (rpAD) patients. The aim of this project was to characterise the special form of AD that is rpAD. This disease is often misdiagnosed as Creutzfeldt-Jakob disease. Patients examined in this study had a median age of 73 and a median survival of 26.4 months from diagnosis. The parameters examined in this study were tau and amyloid beta CSF levels, genetic factors such as *PRNP* (prion protein gene) and ApoE and comorbidity analysis. It was observed that amyloid beta levels were decreased in AD and rpAD compared to healthy controls, while tau levels were increased in AD but not rpAD. The ApoE-E4 distribution was different for rpAD compared to AD in that it was more similar to the healthy controls. In addition, frozen whole blood was analysed for immune profiles. The patients have either AD, rpAD, MCI or other dementias as shown in Table 2.5. T-cell and leukocyte analysis (n = 20) and chemokine receptor surface marker analysis (n = 30) was performed in this sample cohort.

3.4.1 T-cell phenotypes in AD patients and healthy controls

In the previous studies differences in the T-cell subsets were found between AD patients and healthy controls. Therefore also in these cohorts T-cell phenotypes were investigated. Percentage frequencies of CD4+, CD8+ and $\gamma\delta$T-cells within the CD3+ cells were not observed to be different between AD patients and healthy controls (Fig. 3.31). Only in the case of rpAD was a slight trend of more CD8+ and $\gamma\delta$ cells observed (Fig. 3.30). However a more detailed analysis of the T-cell subsets did reveal differences. The expression of CD27 and CD28 on CD4+ (Fig. 3.31) and CD8+ (Fig. 3.33) T-cells differed between the groups. The percentage of early-differentiated CD27+CD28+ cells was reduced in AD patients compared to healthy controls (p = 0.0156) and the percentage of late-differentiated CD27-CD28- cells was also increased (p = 0.054). These results were consistent when using more surface markers to characterise these cell types. Naïve cells - characterised as CD27+CD28+ CD45RA+CD45RO- - are more frequent in healthy controls than AD patients (p = 0.0130) and late memory cells - defined by CD27-CD28-CD45RO+ - less frequent (p = 0.0466) (Fig. 3.32). In the CD8+ compartment, the percentages of late-differentiated T-cells were higher in AD patients compared to healthy elderly (p = 0.019 for CD27-CD28- cells and p = 0.0211 for CD27-CD28-CD45RO+ cells).

Another observation was that of the large range of percentage frequencies of CD27-CD28- cells in the AD patients. It was speculated that this may be explained by different disease stages given that MMSE values ranged from 0 to 27, but statistical analysis showed no correlation. CMV infection status may account for these differences as well, even though CMV is not known to influence CD4+ T-cells. Separating healthy donors according to CMV+ and CMV- status and AD patients into low and high anti-CMV IgG titre failed to explain differences; the percentages of CD27+CD28+ and CD27-CD28- in these groupings did not change appreciably (Fig. 3.35). Within the CD8+ compartment, slight CMV-associated differences were observed, in accordance with previously published studies. In the study by Kujipers *et al.* late-differentiated CD8+ T-cells (defined as CD45RA+CD27-) appeared in association with acute CMV infection and expansion of these cells appeared to be unique for CMV-seropositive individuals [75]. In the present study, the percentages of late-differentiated CD27-CD28- CD8+ cells were observed to be slightly higher in AD patients and healthy controls that showed the highest levels of anti-CMV IgG titre (Fig. 3.36).
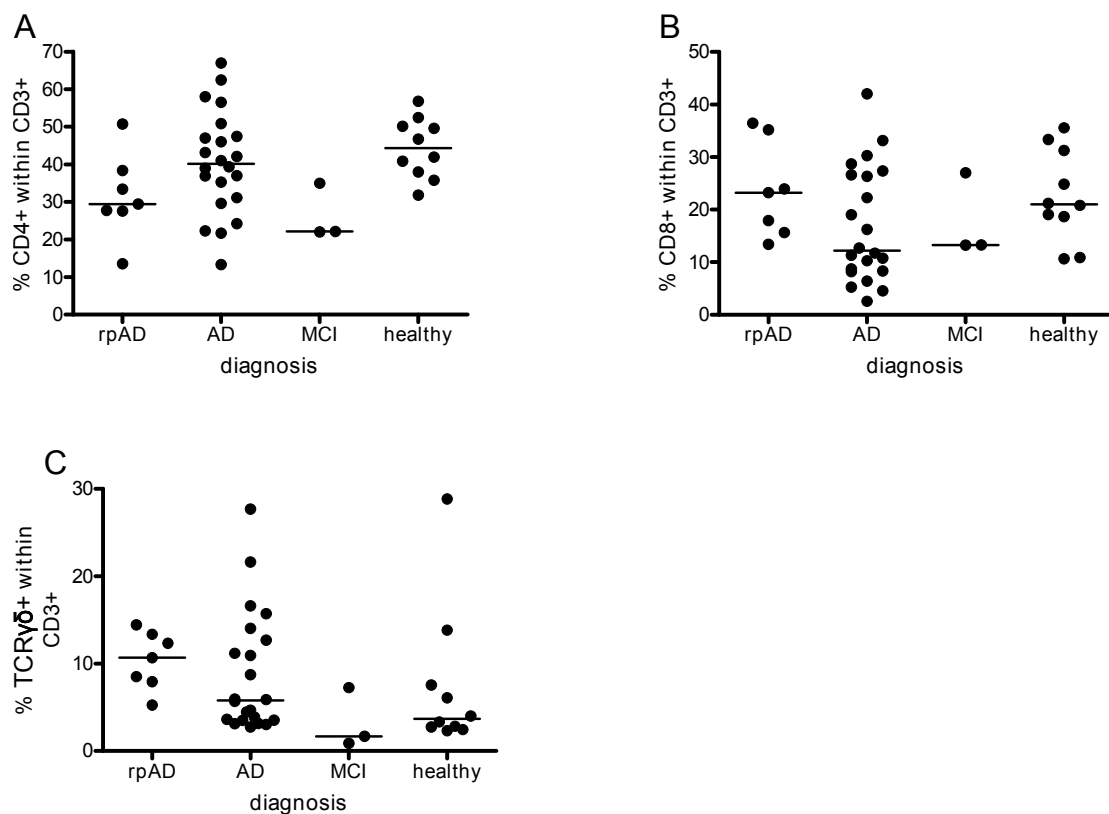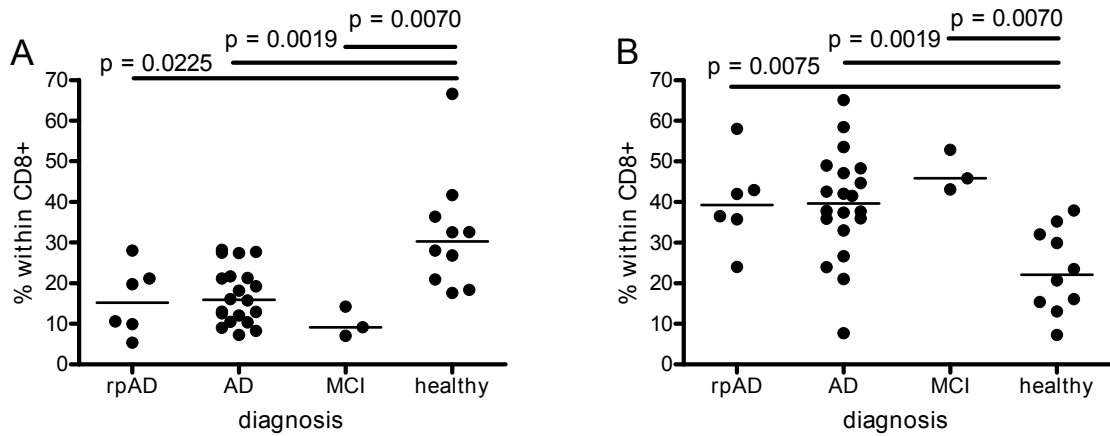

Figure 3.30: **T-cell subsets in AD patients and healthy controls**
Frequencies of CD4+ (A), CD8+ (B) and $\gamma\delta$T-cell subsets in AD patients and healthy controls.
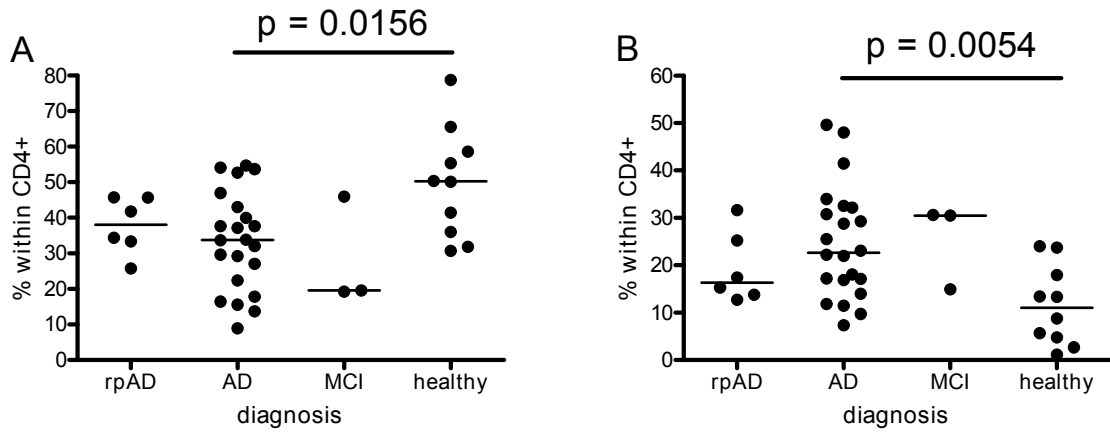
Figure 3.31: **Early and late-differentiated CD4+ T-cells in AD patients and healthy controls**
Frequencies of early-differentiated CD4+ T-cells defined as CD27+CD28+ are shown in (A) and late-differentiated CD27-CD28- CD4+ T-cells in (B). Bars indicate median values. Displayed p values are the result of Mann-Whitney test.
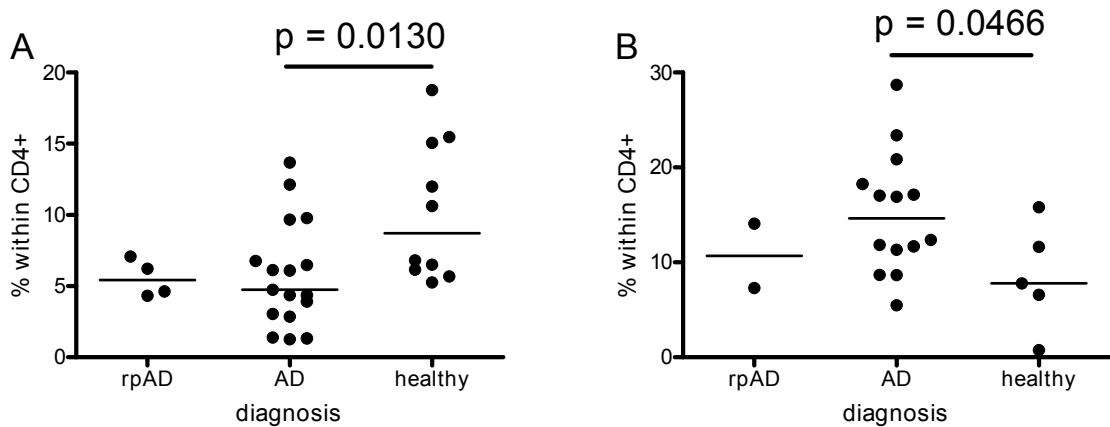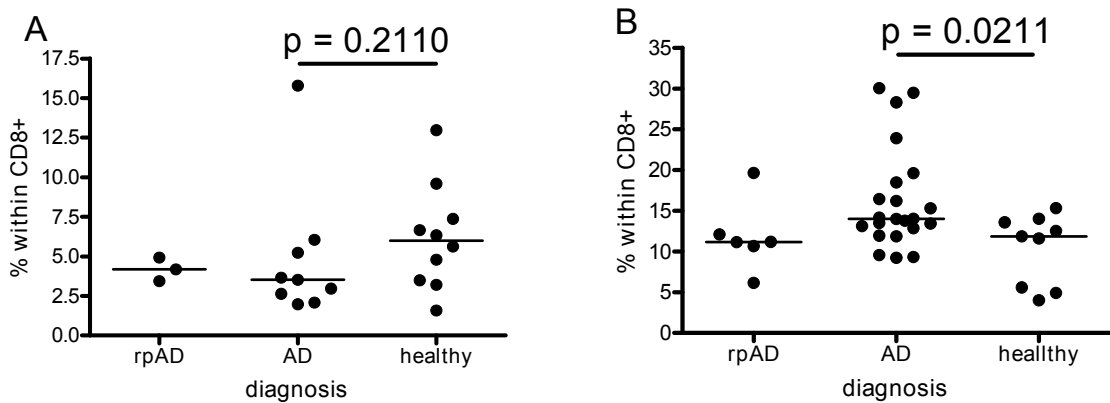

Figure 3.32: **Naïve and memory CD4+ T-cells in AD patients and healthy controls**
Frequencies of early-differentiated CD4+CD27+CD28+CD45RA+CD45RO- T-cells (A) and late-differentiated CD4+CD27-CD28-CD45RO+ T-cells (B) in AD patients and healthy controls. p values derive from Mann-Whitney tests.

