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Review

Herpes Simplex Virus Type 1 and Other Pathogens are Key Causative Factors in Sporadic Alzheimer's Disease

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Abstract. This review focuses on research in epidemiology, neuropathology, molecular biology, and genetics regarding the hypothesis that pathogens interact with susceptibility genes and are causative in sporadic Alzheimer's disease (AD). Sporadic AD is a complex multifactorial neurodegenerative disease with evidence indicating coexisting multi-pathogen and inflammatory etiologies. There are significant associations between AD and various pathogens, including Herpes simplex virus type 1 (HSV-1), Cytomegalovirus, and other *Herpesviridae*, *Chlamydophila pneumoniae,* spirochetes, *Helicobacter pylori,* and various periodontal pathogens. These pathogens are able to evade destruction by the host immune system, leading to persistent infection. Bacterial and viral DNA and RNA and bacterial ligands increase the expression of pro-inflammatory molecules and activate the innate and adaptive immune systems. Evidence demonstrates that pathogens directly and indirectly induce AD pathology, including amyloid-β (Aβ) accumulation, phosphorylation of tau protein, neuronal injury, and apoptosis. Chronic brain infection with HSV-1, *Chlamydophila pneumoniae*, and spirochetes results in complex processes that interact to cause a vicious cycle of uncontrolled neuroinflammation and neurodegeneration. Infections such as Cytomegalovirus, *Helicobacter pylori*, and periodontal pathogens induce production of systemic pro-inflammatory cytokines that may cross the blood-brain barrier to promote neurodegeneration. Pathogen-induced inflammation and central nervous system accumulation of Aβ damages the blood-brain barrier, which contributes to the pathophysiology of AD. Apolipoprotein E4 (ApoE4) enhances brain infiltration by pathogens including HSV-1 and *Chlamydophila pneumoniae*. ApoE4 is also associated with an increased pro-inflammatory response by the immune system. Potential antimicrobial treatments for AD are discussed, including the rationale for antiviral and antibiotic clinical trials.

Keywords: Alzheimer's disease, ApoE4, amyloid, Cytomegalovirus, dementia, Herpes simplex, neurodegeneration, pathogen

THE ALZHEIMER'S DISEASE PATHOGEN HYPOTHESIS

Alzheimer's disease (AD) is an inflammatory brain disease that affects 20 million people worldwide and the incidence is expected to rise. Current medical treatment is not optimal, and thus an effective treatment is very much needed. The disease is associated with a combination of environmental agents and genetic influences leading to inflammation of the brain, neuronal cell death, and progressive dementia [1].

AD is characterized by two main pathological features in the brain: senile plaques and neurofibrillary tangles (NFTs). Senile plaques are extracellular and are

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predominantly made up of amyloid-β (Aβ), a peptide cleaved from the much longer amyloid-β protein precursor (AβPP). Neurofibrillary tangles are intracellular and comprised of abnormally phosphorylated tau protein. Tau protein is normally associated with microtubules in neurons, and contributes to AD pathology in its phosphorylated state [2].

The AD pathogen hypothesis states that pathogens act as triggers, interacting with genetic factors to initiate the accumulation and/or formation of Aβ, hyperphosphorylated tau proteins, and inflammation in the AD brain. Herpes simplex virus type 1 (HSV-1) and other pathogens including *Chlamydophila pneumoniae* and Spirochetes are able to infect the brain, evade the host immune response, and are highly prevalent in the AD brain [3–9]. *In vitro* studies and animal models indicate that pathogens induce formation of Aβ, amyloid plaques, and hyperphosphorylated tau proteins [10–13]. Pathogens induce a glial inflammatory response and can directly and indirectly damage and destroy neurons [14–18]. Significant inflammatory cascades are activated in the brains of AD patients [19, 20]. Together, these processes result in neurodegeneration and disease progression.

This review examines evidence implicating HSV-1 and Cytomegalovirus (CMV), both members of the *Herpesviridae* family, and the bacterial pathogens *Chlamydia pneumoniae*, spirochetes, periodontal pathogens, and *Helicobacter pylori* as causative in the pathogenesis of AD. Limited evidence is also presented regarding the *Herpesviridae* Epstein Barr Virus (EBV) and Human herpes virus 6 (HHV-6) as possible contributing factors in AD pathogenesis. The multi-pathogen AD hypothesis does not exclude toxins or other environmental co-factors that may be involved in the pathogenesis of AD and are reviewed elsewhere [21]. Pathogens were selected based on the degree of significant cumulative evidence identified in an extensive PubMed literature search.