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Figure 3.33: **CD27 and CD28 expression on CD8+ T-cells in AD patients and healthy controls**

The CD27 and CD28 double positive population is shown in (A) and the double negative in (B). p values derived from Mann-Whitney test are given above. Significant differences were only observed when comparing the healthy elderly with the AD patients; patient sub-groups were not observed to be different (p values not shown).
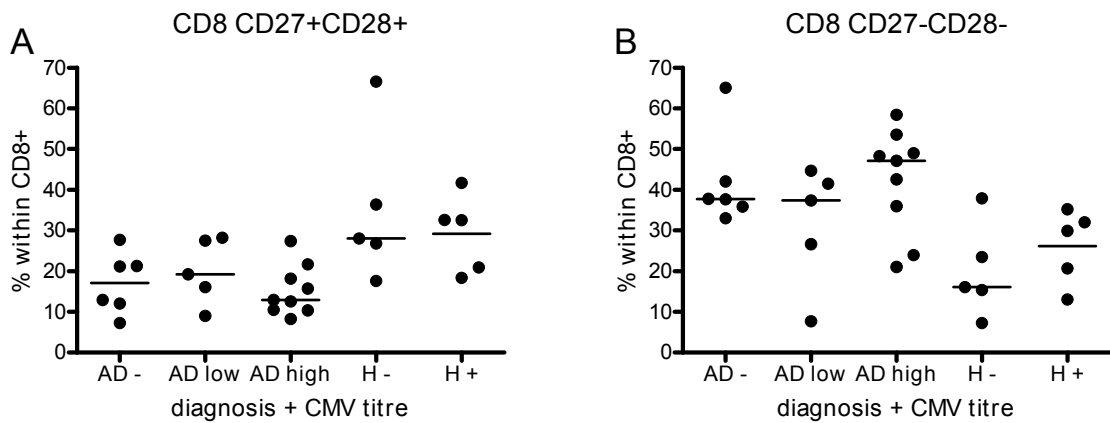

Figure 3.34: **CD8+ T-cell phenotypes in AD patients and healthy controls**

Frequencies of early-differentiated CD8+CD27+CD28+CD45RA+CD45RO- T-cells (A) and late-differentiated CD8+CD27-CD28-CD45RO+ T-cells (B) in AD patients and healthy controls. Bars represent median values
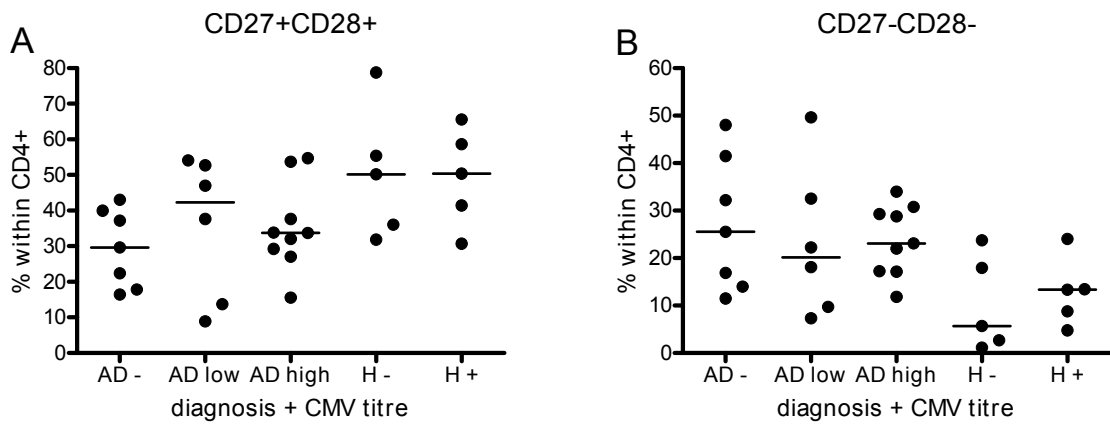
Figure 3.35: **Association of CMV with CD4+ T-cell phenotypes**
Expression of CD27 and CD28 on CD4+ T-cells analysed in association with the CMV infection status. The patients and healthy controls were grouped according their anti-CMV IgG titre: negative < three U/ml < low < five U/ml < high. Due to low sample numbers for the healthy donors "low" and "high" groups were considered together as a single CMV+ group.

Figure 3.36: **Correlation of anti-CMV IgG titre with CD8+ T-cell phenotypes**
CD27 and CD28 expression on CD4+ T-cells in association with CMV status. The patients and healthy controls were grouped according their anti-CMV IgG titre: negative < three U/ml < low < five U/ml < high. Due to low sample numbers for the healthy donors, "low" and "high" groups were considered together as a single CMV+ group.

3.4.2 Tregs

For this part of the investigation, activated and regulatory CD4+ T-cells were analysed. In the inflammatory milieu *in vivo* amyloid beta and cytokines secreted by activated brain cells may stimulate T-cells in the periphery. In the first pilot study lower percentages of CD25+ CD4+ T-cells in patients with mild AD were observed compared to healthy controls. Tregs are positive for CD25, but not all CD25+ cells are Tregs. In order to clearly identify Tregs, more markers were used in this study. Combining Foxp3 with CD127, Tregs can be characterised as CD25+CD127lowFoxp3+. All three markers were included in the antibody panel for this study, but due to the limited cell number in the samples, maximum of two markers were used together. Looking at the CD25+ population within CD4+ T-cells showed a clear increase in AD patients compared to healthy controls. A non-significant trend of increased percentages of CD25+Foxp3+ and Foxp3+CD127low cells in AD patients was observed (Fig. 3.37). Another marker for Tregs that was not included in these experiments is CCR4, but the chemokine receptor panel (Fig. 2.1), that was performed in parallel, indicated that higher percentages ($p = 0.0454$) of CCR4+CD4+ T-cells were observed in AD patients compared to controls (Fig. 3.47).

3.4.3 Other leukocytes

As in the Chieti study, a panel was created for this study in order to analyse different leukocyte populations. After selecting the leukocytes in the FSC-SSC plot, the singlets were gated and the living CD45+ cells selected. This fraction was analysed for the expression of the following markers: CD3 to identify T-cells, CD19 in the case of B-cells, CD14 for monocytes, and CD56 and CD16 for NK-cells. Figure 2.2 shows an example of how these populations were gated.

The percentages of T-cells for AD patients and healthy controls are shown in Figure 3.38A. The percentages of CD3+ cells were increased in AD patients compared to healthy individuals. The CCR panel was not employed to analyse CD3+ and CD45+ cells, but CCR analysis required that these markers be included (Fig. 3.38B). Thus, the CD3 and CD45 frequencies may be compared across two independent experiments. Due to exclusion of FSC high (presumably activated) cells and perhaps due to the use of different CD3 antibodies conjugated to different fluorochromes (CD3-Alexa Fluor instead of CD3-PerCP) the percentages of CD3+ were observed to be higher in general for the CCR experiment, but overall both experiments show similar results in that all patient groups showed higher percentages of T-cells. In the third image (C) in Figure 3.38 the CD3+ frequencies are plotted against the MMSE values of the corresponding patients. For this analysis, all patients have been included, irrespective of dementia type. The results of this analysis indicate that the frequency of T-cells is not altered with disease severity. These results of lower T-cell frequencies in healthy elderly differ from those obtained in the Chieti study (Section 3.3), where no differences between patients and controls were observed with respect to the frequency of CD3+ cells. More studies are needed in order to clarify the relationship between the frequency of T-cells and AD.

The next part of this study investigated NK-cells. In order to exclude NKT-like cells, NK-cells were defined as CD56lowCD16+CD3- (Fig. 3.39B). First CD56 and CD16 were gated and then CD3, but due to limited numbers of CD56lowCD16+ cells not always. As thereby some dif-
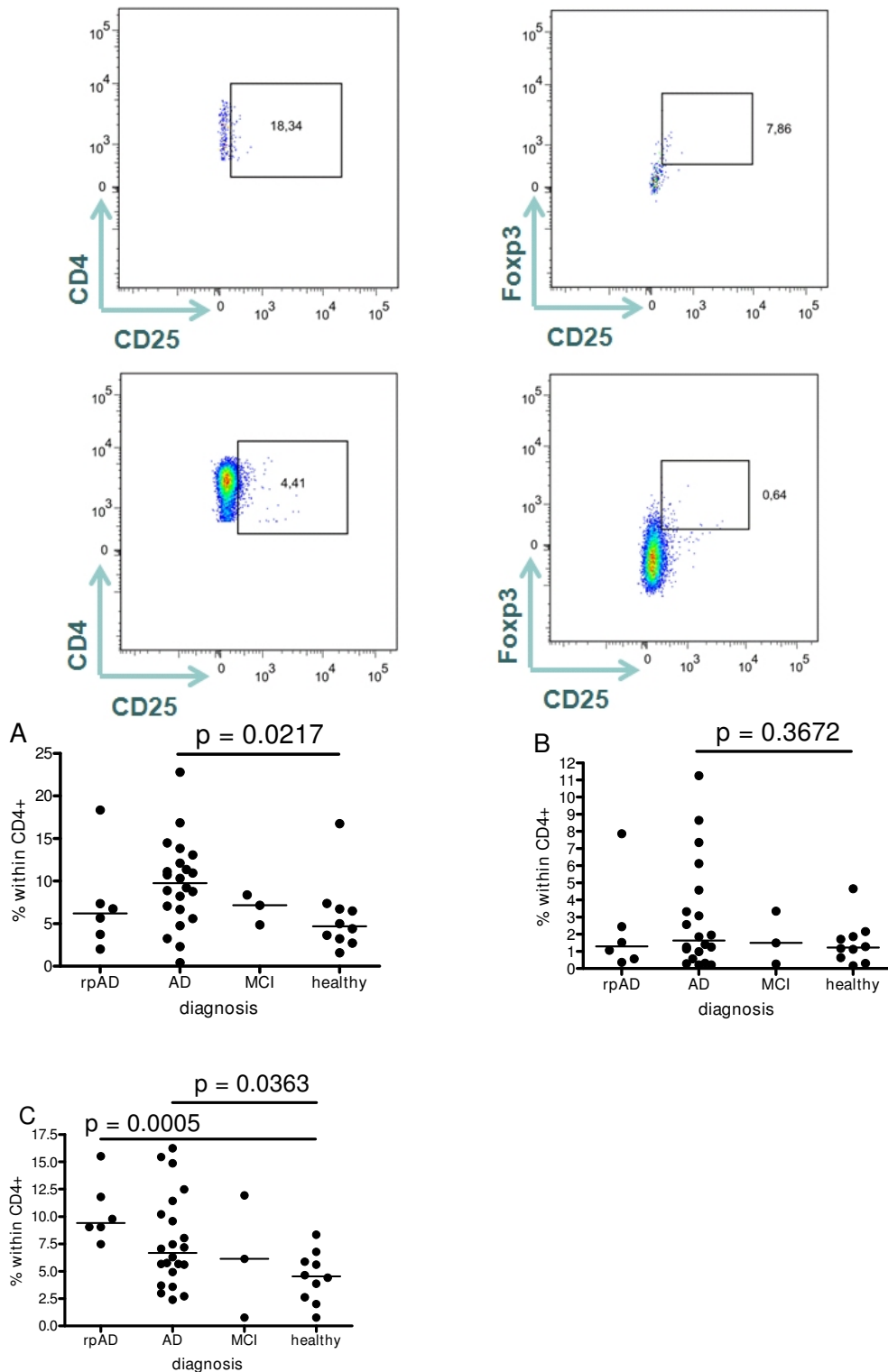
Figure 3.37: **Treg gating strategy and frequencies of Tregs in patients and healthy controls**
FlowJo images with gating applied to PBMCs from a representative AD patient (upper images) and a
healthy elderly control (lower images). Percentages of CD25+ T-cells (A), CD25+Foxp3+ T-cells (B)
and Foxp3+CD127low T-cells (C) within the CD4+ T-cells in rpAD, AD patients and healthy elderly.

ferences might not be detected any more, the frequencies of CD56CD16+ was analysed as well (Fig. 3.39A). This is reasonable, because the frequencies of NKT-like cells only represent a very small fraction of CD56lowCD16+ cells. The results of this analysis indicate that there were no differences between patients and healthy controls with respect to the frequency of NK-cells.

Another leukocyte subset considered were monocytes. From the whole CD45+ population, the percentage frequency of CD14+ cells was determined. The outcome of these experiments indicated an increase in monocyte frequencies in AD patients compared to healthy controls, but after Bonferroni correction was not statistically significant (the p value of 0.014 is slightly above the required significance threshold of 0.01) (Fig. 3.40A). The frequency of CD14+ cells was also investigated in the CCR study (Section 3.4.5) (Fig. 3.40B). A similar trend of higher percentages of CD14+ cells in AD patients compared to the healthy controls, was observed, but with a greater p value (0.0969). Noteworthy is that both studies employed the same antibody (Qdot655-conjugated anti-CD14), therefore these differences may be explained by the use of different PBMC donors from the same cohort of healthy controls. To investigate these results in more detail, the MMSE values from these patients was plotted against the percentages frequencies of CD14+ cells (Fig. 3.40C). The results of this analysis were not supportive of the trend observed in Figure 3.40 A and B. Figure 3.40D shows a more detailed characterisation of monocytes in AD patients and healthy controls which included CD40. The results were not different between AD patients and controls.

### 3.4.4 B-cell phenotype frequencies in AD patients and healthy controls

Frequencies of different B-cell phenotypes were investigated in addition to the previously presented results for T-cells in order to assess potential differences between AD patients and healthy controls. According to a phenotype model that includes IgD and CD27, B-cells were divided into four differentiation states. Naïve cells were characterised as IgD+CD27-. As shown in Figure 3.41A, the percentage frequency of these cells was slightly lower in AD patients compared with healthy controls. In contrast, cells with a phenotype corresponding to late differentiation stages (Fig. 3.41 C and D) were observed to be more frequent in AD patients. For the IgD-CD27+ late-differentiated cells, the non-significant trend of higher frequencies of this cell type was true for all AD patients (Fig. 3.41D), but for the IgD-CD27- late differentiated cells, this was only observed in AD patients - not in rpAD or MCI patients. This trend of a shift towards late differentiated B cell phenotypes in AD patients is consistent with the observation for CD4+ T-cells. Increased frequencies of late differentiated CD4+ T-cells were observed in AD patients compared to healthy controls (Fig. 3.32). The MMSE values for the patients was plotted against the frequencies of early- and late-differentiated B-cells, but no significant correlations were observed (Fig. 3.41 E and F). In addition, CD40 expression on CD19+ B-cells was examined (Fig. 3.42). The results indicate that there was a wide range of percentages of CD40+ B-cells across dementia patients and healthy controls. Comparing the median values for the respective groups, healthy elderly controls were observed to be higher, although this was not statistically significant. There was no association between the frequency of CD40+ B-cells and disease severity as measured by MMSE scores (Fig. 3.42B).
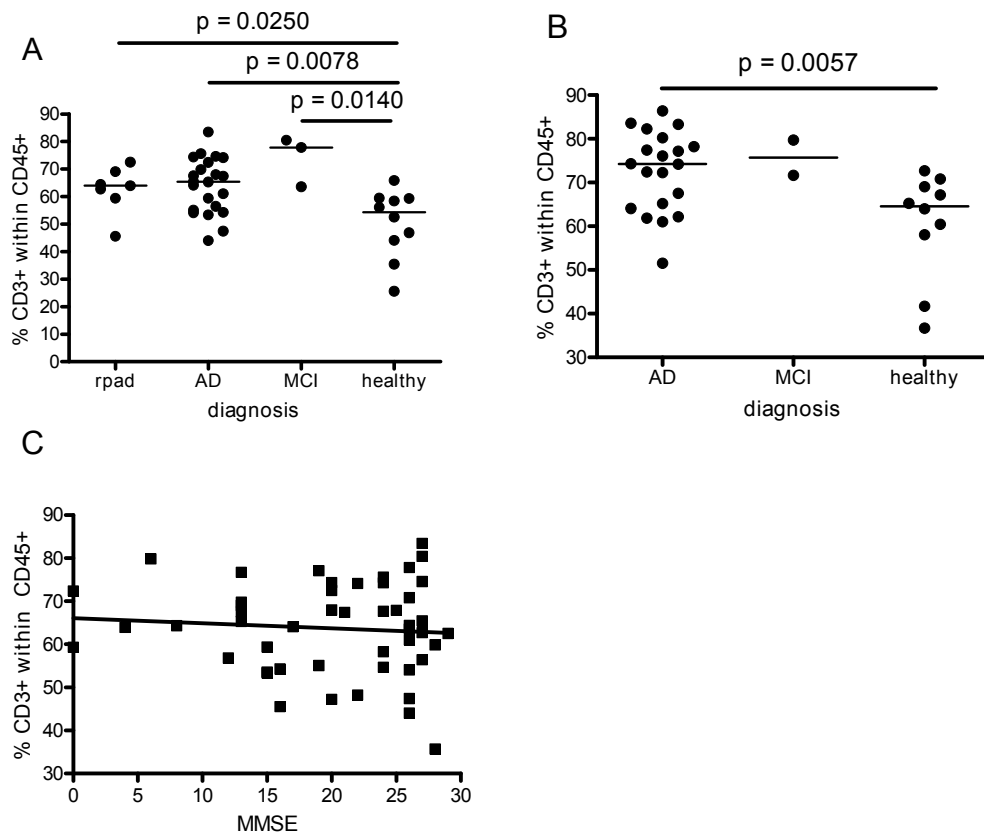
Figure 3.38: **frequency of T-cells in dementia and healthy controls**
Percentages of CD3+ cells within the total CD45+ population in dementia patients and healthy controls.
For A n = 42 and B n= 28.
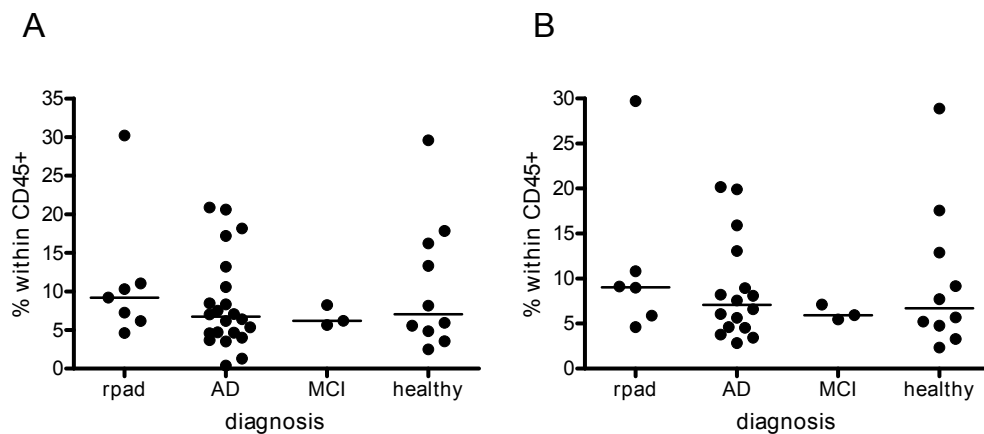
Figure 3.39: **Frequency of NK-cells in dementia and healthy controls**
Percentages of CD56lowCD16+ cells (n = 43) (A) and percentages of CD56lowCD16+CD3- cells (n = 35) (B) from CD45+ cells in patients with rpAD, AD and MCI and in healthy controls. Donors with insufficient CD56lowCD16+ cell numbers (n<100) were excluded from the analysis in panel B.
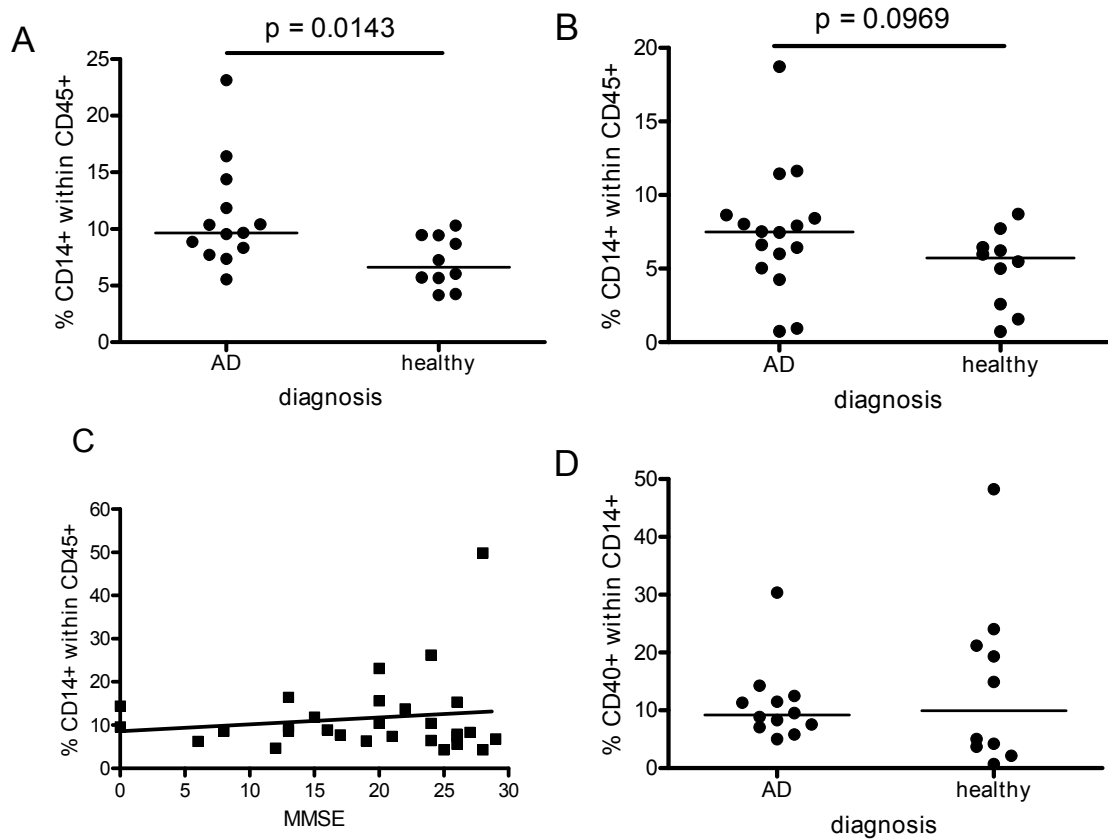
Figure 3.40: **Frequency of monocytes in AD patients and healthy controls**
CD14+ expression in; (A) on PBMCs used in the leukocyte analysis study (n = 23) and (B) on PBMCs in the CCR study. The CD14+ percentage frequencies of all donors of the entire study (n = 29), were plotted against the MMSE values of patients (C). D shows the frequencies of CD40+ cells out of the CD14+ cells in panel A.
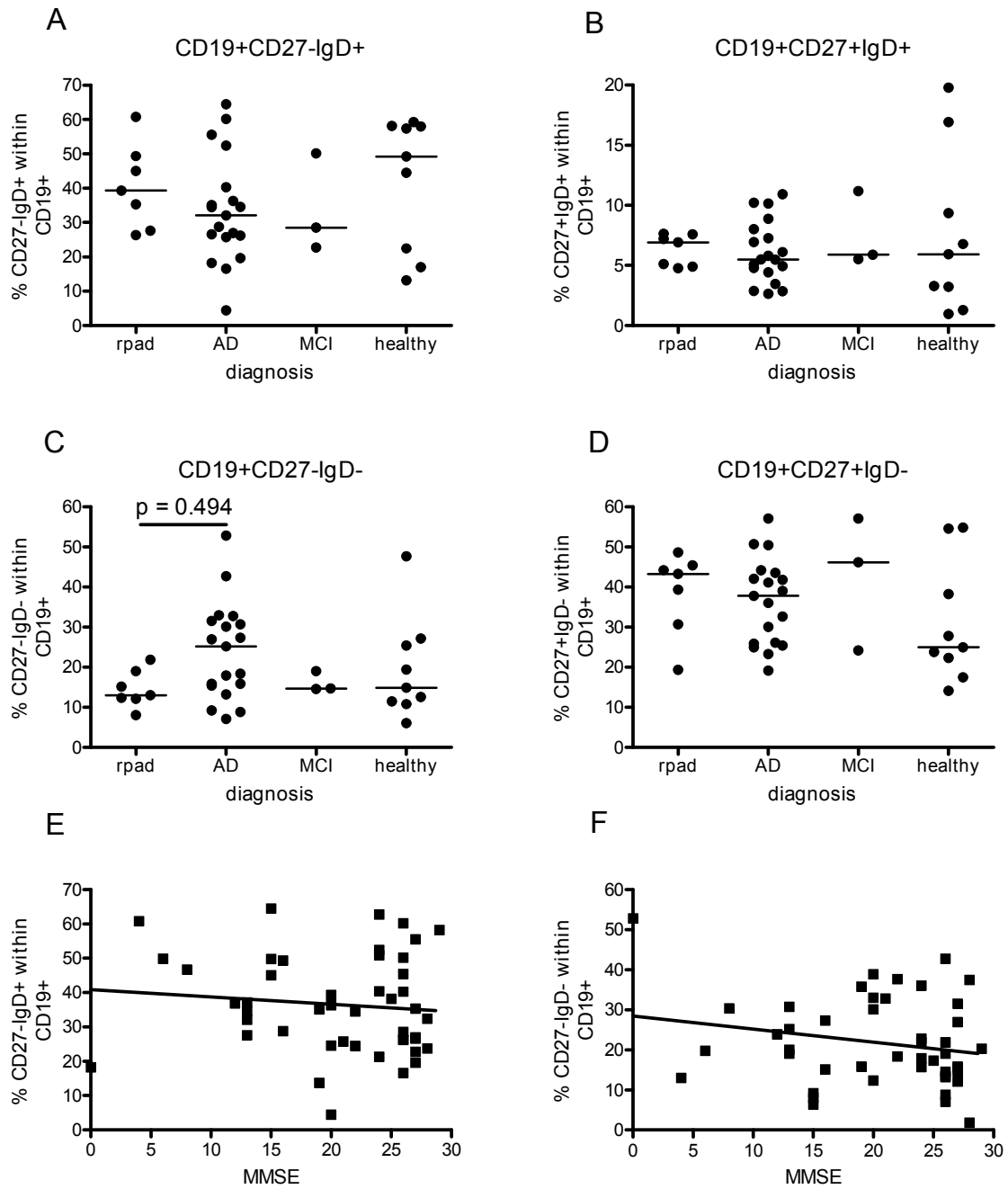
Figure 3.41: **B-cell phenotypes in dementia patients and healthy controls**
Four B-cell (CD19+ cells) differentiation stages defined according to the expression of IgD and CD27. The corresponding gating strategy is shown in Figure 2.2 and the resulted percentage frequencies are presented here in panels A to D. The percentages of E and F show the same values of A and D, but are plotted against the MMSE values of the patients. Figures shown in panel E and F include all 48 patients of this study, not just those in the 4 categories shown in A to D. Thus pseudodementia and Parkinson's Disease patient are also included.
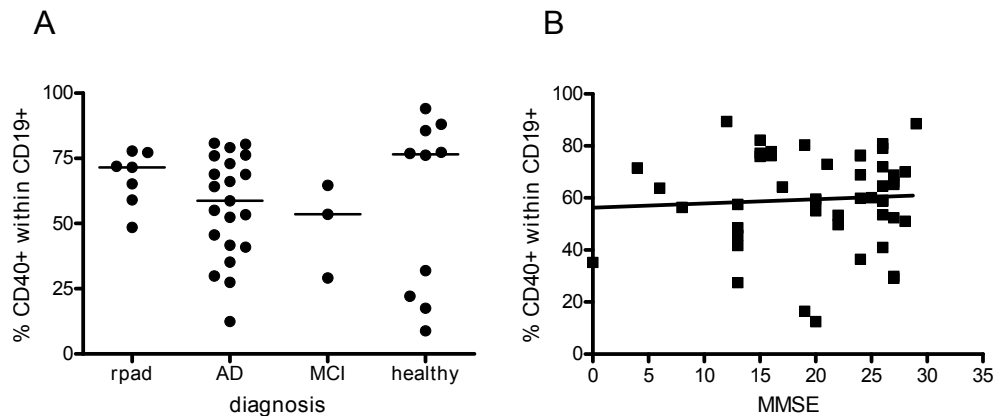
Figure 3.42: **B-cell phenotypes in dementia patients and healthy controls**
Percentages of CD40+CD19+ cells in different dementia groups and healthy controls (rpAD, AD, MCI and healthy elderly) (A) and percentages of CD40+ B-cells plotted against the MMSE values of the patients. For (B), the entire dementia cohort, including pseudodementia and Parkinson's disease, has been analysed.

3.4.5 Chemokine receptor expression

Brain and periphery may be more closely related in conditions such as AD due to a defective blood-brain barrier that is typically associated with AD pathology. This may allow the migration of leukocytes into the brain via chemotaxis that would not normally occur. Therefore, this study investigated the expression of chemokine receptors on different leukocyte populations. The results indicated that CD25+ cells within the CD45+ leukocyte population were increased in AD patients compared to age-matched controls. In addition, the cell exhaustion marker, PD-1, was observed to be more frequent in AD patients (Fig. 3.43). Following this, chemokine receptor expression was investigated. The expression of CCR2 and CCR4 was observed to be slightly higher in AD patients compared to controls (Fig. 3.44). In contrast, a greater difference in the frequency of CCR5 (p = 0.0512) and CCR6 (p = 0.0138) was observed in AD patients and healthy controls (Fig. 3.45). Given the significant result for CCR6, a more detailed investigation took place in which monocytes (CD14+), B-cells (CD19+) and T-cells (CD3+) were analysed separately for the frequency of CCR6 expression. This analysis indicated that CCR6 expression was more frequent on all of these leukocyte types in AD patients compared to healthy controls (Fig. 3.46). Analysing the T-cells in more detail indicated that CD4+ T-cells more frequently express CCR4 (p = 0.0454), CCR5 (p = 0.0490) and CCR6 (p = 0.0009) in AD patients compared to controls, while the same trends were observed for CD8+ T-cells, significant results were only observed for CCR6 (p = 0.0006) (Fig. 3.47).

In addition to the analysis of percentage frequencies, the mean fluorescence values were obtained (data not shown), These results were similar in that CCR2, CCR4, CCR5 and CCR6 tended to be higher in AD patients compared to healthy controls, thereby supporting the results obtained as a percentage frequency.
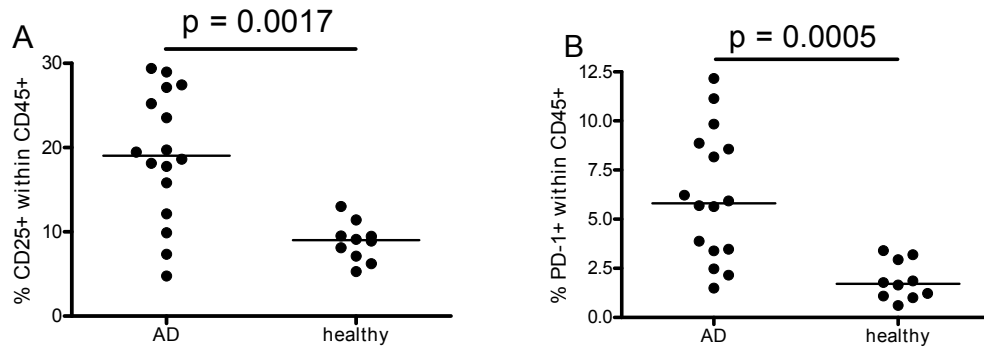
Figure 3.43: **Percentages of activated and exhausted leukocytes in AD patients and healthy controls**

Percentage frequencies of CD25+ (A) and PD-1+ (B) cells within the CD45+ in AD patients and healthy controls are presented.
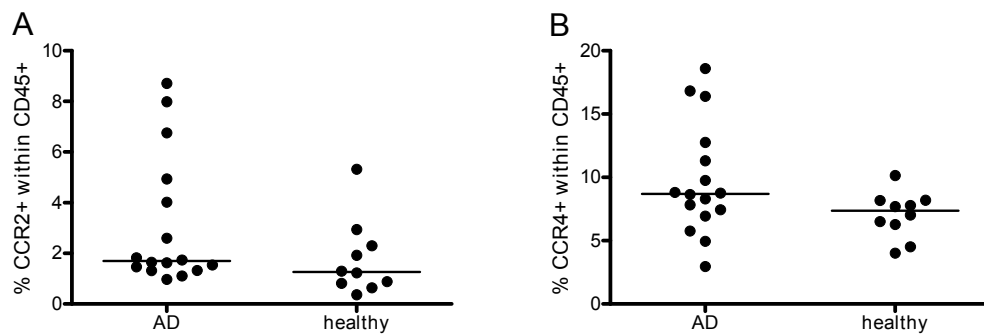
Figure 3.44: **CCR2 and CCR4 expression on CD45+ cells in AD patients and healthy controls**

Percentage frequencies of CCR2+ (A) and CCR4+ (B) cells in total CD45+ leukocyte population. Bars represent median values.
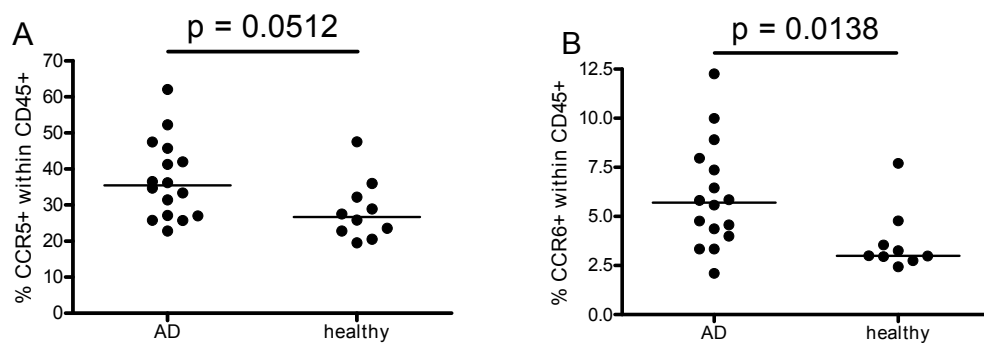
Figure 3.45: **CCR5 and CCR6 expression on leukocytes in AD patients and healthy controls**

Percentage frequencies of CCR5+ (A) and CCR6+ (B) leukocytes (CD45+)
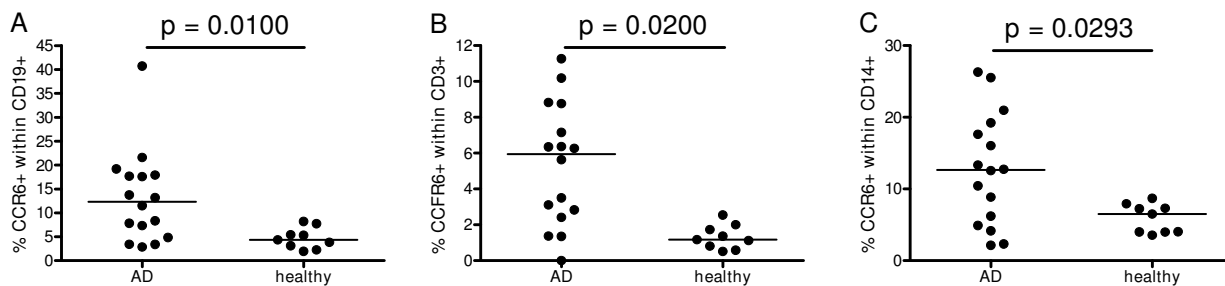
Figure 3.46: **CCR6 expression on leukocyte subsets in AD patients and healthy controls**
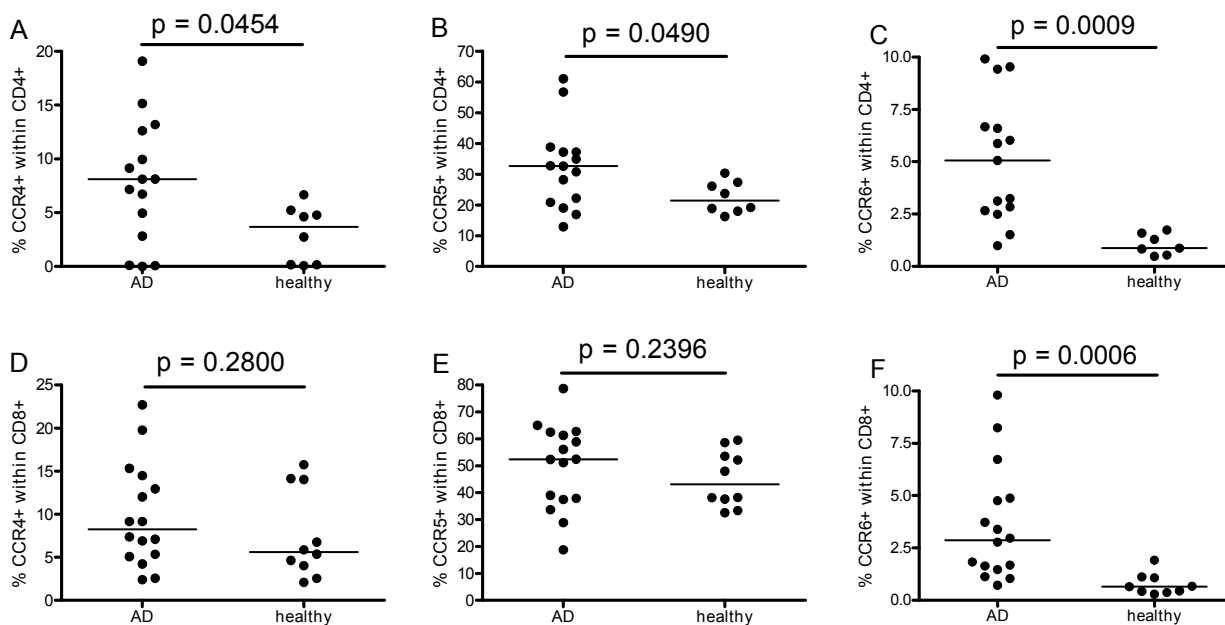Percentage frequencies of CCR6 on CD19+ B-cells (A), CD3+ T-cells (B) and CD14+ monocytes (C).

Figure 3.47: **Chemokine receptor expression on T-cell subsets in AD patients and healthy controls**
Expression of CCR4 (A and D), CCR5 (B and E) and CCR6 (C and F) on CD4+ and CD8+ T-cells.

Table 3.1: Summary of the results obtained in the different studies for this thesis

% of	within	Canada 1	Palermo	Canada 2	Chieti	Göttingen
CD3+	CD45+				=	+, AD*
CD4+	CD3+	-, mAD *	=	=	=	=
CD8+	CD3+	=	=	=	=	=
early-diff.	CD4+	-, mAD*	-, mAD*	-, mAD^t	-, D^t	-, AD*
late-diff.	CD4+	+, mAD*	+, mAD*	+, mAD*	+, AD^t	+, AD*
CD57+	CD4+		=	+, mAD*	=	
KLRG1+	CD4+		+, mAD*	+, mAD^t	=	
early-diff.	CD8+		=		=	-, AD*
late-diff.	CD8+		=		=	+, AD*
CD57+	CD8+		=		=	
KLRG1+	CD8+		+, mAD*		=	
CD25+	CD4+	-, mAD*	+, mAD*		=	+, AD*
Tregs	CD4+		=	+, mAD^t	=	+, AD^t
CD19+	CD45+				=	
early-diff.	CD19+					-, AD^t
late-diff.	CD19+					+, AD^t
CD14+	CD45+				=	+, AD^t
NK-cells	CD45+				=	=
NKTL	CD45+				=	
CCR2+	CD45+					+, AD^t
CCR4+	CD45+					+, AD^t
CCR4+	CD4+			+, AD^t	+, D^t	+, AD*
CCR4+	CD8+				=	+, AD^t
CCR5+	CD45+					+, AD^t
CCR5+	CD4+			+, AD^t		+, AD*
CCR5+	CD8+					+, AD^t
CCR6+	CD45+					+, AD^t
CCR6+	CD3+					+, AD*
CCR6+	CD4+			=	+, D^t	+, AD*
CCR6+	CD8+				+, D^t	+, AD*
CCR6+	CD14+					+, AD*
CCR6+	CD19+					+, AD*

diff = differentiated, NKTL = NKT-like cells, + = higher frequencies, - = lower frequencies, = = similar frequencies, * = significant, ^{t} = non significant trend, mAD = mild or moderate AD, D = dementia (AD, VaD, mixed dementia)

Chapter 4

Discussion

4.1 T-cell phenotypes: a shift from early- to late-differentiated CD4+ T-cells in AD patients

Early literature reports: CD45RA/CD45RO ratio as a diagnostic marker for AD

In AD patients, a state of inflammation is found in brain and in the periphery. As the BBB is compromised, there is an enhanced interaction between the brain and the periphery. Instead of analysing the brain or CSF, this study focused on blood lymphocytes which may have a use for diagnostic and treatment purposes. Amyloid beta is considered to be a chronic antigenic stimulus in AD. Monsonego and coworkers showed increased T-cell reactivity to Aβ-42 in the elderly and in AD patients [103]. When T-cells are stimulated, they become activated and differentiate. To monitor this process, the expression of two isoforms of a receptor that plays an important role in T-cell activation can be assessed. A transition from CD45RA to CD45RO occurs as T-cells undergo differentiation. Tan and collaborators considered this transition as a marker for AD. The authors found lower levels of CD45RA+ on CD4+ T-cells and a higher CD45RO/CD45RA ratio in AD patients, compared to healthy aged-matched subjects. Additionally, this study combined this immune phenotype with Apolipoprotein E (ApoE) genotyping. ApoE is used as a diagnostic marker for AD - the E4 allele is associated with a higher risk of developing AD [94]. The authors showed that assessing the frequency of CD45RA expression in combination with the ApoE-E4 allele or the CD45RO/CD45RA ratio is more predictive for the risk of developing AD than assessing ApoE alone. Therefore, the authors of this study postulated that CD45RA is a potential diagnostic marker for AD [149]. Because analysis of CD45RA expression alone is not sufficient to identify truly naïve T-cells, the studies presented in this thesis included further markers.

Pilot study: T-cell differentiation in AD according to the CCR7-CD45RA model

As reviewed by Appay *et al.*, naïve and memory T-cells can be characterised on the basis of cell surface markers that play roles in homing, proliferation and effector functions [6]. This thesis pilot study was based on a model employing CCR7 and CD45RA (Fig. 1.4, Section 1.2). CD4+

and CD8+ T-cells were divided into four differentiation stages: naïve (CD45RA+CCR7), central memory (CM, CD45RA-CCR7+), effector memory (EM, CD45RA-CCR7-) and terminally differentiated effector memory cells (TEMRA, CD45RA+CCR7). In a small group of well-defined Canadian patients diagnosed with mild AD, significantly lower percentages of naïve CD4+ (CCR7+CD45RA+) cells in AD patients than in controls were found. Reciprocally, significantly more EM and TEMRA cells were observed in AD patients. For CD8+ cells, no significant differences compared to healthy elderly were observed. The only differences were the age- and CMV-related increases in late-differentiated CD8+ cells compared to young individuals that would normally be expected. In addition to this, the expression frequency of CD28 was analysed. These results indicated that the fraction of CD28- cells within CD4+ and CD8+ T-cells was significantly higher in AD samples compared to young and elderly healthy controls. Therefore these results suggest that both the CD4+ and CD8+ T-cells are affected in AD. An explanation as to why no differences regarding the CD8 phenotype were observed between AD patients and controls using the CCR7/CD45RA model may be that the effects of age are so pronounced and thus the frequencies of late-differentiated CD8+ T-cells already so low that it leaves little room for the influence of any additional effects [77]. To investigate whether this hypothesis is true, combinations of other markers where analysed in the subsequent studies.

An extended differentiation model reveals lower frequencies of naïve CD4+ T-cells in AD patients

Following these findings, these results were confirmed in a separate study with a larger cohort from a different population. In patients with mild and moderate AD from Sicily, a shift from early to late differentiated stages in CD4+ but not CD8+ T-cells was also observed. For this study, a different model of T-cell classification was applied, in which the expression of CD27 and CD28 was examined, followed by CD45RA and CD45RO. Naïve cells were defined as CD27+CD28+ CD45RA+CD45RO- and late-differentiated cells as CD27-CD28- CD45RA+CD45RO+. A transition from early- to late-differentiated cells in the elderly compared to the young individuals was predominately observed for CD8+ T-cells. In contrast to this, comparing AD patients and age-matched controls the differences were limited to CD4+ T-cells, but again in case of naïve CD8+ T-cells the frequencies were already so low in the age-matched elderly, that a further reduction due to the AD pathology is not detectable. A reason why this age-related effects are so strong in this cohort compared to others (e.g. Götttingen study participants) is that all young individuals were CMV- and all elderly (controls and AD patients) CMV+.

To support the hypothesis of immune exhaustion and "immunosenescence" caused by chronic stimulation, further markers of immune exhaustion and senescence were investigated. KLRG1 (Killer cell lectin like receptor G1) belongs to a superfamily of inhibitory receptors expressed on NK-cells. Its expression identifies mature CD4+ or CD8+ T-cells capable of secreting cytokines, but with decreased or absent proliferation capacity upon stimulation [125]. CD57 (HNK1) is a receptor that negatively regulates NK-cells that is also expressed on CD8+, and to a lesser extent, on CD4+ T-cells. This has been proposed to be a marker of "replicative senescence" in T-cells [18]. In the cohort of Sicilian AD patients the percentage of CD57+ cells was significantly higher

within T-cells of CMV+ elderly compared to CMV- young. Within the age-matched controls we could identify two groups, one with over ten percent of CD57+CD4+ T-cells and one with under ten percent, but still higher frequencies than in young individuals (3.4). One explanation could be that all age-matched individuals are CMV+, but some possess a higher anti-CMV IgG titre. Because this wasn't determined in this study the answer to this question remains to be investigated. Comparing AD patients and elderly controls no differences were observed, neither within CD4+ nor CD8+ T-cells. The frequency of KLRG1+ CD4+ T-cells was found to be significantly higher in AD patients than in healthy controls (3.4). Regarding CD8+ T-cells the difference was a non significant trend [111].

Confirmation of previous findings; more advanced CD4+ T-cell differentiation in AD

To examine and expand upon the reported finding of higher frequencies of more advanced T-cell differentiation in AD, cohorts with different genetic backgrounds and environments were used to perform three further studies for this thesis. PBMC and blood samples of AD patients from different locations (Canada, Germany and Italy) were collected. For these studies, the sample cohort was extended to include patients with severe AD, VaD and mixed dementia. The T-cell analysis was based on models relying on markers employed in previous studies [6, 111]. The results of these studies indicated that CD4+ T-cells expressing the costimulatory molecules CD27 and CD28 occur in higher percentages in healthy controls than in AD patients. Parallel to this, the CD27 CD28 double negative population of late-differentiated T-cells was increased in AD patients, although these results were not all statistically significant. The trends were consistent when the CD45 isoforms were included in the analysis. The percentages of CD27+CD28+CD45RA+CD45RO- CD4+ T-cells were found to be lower in the AD cohorts compared to healthy controls. A shift from early- to late-differentiated CD4+ T-cells was determined, consistent in all cohorts. To investigate whether this is a phenomenon of early onset which increases in late AD, patients of different AD stages were analysed.

T-cell phenotypes in different disease stages

While the initial studies included patients with mild and moderate AD, subsequent investigations with the Chieti cohort included patients with more severe disease (indicated by low MMSE scores). Correlating the MMSE scores with T-cell phenotypes showed no significant relationships. In the Canadian study cohort, patients with mild, moderate and severe AD were recruited. Those with mild and moderate AD showed similar immune signatures, while patients with severe AD were more similar to the healthy elderly controls in that they showed slightly higher frequencies of naïve T-cells and lower frequencies of late-differentiated cells. A follow-up of the Göttingen AD patients hinted towards a reversal of these differences. The frequencies of naïve CD4+ T-cells were higher after the follow-up period, but follow-up was only available for five patients which did not allow the application of statistical analysis (data not shown). If inflammation is the driving force for enhanced T-cell differentiation in AD, a chronically stimulated and exhausted immune system with reduced production of inflammatory cytokines might explain the

decreased frequencies of late-differentiated T-cells observed upon follow up. Supporting this idea of immune exhaustion is a study that reported LPS stimulated blood cells to release less IL-6 and IL-1β in patients with severe AD [168].

Impact of CMV on T-cell phenotypes

Given the observation that CMV impacts the immune system in addition to the impacts exerted by AD, CMV+ and CMV- individuals were investigated separately. In the Chieti and Canadian cohorts, the number of CMV- participants was too low to allow for statistical analysis of these groups separately. The Göttingen cohort was analysed while considering CMV status, but no significant differences were observed. Only a trend of higher frequencies of late-differentiated CD8+ T-cells in CMV+ healthy elderly controls and AD patients compared to their CMV- counterparts.

Suggestions of the effect of CMV were provided by Wikby *et al.* in their immune profile analysis of an elderly Swedish population. In this four year follow-up study, cognitive impairment together with an "immune risk profile" (IRP) predicted 58% of observed mortality. Suggesting an allostatic overload amongst the IRP individuals, all those with cognitive impairment did not survive the follow-up period. The IRP markers included were an inverted CD4/CD8 ratio, higher frequencies of late differentiated CD8+ T-cells and persistent CMV infection. Analysis of late-stage CD8+ cells (CD27-CD45RA+) in IRP individuals revealed lower numbers of these cells in those with cognitive impairment. On the other hand, higher numbers of CD8+ T-cells with an intermediate phenotype (CD27+CD45RA- and CD27-CD45RA-) were found. The authors suspected impaired maturation of CD8+ T-cells in individuals with the IRP and point to studies in which reduced effector cells were associated with decreased immunity to latent EBV and CMV infections in immuno-suppressed individuals. Once activated, cells of individuals in the IRP category produced reduced levels of the growth factor IL-2 and differentiated poorly, compared to their counterparts in the non-IRP group [168].

Phenotype of brain infiltrating T-cells in AD

That memory cells are present in the brains of AD patients was demonstrated by Togo and colleagues who examined the expression of CD45 isoforms on cells in the brain. The authors observed that CD45RA+ cells (defined as naïve cells in that study), were infrequently found among cells in the brains of AD patients, whereas CD45RO, a marker for activated or memory cells, was commonly observed on cells in the brain of AD patients. The authors of this study also examined the expression of CD27. In the hippocampus it was observed that CD3+CD27- cells were more frequent than CD3+CD27+ cells in the brains of AD patients [151]. Although this study did not employ a comprehensive naïve cell phenotyping strategy, instead relying on CD45RA, these findings agree with those of studies examining T-cells in the peripheral immune system of AD patients. The observation that memory T-cells but not naïve cells migrate to the brain [163], implies that T-cells undergo stimulation in the brain and the periphery separately independently. One hypothesis to explain a parallel stimulation is that Aβ, which can cross the disrupted BBB

in AD, is responsible for T-cell activation and differentiation in both compartments. On the other hand, CSF levels of Aβ in the cohort of AD patients in this study were, in agreement with current literature, decreased compared to healthy controls, which questions its role as a stimulus [136]. Soluble Aβ applied in the T-cell *in vitro* stimulation assay in this study did not result in cytokine production. Therefore other candidates responsible for immune stimulation, for example CMV might be considered. In healthy individuals, CMV infection was shown to cause chronic antigenic stress, resulting in higher frequencies of late differentiated T-cells [30] and also in this study AD patients with an high anti-CMV IgG titre tended to have higher frequencies of late-differentiated T-cells compared to CMV- patients. As CMV-infected cells were shown to occur in the brains of AD patients by Lin *et al.*, it may act as an immune stimulus within the brain [83]. The BBB is composed of endothelial cells and van de Berg *et al.* reported endothelial cell damage caused by CMV-induced effector T-cells [155]. The associated chronic endothelial cell inflammation is characterised by the production of the chemokine fractalkine (CX3CL1) by these cells. Responsible for this endothelial cell activation are pro-inflammatory cytokines, such as TNF and INFγ, secreted by T-cells [15]. Bolovan-Fritts *et al.* further demonstrated that this effect is limited to CD4+ T-cells which were stimulated by CMV antigens. CD8+ T-cells, NK-cells and monocytes did not induce fractalkine production, but were attracted by the chemokine, possibly contributed to the vascular damage and migrated through endothelial cells in an *in vitro* model [15, 14]. Induction of adverse effects on the BBB could explain why a higher anti-CMV IgG titre is considered a risk factor for AD (see Table 1.1). Collectively, these data indicate that immunological stress may contribute to AD and the inflammation that is typically observed in AD patients.

Conclusions

A study in elderly individuals with impaired functional status reported fewer B-cells and CD4+ T-cells and more CD8+ T-cells and NK-cells and a resultant decreased CD4:CD8 ratio. In addition to lower responses to CD3 stimulation, higher CMV responses were reported. These observations were attributed to the limited leukocyte and B-cell repertoire and greater differentiation of these cells, caused by CMV infection. In addition, anti-CMV titres were higher in the group with poor motor ability. The authors concluded *"that the functional decline of elderly individuals was clearly associated with the aging of their immune system, and the intensity of the response to CMV"* [101]. Negative CMV effects could also be suggested for AD patients.

Another contributor to immune cell differentiation and health status could be Aβ. Jozwik *et al.* observed higher percentages of activated (CD69+, HLA-DR+) CD4+ T-cells in PBMCs of AD patients with a high proliferative response to Aβ compared to those with a low response [69]. Their report indicates that Aβ has a direct influence on leukocytes. A suggestion that increased T-cell differentiation leads to higher patient mortality is evidenced by the IRP. The immune risk profile was established in the longitudinal OCTO NONA studies in a Swedish population. In addition to earlier death, participants in the risk category showed lower leukocyte proliferative capacity, lower B-cell counts, fewer naïve CD8+ cells and CMV seropositivity [168].

4.2 Distribution of regulatory T-cell subsets in AD patients

Frequencies of activated T-cells in AD

Together with systemic inflammation, many other immunological alterations can be observed in AD. One of these is an increase in activated leukocytes in the brain and periphery [154, 81]. Two markers of early stage activation are CD69 (a human transmembrane C-Type lectin protein) and CD25 (the alpha chain of the IL-2 receptor). Schindowski *et al.* investigated CD69 expression in AD patients and demonstrated an increased frequency of activated CD4+ and CD8+ T-cells in AD patients compared to healthy elderly controls [134]. Pellicanó *et al.* observed a significant increase in CD69+ T-cells only after stimulation with Aβ-42 in vitro. In addition, the authors found no significant differences in CD69 and CD25 expression frequency in B-cells between AD patients and healthy controls, showing a limitation of the activation among immune cells [110]. Jozwik *et al.* divided AD patients and healthy elderly into two groups according to their proliferative response to Aβ peptide *in vitro*. These authors found an increase in HLA-DR and CD69 expression frequency, but not CD25 on CD4+ T-cells in patients who showed a greater response to Aβ stimulation compared to those who showed less of a response [69].

Conflicting studies regarding the change in CD25 expression frequency have been reported. Some studies have reported an increase in CD25+ T-cells in AD patients [88, 110], while in the pilot study in this thesis lower levels were found (Section 3.1.1)[77]. In addition, the phenotype of CD4+CD25high T-cells was determined using a model with CCR7 and CD45RA. In this study it was found that effector memory cells (CCR7-CD45RA-) dominate in AD patients and that the frequencies of earlier differentiation stages were lower compared to healthy young and old controls. This trend is similar for the phenotypes of all CD4+ T-cells in AD patients compared with healthy controls (Section 3.1.1)[77]. The frequency of CD4+CD25+ T-cells was not different between AD patients and controls in the Chieti cohort, while higher frequencies were observed in AD patients in the Göttingen cohort. The differences between the studies may be attributable to differences in the AD patient cohorts. One explanation is that in MCI and early AD the percentages of activated cells increases but due to stress and immune exhaustion in AD patients, the frequency of these cells is lower. This hypothesis could not be confirmed in the present studies, as no correlation between disease stage according to patient MMSE scores and CD25 expression was detected. For true proof longitudinal studies are needed.

Treg frequencies and phenotypes in AD patients

The population of CD25+ T-cells includes regulatory cells. To define the populations of Tregs, Rosenkranz *et al.* measured the Foxp3 expression frequency in healthy young and old individuals as well as dementia patients. The authors only observed differences in CD4+ Foxp3+ percentages between young and old individuals, not between healthy elderly and patients with AD or Parkinson's disease [127]. The inclusion of the CD127 protein re-confirmed these results. As determined in a pilot study of this thesis, the percentages of CD4+CD25+CD127lowFoxp3+ Tregs were increased in elderly compared with young individuals in a Sicilian population. This

study failed to observe differences between healthy elderly and age-matched patients with mild or moderate AD (Section 3.1.2) [111]. As well in the following three studies presented in this thesis (Chieti, Canada and Göttingen) no significant differences in Treg percentages between patients and controls were found using CD25, Foxp3 and CD127 proteins to characterise Tregs, but a non-significant trend of increased Foxp3+ CD4+ T-cells in CMV+ compared with CMV-AD patients was observed.

Even if the percentages of Tregs are not altered in AD, these cells may still play an important role in AD pathology. Rosenkranz *et al.* observed that Tregs from AD or Parkinson's patients, compared to healthy controls, more effectively suppressed T-cell proliferation [127]. A study reported by Saresella *et al.* showed that Aβ-stimulated-T cell proliferation was reduced in MCI patients compared to AD patients and Treg-mediated suppression was more efficient in MCI patients compared to AD patients. In healthy elderly controls, stimulation with immunogenic Aβ peptides did not elicit the generation of Aβ-specific T-cells. This study indicates a potential connection between Treg activity and disease progression. Furthermore the authors examined the Treg phenotype and observed PD-1- Tregs to be increased in MCI patients. PD-1-expressing Tregs cells are considered to possess the highest suppressive capacity. These results suggest that in AD patients the immune system is attempting to limit the degree of inflammation by enhancing Treg activity, although given that increased inflammation is commonly observed in AD patients this would suggest that the immune system is unsuccessful in this attempt. At early stages of dementia (MCI patients), the activity of Tregs increases, but later drops in AD patients, at least in the study of Saresella *et al.* In contrast to previous reports, the results from their study indicate that the total percentages of CD25highFoxp3+ CD4+ T-cells were diminished in healthy elderly compared to middle aged controls, MCI and AD patients [132]. These differences compared to our pilot study may be explained by the greater age range of the controls, and the use of different Treg marker proteins.

Given that in the pilot study presented in this thesis (Section 3.1.1), the phenotype of CD4+ CD25high T-cells shifted to a predominantly memory type in AD patients, CD45RA and CD45RO were included in subsequent Treg analyses. By gating resting and activated Tregs according to the models published by Miyara *et al.* and Booth *et al.* [98, 16] several Treg populations were observed, but there were no significant differences between patients and controls. In the Chieti study of chemokine receptors, trends of higher CCR expression on CD25+ T-cells of AD patients compared with controls were observed. Although these results were not significant, they suggest an association between activated T-cells and AD. As possible reasons several factors could account for this (viral infection, systemic inflammation, tau and Aβ), but so far no definite evidence has been provided.

4.3 Leukocytes in healthy controls and AD patients

4.3.1 Controversial results concerning the frequency of T-cells in AD

Previous studies have observed a decrease in the total frequency of CD3+ T-cells in AD patients compared to age-matched controls [124], but analysis of T-cell subsets led to contradictory re-

sults. In the pilot study presented in this thesis (Section 3.1.1) significantly reduced CD4+ T-cells were observed in AD patients. This resulted in a lower CD4+/CD8+ ratio in the T-cell population in patients with mild AD compared to healthy age-matched controls [77]. This observation might be explained by greater susceptibility to apoptosis of CD4+ T-cells in AD patients compared to controls. This is supported by a study describing apoptosis of CD4+ T-cells and NK-cells in AD patients [135]. In contrast, Richartz-Salzburger *et al.* found no difference in the CD4+/CD8+ ratio between AD patients and healthy controls [124], while other reports have observed a higher CD4+/CD8+ ratio [138]. The cause of these discrepancies is not known, but may be associated with disease stage of the patients analysed, or with the failure to exclude NK-cells that express CD8 from the analysis. Therefore the present studies included NKT-like cells in the analysis. In the Chieti and Göttingen cohorts, no differences in the percentages of CD56lowCD16+CD3+ NK-T-cells within the total CD45+ cells were seen between AD patients and controls. This observation led to the conclusion that the frequency of NKT-like cells does not influence the percentages of CD8+ T-cells in AD patients compared with controls. In addition, in three of the four studies in this thesis, no differences in the percentages of CD4+ and CD8+ T-cells in patients and controls were detected. Another T-cell subset of interest was $\gamma\delta$T-cells. These cells are predominately found among CD4-CD8- T-cells and relevant, because $\gamma\delta$T-cell subsets were shown to produce predominantly pro-inflammatory cytokines or function as APCs to induce a Th2 response of CD4+ T-cells [102] and so might contribute to inflammation in AD. Another point is their ability to *"operate as a professional phagocyte"* [173]. $\gamma\delta$T-cells were investigated in the Canadian cohort. No significant differences were found in the percentages of CD4-CD8- and CD4-CD8-TCR$\gamma\delta$+ cells when comparing AD patients with healthy elderly controls. In the CD4-CD8-TCR$\gamma\delta$+ population, a marginal increase in the frequencies of these cells was observed in AD patients, which could not be confirmed in the cohort of donors from Chieti and Göttingen. Besides antibody opsonisation, CD16 plays a main role in phagocytosis [173]. Therefore the hypothesis could be investigated, that the frequency of the whole $\gamma\delta$T-cell population does not differ between patients and controls, but that percentages of individual subsets, such as CD16+ $\gamma\delta$T-cells, are altered. Phagocytic capacity was demonstrated for bacteria and synthetic beads, but the uptake of Aβ is one of the many open questions in this field to be investigated. In addition a phenotype analysis could reveal differences as shown for CD25+ T-cells [77].

4.3.2 Reduced frequencies of naïve B-cells in AD patients compared with healthy controls

The frequency and phenotypes of B-cells

Few studies have investigated B-cells in AD patients. While some studies led to the conclusion that the frequency of CD19+ B-cells is decreased in AD patients, e.g. [124], other studies have observed no differences in the frequency of CD19+ B-cells in patients with MCI or AD, as reviewed by Rezai-Zadeh *et al.* [123]. Patients from the Chieti cohort in the present study showed no differences in the percentage of CD19+ within the CD45+ leukocyte population across the different dementia types examined.

Another aspect that was investigated was the lymphocytic expression of APP - within lymphocytes primarily related to B-cells - which was found to be increased in MCI patients. This finding is thought to be related to dysregulated APP metabolism in the brain. These findings hint at the systemic failure of B-cells to activate and effectively process APP, thus they contribute to AD pathology [123]. As reviewed by Rezai-Zadeh et *al.*, further B-cell analysis included measurements of CD69 on the surface of B-cells following mitogenic treatment. The results indicated that the expression of CD69 was decreased in AD patients compared with controls. The consequences of CD69 on AD have so far not been investigated. One consideration is the production of Aβ autoantibodies, which may be related to Aβ neurotoxicity. Several studies have been performed by separate investigators, but the outcomes have been contradictory, perhaps reflecting the different experimental techniques employed [123]. The production of antibodies is predominately by differentiated B-cells. In a more detailed analysis, this study assessed B-cell phenotypes. Four differentiation stages were characterised according to the expression of IgD and CD27. The results indicated no significant differences in the frequency of these phenotypes between AD patients and healthy controls, but a slight trend of lower frequencies of naïve IgD+CD27- B-cells, and a slightly higher level of late-differentiated (IgD-CD27+ and IgD-CD27-) B-cells in AD patients compared to healthy elderly was observed. This trend is similar to the finding of higher frequencies of more differentiated T-cells in AD patients and counts as another sign for ageing of the immune system in AD.

Relevance of CD40

CD40 is a glycoprotein and member of the TNF-receptor superfamily that is upregulated by the pro-inflammatory cytokine IFNγ. Ramirez *et al.* investigated CD40 expression on brain endothelial cells of patients affected by HIV-1 encephalitis (HIVE). The authors concluded that there was an important role for CD40 and its ligand CD40L in BBB regulation [118]. When examining HIVE patients with cognitive impairment, CD40-CD40L interactions led to increased inflammation and production of adhesion molecules in endothelial cells. Levels of reactive oxygen species and as a consequence chemokine secretion were enhanced as well. Soluble CD40L can decrease levels of ATP. Appropriate ATP levels are required for maintenance of tight junction permeability in the BBB [27]. Other reports have demonstrated that CD40 expressed on B-cells resulted in their activation and differentiation via T-cells [47]. B-cell-T-cell interactions are probably influenced by CMV, as mouse CMV glycoprotein m155 was shown to inhibit CD40 expression [86]. The results of the study performed in this thesis suggest that a more differentiated B-cell phenotype is found in AD patients, which might be related to CD40. Therefore the analysis of CD40 expression was included in this study. In the Göttingen cohort, CD19+ cells in AD and MCI patients did not show significant differences in the frequency of CD40+ cells compared to controls. The values show a broad range with a slightly higher median in case of the healthy elderly, although this trend did not reach significance.

4.3.3 No difference in the frequency of NK- and NKT-like cells in AD patients

The percentages of NK-cells were not shown to be different between dementia patients and controls in earlier reports [123]. Both, the Chieti and the Göttingen studies from this thesis, confirm these reports. The percentages of CD56low CD16+CD3- cells within the CD45+ population did not differ between AD patients and controls. This means that either NK-cells do not play an important role in AD, or that sufficient NK-cells are produced to compensate NK-cell exhaustion in the brain. A third hypothesis is that NK-cell numbers are not affected by AD, but instead their functionality is altered with disease progression. This is supported by studies that have found increased or decreased NK-cell activity in AD patients compared to healthy controls [123].

A less frequent leukocyte subset, the NKT-like cells, was also investigated but the percentage of these cells (defined as CD56lowCD16+CD3+) did not differ between patient and control groups. Because NKT-like cells respond to cytokines and have a role in modulating the immune system, they might be associated with AD pathology. The hypothesis proposed for NK-cells may also apply to NKT-like cells - the frequency of these cells does not vary, but their functional capacities might be altered. This remains to be investigated in future studies.

A previously reported finding that may be an important consideration is that NK-cells play a role in the recognition and regulation of virally infected cells. They are also particularly important in immunosurveillance against CMV [101]. NK-cell numbers increased with age and this has been proposed to be due to an increased response against CMV. Greater frequencies of NK-cells with reduced functional status have been reported in the elderly. In these elderly individuals higher anti-CMV antibody titer was also observed [101]. Originally, it was an aim of this thesis to investigate if NK-cells differ between CMV- and CMV+ AD patients, but due to insufficient participant numbers this outcome was not achieved.

4.3.4 No significant differences in the frequency of monocytes between dementia patients and healthy controls

Characterisation of the monocyte population in AD patients

To the best of my knowledge, the total number of monocytes in dementia patients has not previously been assessed. Here the frequencies of monocytes defined as CD14+CD45+ cells were determined. In the Chieti study cohort no differences between patients and controls were detected, while in the Göttingen cohort a slight decrease of CD14+ cells within the CD45+ population was observed in AD patients. In previous studies using a transgenic murine AD model, the loss of CD14 expression was associated with enhanced expression of genes encoding the pro-inflammatory cytokines TNF and IFNγ [122] and a systemic inflammation is characteristically in AD patients. CD14 - an LPS receptor - was demonstrated to interact with Aβ and to create an inflammatory response in mice. One hypothesis is that aggregated Aβ fibrils mimic pathogen-associated microbial patterns that are biophysically similar. Other promoters of LPS-mediated inflammation are scavenger receptors and CD11b/CD18. In hybridisation experiments, CD14 mRNA from APP23 transgenic mice was more highly expressed compared with wild type mice

[37]. These results show that in addition to the frequencies of CD14+ cells as analysed in the Chieti and Göttingen studies, the relative proportion of different monocyte subsets could be of importance. Seidler *et al.* showed that healthy elderly compared to healthy young did not differ in terms of total leukocyte or monocyte frequencies, but the proportion of the subsets of these cell types did. *"Non-classical CD14+CD16+ monocytes significantly increased with age, but displayed reduced HLA-DR and CX(3)CR1 surface expression in the elderly"*. But the authors state that there was no significant correlation between *"Classical CD14++CD16-"* monocyte frequencies or counts and age [137]. It is possible that certain monocyte subtypes are present in differing proportions in AD patients or that their activation status or functionality is different, even if the frequency of monocytes is constant or diminished due to AD-associated immune exhaustion.

This may also be the case for CD11b and the scavenger receptor CD36, which helps to mediate microglial and macrophage response to Aβ fibrils and which seems to play a key role in AD-associated inflammation [48]. In the Chieti study cohort, the frequencies of monocytes did not differ significantly between patients and controls, but the mean fluorescence intensities were not considered. Considering this, it is possible that even though the proportion of cells was not different, the expression of the proteins of interest on each monocyte may have been different in the AD patients compared to the controls. More detailed studies are required, particularly considering that CD11b+ cells and CD11c+ cells have been reported to be localized with Aβ plaques in APP transgenic mice. CD11b+ are phagocytic cells which produce pro-inflammatory cytokines including IL-1β, TNF in addition to promoting NO, free radicals, chemokines and activating complement. After incubation with IFNγ, microglia differentiated into CD11b+ and CD11c+ cells that served as APCs for both Aβ 1-40 and Aβ-42 and supported CD86-dependent proliferation of Aβ-reactive T-cells. In addition, IFNγ and Aβ-stimulated microglia and astrocytes led to NO-mediated toxicity that affected T-cells resulting in apoptosis [99]. This may also account for the observed decrease of CD14+ leukocytes in the Göttingen cohort.

CD40 expression on monocytes

Lymphocytes as well as microglia are activated in AD. One mediator for this microglia activation is an interaction between CD40 and CD40 ligand (CD40L). These two molecules are typically up-regulated in AD [123]. Both CD40 and CD40L are present as soluble and membrane-bound forms. In addition to their role in costimulation of immune cells, these interaction partners are reported to enhance neuroinflammation, modulate APP processing and reduce Aβ clearance from the brain. When microglia were cocultured with primary neurons in the presence of Aβ peptide, together with IFNγ or CD40L, microglia secreted high levels of TNF, which led to neuronal injury [47]. Given that reactive microglia in the brain express CD40 and that vascular CD40 expression has been found to be elevated in AD-associated brain lesions [152], the expression of CD40 on monocytes was investigated in the Göttingen AD cohort in this thesis. This study showed similar frequencies of CD14+CD40+ cells in AD patients and healthy elderly controls. The results were similar in the case of CD40+ B-cells. Given that this study only examined the frequency of these cells, the degree of CD40 expression may have been different between the two groups. Another aspect that could be examined in future studies could be the characterisation of

CD40 on monocyte subsets (CD16+ monocytes, for example). But it may also be the case that CD40 on monocytes and B-cells does not play an important role in AD.

Activated monocytes in AD

To investigate the activation state of monocytes, Kusdra *et al.* assessed the expression of CD69 on monocytes and macrophages. The results indicated that the percentages of CD14+CD69+ cells were higher in AD patients compared to healthy controls. The Chieti study cohort investigated in this thesis did not confirm the results of Kusdra *et al.* No differences between patients and controls were seen in the frequency of CD14+CD69+ cells. Another suggestion of the importance of CD69 in AD was provided by Schindowski *et al.*, who reported a higher percentage of CD8+CD45RO+CD69+ T-cells after mitogenic stimulation in AD patients compared to healthy elderly [134]. Given that in the study by Schindowski *et al.*, the basal levels of CD69+ T cells were similar between the two groups, the Chieti cohort studied in this thesis may show differences between AD patients and controls in the frequency of CD69-expression T cells after exposure to a stimulus. Chronic immune stimulation might be the normal state in AD patients. This is supported by several studies such as that by Pellicanó *et al.* This study showed that CD69-expressing T-cells in AD patients in a Sicilian population were slightly more frequent in comparison to those in healthy controls. These slight increases were statistically significant when PBMC samples were cultured with Aβ-42 protein [110]. Given that these differences were not seen in CD19+ cells, it is not possible to comment on whether or not these results are indicative for other leukocyte cell types. These experiments remain to be performed in future studies. The conditions for these experiments should be carefully chosen as a wide range of conditions have been employed by other investigators. For example Stieler *et al.* incubated PBL with phytohaemagglutinin (PHA, 12 μg/ml), pokeweed mitogen (PWM, 4 μg/ml), Protein A (50 μg/ml) or nerve growth factor (NGF, 100 ng/ml). Stieler *et al.* used siliconized glass tubes to prevent the adhesion of isolated cells on the surface of the tubes during the incubation time of 4 h. Whether the authors investigated CD3+CD4+ or CD19+ lymphocytes, the stimulation index (defined as the ratio of CD69 expression after mitogenic stimulation to unstimulated control) correlated inversely with patient MMSE scores, irrespectively of the stimulus applied. PHA was reported to be the strongest stimulus. For other leukocyte cell types studied (CD45+, CD3+ or CD8+) the observed trends did not reach significance [145]. The conditions contrast with the study by Pellicanó *et al.* in which 24-well plates for 48 h of stimulation at 37°C were used [110] and this could explain different results.

4.4 Degree of T-cell cytokine production does not differ between AD patients and healthy controls

Along with age the level of C-reactive protein and pro-inflammatory cytokines increase. The resulting low-grade systemic inflammation termed "inflammageing" is associated with several

age-related diseases including cardiovascular disease, type 2 diabetes and dementia [8]. The OCTO and NONA longitudinal studies demonstrated that in a Swedish cohort low-grade inflammation predicted two-year mortality in very old individuals independently of their health status [169]. In addition, levels of the pro-inflammatory cytokine IL-1 and the "multifunctional" IL-6 were suggested to be associated with impaired cognition [168]. To place their results in the context of AD, the authors referred to studies in which intracerebral injection of an APP peptide in mice resulted in increased brain and plasma-IL-6. Based on these and other findings, Wikby *et al.* suggested to characterise late life neurodegeneration as chronic inflammatory condition where microglia are activated. In their study of predictors of mortality the authors established an immune risk profile (IRP) with CMV seropositivity as one main hallmark. To control persistent CMV infection CD8+CD28- T-cells are generated and the number of these differentiated CD8+ cells were elevated in individuals of the IRP cohort. A correlation between the IRP and IL-6 levels was not found; both parameters predicted survival independently [168, 169]. Bartlett *et al.* investigated the role of CMV in inflammageing over ten years and concluded that *"CMV infection is not a primary causative factor in the age-related increase in systemic inflammation"* [8]. This indicates that CMV does not act as a stimulus that causes the inflammation present in AD, but adds an additional burden. In the OCTO and NONA studies all individuals with cognitive impairment which were in the IRP group were deceased at follow-up [168]. As an alternative factor, in this thesis a possible role of Aβ as driving force for the inflammation was investigated. The expression of cytokines by leukocytes in the peripheral immune system and the best method for their assessment remains a controversial topic. A review of the literature was provided by Lee *et al.* [78]. The authors report that amyloid beta synthesis was induced by cytokines secreted from microglial cells, astrocytes and/or neurons. Aβ, in turn, induced the production of IL-1β, TNF and IL-6 in astrocytes and microglia *in vitro*. AD is a disorder of the brain, thus CSF is the ideal source to measure cytokines. But it is far easier and more cost effective to obtain blood from patients as it is tested as part of routine clinical examination. Therefore, biomarkers for AD that are present in the blood would be very useful for diagnosis and for the determination of disease progression. Given that the inflammatory state in AD is not only limited to the brain but is systemic, blood may provide relevant information regarding the disease. Damage to the BBB is common in AD patients, which allows the passage of small molecules. Aβ may be able to pass the BBB and go on to damage lymphocytes in the periphery. This could result either in reduced cytokine production, or may result in activation with a subsequent increase in cytokine production, as Blurton-Jones and La Ferla postulate for what may be occurring in the brain [13]. A potential clue is provided by Fiala *et al.* - they show that Aβ stimulates monocytes to secrete cytokines [40].

In the present study cytokine production by PBMCs from healthy donors and AD patients was analysed. PBMCs may migrate to the brain where PBMCs along with the cytokines they produce may play an important role in the pathology of AD. Therefore it was of interest to investigate the capacity of these cells to produce cytokines. The experiments summarised in Section 3.2.4 showed no reduction in cytokine production, which if observed, may have been due to Aβ toxicity in the blood. The live:dead cell discriminator EMA did not indicate a large increase in the proportion of dead cells after Aβ stimulation (data not shown). PMA / ionomycin stimulation resulted in a similar increase in cytokine production in both AD patients and controls. Future

studies could examine not only the frequency of cytokine producing cells, but also the degree by which the positive cells express the cytokines (either by using the MFI with flow cytometry or by measuring cytokine levels in the plasma).

Additionally, a co-culture experiment with PBMCs and Aβ protein and stimulation with PMA and ionomycin may assist in investigating if Aβ protein is capable of inhibiting cytokine production. Other experiments performed in this study do not confirm another hypothesis that there is increased cytokine production in AD patients due to Aβ-reactive T-cells as incubating PBMCs with Aβ did not result in an appreciable increase in cytokine production. Despite these results, Monsongeo *et al.* observed increased T-cell reactivity to different amyloid beta peptides measured by proliferation and cytokine production, in elderly individuals and AD patients. But the authors of this study used longer incubation times (30 h compared with the 17 h in this thesis) and did not detect the production of all cytokines (for example, low levels of IL-4 and IL-10 were observed) [103]. A review of T-cells and cytokines in AD patients is presented by Town *et al.* The authors point out that clinical trials with vaccination of Aβ in humans (in contrast to mice) led to a strong Th1 response and production of inflammatory cytokines such as IL-2 and IFN-γ. This was thought to result in the observed side effects such as meningoencephalitis, resulting in termination of the trial [154]. Other factors might be considered, for example the basal level of cytokines appears to be slightly higher in a proportion of AD patients compared to healthy elderly controls. This might be due to other stimuli in the blood of AD patients. The difference was marginal; the employed technique of flow cytometry was not able to clearly detect the difference. Follow-up experiments to more thoroughly investigate this potential difference could use either a longer incubation time, *in vitro* stimulation with other potential immune stimulants, or an assay capable of assessing plasma cytokine levels in AD patients. In summary, this study observed that amyloid beta did not influence PBMC cytokine production. Future studies should employ modified experimental parameters in order to investigate this topic in more detail.

4.5 Higher chemokine receptor expression in AD patients compared with healthy controls

In AD patients, cells of the immune system migrate along a chemokine gradient from the periphery, through the blood-brain barrier, to the brain. Due to this, this study investigated chemokine receptor expression on PBMCs of AD patients compared with healthy controls. Reale *et al.* postulate that chemokine receptors are present and some up-regulated in the brain of AD patients by comparison with healthy individuals. The system of chemokines and their receptors is thought to lead to an accumulation of microglia in senile plaques and may play a role in a cascade which results in neuronal death. Increased concentrations of cytokines and chemokines in AD have been reported for the periphery and the brain, but which of these is the primary driving force and which is altered in consequence is still under debate. Using genetic and cytofluorimetric analysis, Reale *et al.* showed that CCR2, CCR5 and CCL5/RANTES were more highly expressed, while CCL2/MCP-1 showed lower expression in AD patients compared to controls. The results of this study indicated that CD4+ CCR2- and CCR5-expressing T-cells were more frequent compared

to CD8+ T-cells. An increase in CCR5, CCL5, CCL2 and IFNγ was also observed when PBMCs were stimulated *in vitro* with Aβ-42 [119].

The present study analysed CCRs from a different perspective. This study investigated the expression of CCRs on PBMC and the results confirmed those reported by Reale *et al.* Additionally, this study included CCRs that were not studied by Reale *et al.* In the Chieti sample cohort, the expression frequency of CCR4 and CCR6 was analysed on T-cells. The results indicated a slight trend; CD4+ T-cells of AD and VaD patients tended to express CCR4 and CCR6 more frequently, particularly when these cells were activated (that is, positive for CD25). This analysis was repeated with the inclusion of CCR5 in the Canadian cohort. The results showed that CCCR4 and CCR5, but not CCR6, was expressed more frequently on CD4+ T-cells in AD patients compared to healthy controls, although the results did not reach statistical significance. These results were independent of disease severity.

Another study indicated increased CCR2 and CCR5 expression frequency on T-cells and B-cells after the stimulation of PBMCs from AD patients and healthy controls with Aβ-42 protein. In the case of CCR5, T-cells from AD patients expressed this protein in higher amounts than in controls [110]. This study also investigated the expression frequency of CCRs on other leukocyte cell types. In the Göttingen AD cohort, CCR2, CCR4, CCR5 and CCR6 were slightly more frequently expressed on the entire leukocyte population (defined as CD45+) in AD patients compared with controls. Greater differences were observed when the leukocytes were divided into subsets. CCR6 was observed to be more frequently expressed on T-cells (CD3+), B-cells (CD19+) and monocytes (CD14+) in AD patients compared with controls. Within the CD4+ and CD8+ T-cell subsets, the difference of higher CCR4 and CCR5 expression was more pronounced in the CD4+ subset compared to the CD8+ subset. This is in line with previous studies showing that CD4+ T-cells are more differentiated than CD8+ T-cells in AD patients [77, 111]. A particularly important observation by previous investigators is that of higher expression of the CCR5 ligand MIP-1α by T-cells. CCR5 expression on human brain microvascular endothelial cells (HBMECs) may provide a mechanistic explanation for T-cell migration through the BBB to the brain [110].

Lee *et al.* detected higher Aβ-42 and beta-secretase levels in the brain of CCR5 knockout mice compared with wild-type mice. The authors hypothesised that the absence of CCR5 leads to an activation of CCR2, which through astrocyte activation in turn leads to Aβ deposition and memory dysfunction [79]. The CCR5 signalling pathway also influences the expression of other chemokine receptors. In CD4+ T-cells of CCR5 knockout mice, the expression of CCR1, CCR2 and CXCR3 is reduced. These mice showed altered CNS trafficking [49]). Therefore, these results suggest that CCR5 may act to suppress the development and progression of AD pathology. The more frequent CCR5 expression on leukocytes in AD patients is perhaps a mechanism by which the body is attempting to counteract AD pathology. Further support for this hypothesis is given by a previous study that reported a possible neuroprotective role for CCR5 [144]. Thus, the observation in this study that PBMCs express CCR5 more frequently may reflect attempts by the body to counteract the pro-inflammatory nature of AD pathology mediated by other CCRs

such as CCR6.

It is thought that CCR6 may also play an important role in this context. Together with adhesion molecules such as selectins and integrins, CCR6 influences T-cell migration through the choroid plexus into the CSF [129]. Previous studies have reported higher CCR6 expression in the brain and periphery of transgenic mice with AD-like disease. The authors of this study suggested that this was due to the systemic inflammation that is present in AD [147]. The findings presented in this thesis indicate that AD in humans is similar to AD-like disease in mice in relation to its inflammatory nature. One hypothesis is that certain immune cells which infiltrate the brain contribute to inflammation while exerting anti-disease activity. Thus, limiting trafficking of immune cells to the brain which secrete pro-inflammatory cytokines might help to reduce inflammation. Additionally, brain-recruited microglia might secrete neurotoxins, causing neurodegeneration [34]. This could be reduced by preventing those microglia from entering the brain (Fig. 4.1). On the other hand migration of immune cells with anti-disease activity should be promoted.

An advantage of studying several chemokine receptors simultaneously is that different leukocyte subsets may not express all of the receptors. This may allow the identification of expression patterns on leukocyte subsets so that it may be possible to develop therapies that specifically target subsets that play a role in AD pathology. For example, B-cells may play a role in controlling CNS inflammation through IL-10 secretion [41] in addition to releasing nerve growth factors that promote regeneration of brain cells [153]. Aβ activates neuroprotective microglia to amyloid plaques via a signalling cascade that begins with the scavenger receptor CD36 [104]. Aβ can be phagocytosed by macrophages which in turn reduces the negative effects of Aβ (Fig. 1.2). Experiments from Zaghi *et al.* showed that *"AD macrophages ingested and cleared less, and underwent apoptosis upon exposure to soluble, protofibrillar, or fibrillar Aβ"* [176]. Another study by Fiala *et al.* showed that in contrast to those from controls, monocytes from the blood of AD patients take up Aß but insufficiently mediate its clearance, have less capacity for differentiation into macrophages, and more readily undergo apoptosis [39]. In transgenic mice, experiments with GFP-labelled Aβ showed that blood-derived monocytes enter the brain and phagocytose Aβ [92, 142]. Because these experiments were performed in chimeric mice in which the host animal was irradiated and the bone marrow replaced with that of transgenic mice in which lineage markers or selection genes had been introduced, the brain infiltration of bone marrow-derived cells might be artifactual (reviewed by Cameron *et al.*) [22]. However, the data presented in thesis observed increased expression frequency of CCR6 in monocytes and B-cells in AD patients, suggesting that they may have an immune regulatory role in the brain.

A direct influence of CCR2 on disease progression was shown by El Khoury *et al.* [34]. In mice lacking CCR2, the authors observed less microglial accumulation and Aß clearance in addition to the promotion of early disease progression [34]. On the other hand, it has been stated that *"Hematopoietic CC-chemokine receptor 2 (CCR2) competent cells are protective for the cognitive impairments and amyloid pathology in a transgenic mouse model of Alzheimer's disease"* [100]. A correlation between CCR2 expression and MMSE scores in an elderly cohort was reported by Harries *et al.* along with data associating CCR2 expression with ApoE haplotype status - an AD risk factor [59]. Evidence in mice has been reported that suggests Ly-6C(hi)

CCR2+ monocytes are recruited to brain lesions which then differentiate into microglia [96]. Regarding CCR2 ligands, Westin *et al.* observed a correlation between increased CCL2 levels in the CSF and more rapid cognitive decline in MCI patients that had developed prodromal AD upon follow-up [164].

The results presented in this thesis confirm differences in the frequency of chemokine receptor expression on PBMCs of AD patients compared to controls, potentially linking inflammatory cells of the immune system to AD-associated brain damage. The results of this study are not able to determine whether these differences are beneficial or harmful with respect to the development and progression of AD. In order to determine this, follow-up studies, which are already underway, are required. It is speculated that interactions between certain immune cell types may be beneficial at the onset of disease but harmful in later stages of disease, as evidenced by several studies [41, 34, 176, 153]. In future experiments it would be of interest to investigate whether blocking or enhancing the migration of peripheral leukocytes to the brain is associated with changes in brain-associated inflammation, amyloid plaque accumulation or immune exhaustion, or whether this results in adverse effects. The present studies suggest several novel therapeutic targets (Fig. 4.1) based on proteins observed to be more frequently expressed on peripheral blood cells from AD patients than in healthy elderly controls.
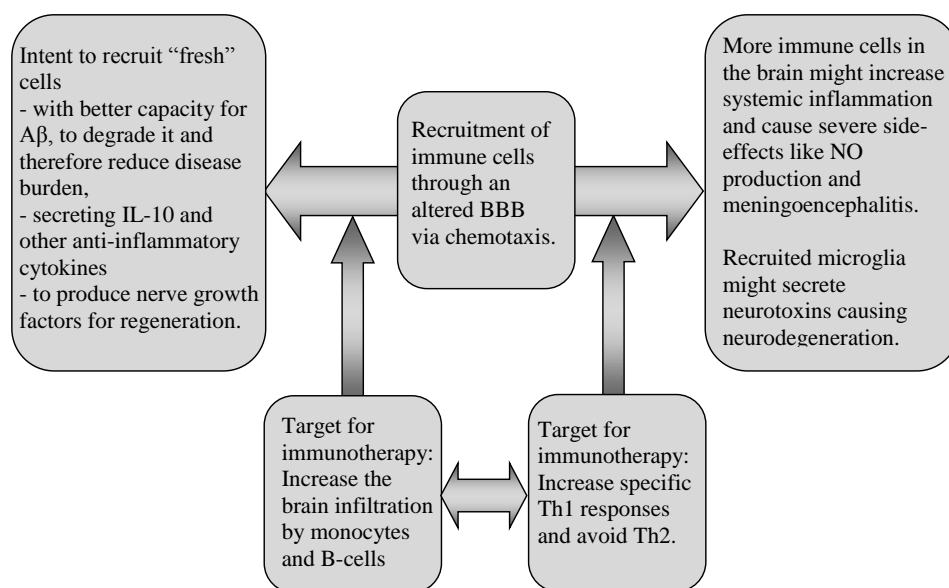
Figure 4.1: **Overview of AD immunotherapy opportunities in AD**
Beneficial and negative effects of enhanced cell migration in AD due to an altered blood-brain barrier, systemic inflammation and enhanced chemokine expression by activated brain cells resulting in possible targets for immunotherapy.

Chapter 5

Summary

This study investigated the immune profiles of patients with dementia (AD, VaD, mixed dementia). The focus of this study was on leukocyte populations found in the blood. Initial experiments assessed the frequency of major leukocyte subsets in AD patients and healthy controls, but no differences were observed - the frequencies of B-cells, NK-cells and NKT-like cells were similar for all groups. The monocyte marker CD14 tended to be less frequently expressed in control subjects compared to AD patients, but the results differed across the cohorts that were studied.

In the second phase of this study, the phenotype of different leukocyte populations was analysed. B-cells showed a tendency towards a lowered frequency of CD27-IgD+ naïve cells in AD patients compared to healthy controls. This trend was even more pronounced for T-cells, notably in the case of CD4+ T-cells. This decrease was observed in pilot studies using samples from the Canadian and Sicilian cohorts, but also in studies in the three other cohorts examined. In these studies, different phenotype models were applied. Naïve T-cells were either characterised as CCR7+CD45RA+ or as CD27+CD28+CD45RA+CD45RO-. In both cases the percentages of CD4+ T-cells were lower in AD patients than in controls. Parallel to this, the percentages of late-differentiated CD4+ T-cells were found elevated in AD patients. In addition, the frequency of CD57- and KLRG-positiv cells tended to be greater in patients than controls, but these differences were only statistically significant in some cohorts. Another observation, indicative of immune exhaustion, was an increased level of PD-1 in T-cells of AD patients compared with controls, although this was also not statistically significant. The question remains, which is the factor causing these immune changes in AD. One possible explanation is that Aβ is causing chronic antigenic stress and driving T-cell differentiation.

Another aspect that was studied was the functionality of the PBMCs in AD patients compared with healthy controls. The aim was to assess the capacity of T-cells to produce cytokines and control inflammation. If the cells are still able to produce anti-inflammatory cytokines, they could serve as a target for immunotherapy. Another option to reduce inflammation is to identify and neutralize the antigen triggering pro-inflammatory responses in AD. Aβ is one candidate which was investigated in this study. After overnight stimulation with either PMA and ionomycin or Aβ-42 together with brefeldin A, the levels of intracellular cytokines in CD4+ and CD8+ T-cells were determined with flow cytometry. The results of these experiments showed no major differences between controls and AD patients. They were also independent of the disease stage.

Further studies investigated chemokine receptor expression. In AD, a compromised and potentially leaky blood-brain barrier results in an exchange between the periphery and the brain that does usually not occur. A consequence is that cytokines, Aβ protein and cells may migrate through the BBB. One consequence of this is systemic inflammation. One aspect of AD pathology is the potential recruitment of immune cells to the brain. To elucidate the mechanisms behind these processes, the frequency of peripheral leukocytes expressing CCR2, CCR4, CCR5 and CCR6 was determined. The results indicated that leukocytes from AD patients more frequently expressed these proteins, although the differences were not always statistically significant in all cohorts. In the case of CCR6, it was observed that this receptor was not only more often expressed on a single leukocyte subset, but expressed more frequently on B-cells, monocytes, CD4+ and CD8+ T-cells as well.

In conclusion, this work provides evidence that hints toward a more differentiated and exhausted immune system in AD patients. What still remains unclear is whether the recruitment of immune cells to the brain is a consequence or cause of the disease. Experiments using *in vitro* models where the entry of immune cells to the brain can be blocked may provide insight into this lingering question. These experiments may also assist in determining if the migration of immune cells results in beneficial effects, for example by phagocytosing Aβ protein, or if they function to promote disease progression assuming that *in vitro* models accurately parallel human disease. A way of investigating this could be to enhance the chemokine gradient *in vivo*, as the immune cells examined in this study demonstrated increased CCR expression. In the event that immune cells are found to promote AD, it may be possible to treat patients with CCR-blockers in order to reduce immune cell migration to the brain. Using this approach, it may be possible to selectively block B-cell migration as they were observed to express CCR6, but not CCR4 or CCR5. After determining which leukocyte subset contributes to the promotion of disease, it may be possible to employ antibodies specific to CCRs that are predominately expressed on this subset.

Previous studies in healthy elderly individuals showed that CMV serves as a chronic stimulus for the immune system, particularly for CD8+ T-cells. In AD patients, Aβ might play a similar role. In the present study, the cytokine production of T-cells stimulated with Aβ-42 was assessed, but this did not result in appreciable cytokine production. Aβ may influence the immune system through more than one mechanism, and it is possible that Aβ contributes to the observed shift from early-differentiated to late-differentiated T-cells and B-cells in AD patients. This could be tested by culturing T-cells with and without Aβ protein and analysing the resultant immune profile. It is hypothesised that a rejuvenation of the immune system may be beneficial for AD patients. This might be achieved through improving Aβ clearance, possibly by enhancing monocyte migration from the periphery to the brain. Reduced Aβ load may result in reduced T-cell differentiation, i.e. fewer memory T-cells and more naïve T-cells. It is thought that in AD patients as in healthy elderly individuals that CMV is the driving force of CD8+ T-cell differentiation and Aβ may have a similar effect in AD patients. Whether the effects of CMV and Aβ are additive remains to be determined. A larger cohort of study participants - as recruited here - including sufficient CMV- and CMV+ AD patients and controls would be necessary to investigate this. This question may be of particular importance given that CMV may contribute to the inflammatory state in AD patients and thus provide another burden to the functioning of the immune system thereby decreasing the ability of the body to control the disease. The role that CMV may play

in the functional decline of the immune system may also be related to cognitive decline, such as that in AD.

5.1   Summary in German

In dieser Studie wurden Immunprofile von Patienten mit verschiedenen Demenzformen (AD, VaD, mixed dementia) erstellt. Der Fokus lag dabei auf verschiedenen Blutleukozyten. Die Prozente an B-Zellen, NK-Zellen und NKT-Zellen waren ähnlich in allen Gruppen. Der Monozytenmarker CD14 scheint in Kontrollen weniger exprimiert zu sein als in Alzheimer-Patienten, doch die Aussagen sind nicht schlüssig, da verschiedene Studien zu unterschiedlichen Ergebnissen geführt haben.

In einem zweiten Teil wurde der Phänotyp der Leukozyten untersucht. B-Zellen wiesen dabei tendenziell geringere Prozentzahlen an CD27-IgD+ naiven Zellen in Alzheimer-Patienten im Vergleich zu gesunden Kontrollen auf. Dieser Trend ist stärker ausgeprägt in T-Zellen, vor allem in den CD4+, und wurde durchgängig in allen Studien festgestellt. Dabei wurden verschiedene Differenzierungsmodelle angewandt, in denen naive T-Zellen als CCR7+CD45RA+ oder als CD27+CD28+CD45RA+CD45RO- charakterisiert wurden. In beiden Fällen war die Häufigkeit in Patienten niedriger als in Kontrollindividuen. Parallel dazu war die Frequenz an weit differenzierten CD4+ T-Zellen höher in Alzheimer-Patienten. Zusätzlich war die Häufigkeit von CD57- und KLRG1-positiven T-Zellen in Patienten höher, wenn auch nicht immer signifikant. Ein weiteres Anzeichen für ein erschöpftes Immunsystem ist das nicht signifikant höhere Level von PD-1 auf T-Zellen von Patienten der Alzheimer-Krankheit. Daraus stellt sich die Frage, was für die immunologischen Veränderungen in Bezug auf die Alzheimer-Krankheit verantwortlich ist. Eine Hypothese ist, dass Aβ als antigener Stressor T-Zell Differenzierung vorantreibt.

Ein weiterer untersuchter Aspekt war die Funktionalität von PBMC. Ziel war es, die Kapazität von T-Zellen, Zytokine zu produzieren, zu untersuchen. Falls diese Zellen in der Lage sind, ausreichend anti-inflammatorische Zytokine zu produzieren, so könnten sie als Ziel für eine Immuntherapie dienen. Eine weitere Maßnahme, um die Entzündung zu reduzieren, ist die Identifikation und Neutralisation des Antigens, was Zellen stimuliert, pro-inflammatorische Zytokine auszuschütten. Aβ ist ein möglicher Kandidat, dessen Wirkung auf T-Zellen in dieser Studie untersucht wurde. Nach Übernachtstimulation mit PMA und Ionomycin oder Aβ-42 zusammen mit Brefeldin A wurde die Expression von verschiedenen Zytokinen in CD4+ und CD8+ T-Zellen mittels intrazellulärer Färbung für Durchflusszytometrie gemessen. Ergebnisse dieses Versuches zeigen keine größeren Unterschiede zwischen der Kontrollgruppe und den Patienten auf, unabhängig vom Schweregrad der Krankheit.

In weiteren Versuchen wurde die Expression von Chemokinrezeptoren untersucht. In AD führt eine in Mitleidenschaft genommene Blut-Hirn-Schranke zu einem intensiven Austausch zwischen Peripherie und Gehirn, der so normalerweise nicht stattfindet. Zytokine, Aβ und Zellen passieren die Schranke und eine der Konsequenzen ist eine systemische Entzündung. Ein entscheidender Punkt ist dabei die Rekrutierung von Immunzellen ins Gehirn. Um dies zu veranschaulichen, wurde die Expression von CCR2, CCR4, CCR5 und CCR6 auf Leukozyten im Blut

bestimmt. Dabei wurde eine höhere Expression auf Leukozyten von Patienten der Alzheimer-Krankheit vorgefunden, wenngleich auch nicht alle Unterschiede signifikant waren. Am Beispiel von CCR6 konnte gezeigt werden, dass die höhere Expression sich nicht auf eine bestimmte Zellgruppe beschränkt, sondern bei B-Zellen, Monozyten, CD4+ und CD8+ T-Zellen auftrat.

Insgesamt bietet diese Arbeit Hinweise für ein erschöpftes Immunsystem mit weiter differenzierten Zellen. Was dabei noch unklar ist, ist die Frage, ob die Rekrutierung von Immunzellen ins Gehirn Folge oder Ursache der Erkrankung ist. Dies zu beantworten benötigt weitere Untersuchungen. Ein Beispiel könnte die Blockade des Gehirneindringens in Modellsystemen sein. Dies würde auch zum Verständnis beitragen, ob die Unterstützung der Zellmigration den Aβ Abbau fördert und Neurodegeneration verlangsamt oder ob diese Zellen weiter zu einer verstärkten Entzündung beitragen. Als Untersuchungsmethode könnte die Erhöhung des Chemokingradienten dienen. Im Falle von negativen Effekten von Immunzellen im Gehirn könnte eine Behandlung von Patienten mit CCR-Blockern die Zellanhäufung vermindern. Dabei kann man auch gezielt bestimmte Zellen anvisieren und andere ausschließen durch die Wahl des zu blockenden Rezeptors. B-Zellen exprimieren z.B. CCR6, aber nicht CCR4 und CCR5. Nach Experimenten, die zeigen, welche Zellpopulation zur Entzündung beiträgt, könnte man spezifische Antikörper gegen Rezeptoren verwenden, die vorwiegend auf diesen Zellen exprimiert werden.

Frühere Studien an gesunden, älteren Menschen zeigten, dass CMV als chronischer Stimulus für das Immunsystem wirkt, vor allem für CD8+ T-Zellen. In AD könnte Aβ diese Rolle übernehmen. In dieser Studie wurde die Zytokinproduktion von T-Zellen nach Stimulation mit Aβ-42 analysiert, jedoch keine nennenswerte Produktion nachgewiesen. Ein anderer möglicher Effekt von Aβ könnte die Verschiebung von früh zu spät differenzierten Zellen sein. Dies könnte in Experimenten untersucht werden, in denen Zellen mit und ohne Aβ inkubiert werden und anschließend Immunprofile analysiert werden. Man könnte vermuten, dass eine Verjüngung des Immunsystem hilfreich für AD Patienten sein könnte. Ein Weg, dies zu erreichen, könnte das Verbessern des Aβ-Abbaus sein, zum Beispiel durch Erhöhung der Infiltration von peripheren Monozyten ins Gehirn. Eine Reduktion von Aβ könnte die Differenzierung von naiven zu Gedächtniszellen stoppen. Wie bei gesunden älteren Menschen CMV die Rolle der treibenden Kraft der CD8+ T-Zelldifferenzierung zugedacht wird, so könnte Aβ einen ähnlichen Effekt in AD besitzen. Ob CMV und Aβ Effekte additiv sind, bleibt noch zu untersuchen. Dafür wäre eine größere Anzahl an Studienteilnehmern als hier untersucht nötig, die genügend CMV- und CMV+ AD Patienten und Kontrollen beinhaltet. Diese Fragestellung ist von besonderer Bedeutung, da CMV zur Entzündung beitragen und somit eine weitere Belastung des Immunsystems darstellen könnte, welche die Kontrolle der Krankheit erschwert. Der Effekt von CMV auf das Immunsystem in funktionellem Verlust könnte auch bei kognitivem Verlust relevant sein.

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Glossary

Aβ
  Amyloid β.

AD
  Alzheimer's Disease.

ADDTC
  The State of California Alzheimer's Disease Diagnostic and Treatment Centers.

APC
  Antigen presenting cell.

ApoE
  Apolipoprotein E.

APP
  Amyloid precursor protein.

BBB
  Blood-brain barrier.

CCL
  CC chemokine ligand.

CCR
  Chemokine receptor.

CMV
  Cytomegalovirus.

CNS
  Central nervous system.

CRP
  C-reactive protein, an inflammation marker.

CSF

Cerebrospinal fluid.

CT

X-ray computed tomography.

DC

Dendritic cell.

DMSO

Dimethylsulfoxide.

EDTA

Ethylenediaminetetraacetic acid.

ELISA

Enzyme-linked immunosorbent assay.

EMA

Ethidium monoacide bromide used to label dead cells.

FBS

Fetal bovine Serum.

Foxp3

Forkhead box P3, a marker for regulatory T-cells.

HBMECs

Human brain microvascular endothelial cells.

HSV

Herpes simplex virus.

ICD-10

International Statistical Classification of Diseases criteria, including six subtypes of VaD.

IFNγ

Interferon gamma, a pro-inflammatory cytokine.

Ig

Immune globulin.

IL

Interleukin, interleukins are a group of chemokines.

IRP

Immune risk profile, set of immune parameters which correlate with survival.

KLRG1

Killer cell lectin-like receptor subfamily G member 1.

LPS

Lipopolysaccharide, an endotoxin.

MHC

Major histocompatibility complex.

MMSE

Mini mental state examination; 30 point questionnaire established 1975 by Folstein to diagnose cognitive impairment.

MRI

Magnetic resonance imaging (MRI), nuclear magnetic resonance imaging (NMRI), or magnetic resonance tomography (MRT).

NFT

Neurofibrillary tangles.

NINCDS-ADRDA

NINCDS-ADRDA National Institute of Neurological and Communicative Diseases and Stroke - Alzheimer's Disease and Related Disorders Association.

NINDS-AIREN

National Institute of Neurological Disorders and Stroke - Association Internationale pour la Recherche et l'Enseignement en Neurosciences.

NK-cell

Natural killer cell.

PBMC

Peripheral blood mononuclear cell.

PBS

Phosphate buffered saline.

PD-1

Programmed cell death protein 1, a marker of immune exhaustion.

PET

Positron emission tomography.

PMA

Phorbol 12-myristate 13-acetate.

PS

Presenilins, a family of transmembrane proteins that are found in the complex which processes APP to $A\beta$.

rpAD

Rapidly progressive Alzheimer's disease.

TCR

T-cell receptor.

TNF

Tumour necrosis factor, a pro-inflammatory cytokine.

Treg

Regulatory T-cell.

VaD

Vascular dementia.

XVIVO-15

Culture medium for PBMC.