HERPES SIMPLEX VIRUS TYPE 1

HSV-1 is a neurotropic virus that infects most humans, attaining 90% prevalence by the sixth decade of life. Infection is life long, as the virus resides in the trigeminal ganglia of the peripheral nervous system in latent form with viral genome but no virions present. Reactivation leads to viral replication and acute infections known as herpes labialis, commonly referred to as cold sores [22].

In 1982, Melvin Ball hypothesized that HSV-1 was causative in AD. He proposed that latent HSV-1 located in the trigeminal ganglia could reactivate and ascend along known nerve pathways into the limbic system and areas of the brain most affected in AD [23].

Herpes simplex encephalitis and AD affect the same brain regions, including the frontal lobes, temporal lobes, and hippocampus. Herpes simplex encephalitis survivors show cognitive, memory, and behavioral decline. Other viruses implicated in neurological disease include measles in subacute sclerosing panencephalitis and human immunodeficiency virus in HIV-associated dementia [22]. As with AD, both subacute sclerosing panencephalitis [24] and HIV infection [25] are associated with the formation of phosphorylated tau protein and NFTs in the brain.

EPIDEMIOLOGICAL STUDIES: HSV-1 HUMORAL RESPONSE, COGNITIVE DECLINE, AND AD

Epidemiological studies show an association between viral infectious burden (IB) and cognitive decline. IB is defined as a composite serological measure of exposure to common pathogens [27]. Strandberg *et al*. measured seropositivity to HSV-1, HSV-2, CMV, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae* in 383 elderly patients with cardiovascular disease. Assessments including the Mini-Mental Status Examination (MMSE) and the Clinical Dementia Rating were used to define cognitive impairment. Having three positive viral titers was associated with a 2.5 times higher risk for cognitive impairment after 12 months [26]. Katan *et al*. [27] found an association between *Herpesviridae* and cognitive decline using a composite serologic measure of exposure to both bacterial (*Chlamydia pneumoniae* and *Helicobacter pylori*) and viral (CMV, HSV-1, and HSV-2) pathogens. As reviewed by Strandberg, the association was primarily driven by viral IB [28].

Letenneur *et al*. studied the risk of developing AD according to the presence or absence of serum anti-HSV IgG and IgM antibodies by following 512 elderly patients initially free of dementia for 14 years. The presence of anti-HSV IgM antibodies is associated with primary infection or recent reactivation of HSV. In contrast, the presence of anti-HSV IgG antibodies indicates lifelong HSV infection [29]. Subjects who were IgM-positive at baseline showed a significantly higher risk of developing AD (hazard ratio = 2.55). No significant increased risk for AD was found in IgG-positive

subjects. Among the 43 IgM-positive subjects, only 2 were IgG-negative, which supports recent HSV reactivation rather than primary infection in most of the IgM-positive subjects [29]. Similar results were obtained in a longitudinal study by Lövheim *et al*. involving 3,432 elderly patients with a mean follow-up time of 11.3 years. Baseline increased serum levels of anti-HSV IgM antibodies were associated with increased risk of developing AD by a factor of 2 [30]. Thus, HSV reactivation, as indicated by the presence of anti-HSV IgM antibodies, is highly correlated with incident AD [29, 30].

Kobayashi *et al*. [31] used the avidity index of anti-HSV-1 IgG antibodies as an indicator of HSV-1 reactivation. The study, involving patients with amnestic mild cognitive impairment (MCI), AD, and healthy controls evaluated the relationship between HSV-1 reactivation and the degree of cognitive impairment in AD. The avidity index is defined as the strength with which IgG attaches to antigen [32]. HSV reactivation is characterized by increased levels of high-avidity anti-HSV IgG antibodies compared to lower levels seen with initial HSV infections [31]. MMSE and frontal assessment battery were used to assess cognition. MCI patients had a higher anti-HSV-1 IgG antibody avidity index than AD patients or healthy controls implying that HSV-1 reactivation occurs more frequently in the MCI group than in the AD group or healthy control group. Differences in anti-HSV-1 IgG antibody titer and anti-HSV-1 avidity index readings between the MCI group and healthy controls also suggests that reactivation of HSV-1 contributes to progression from healthy state to MCI [31].

In a longitudinal nested case–control study, Lövheim *et al*. measured plasma HSV antibody samples taken on average 9.6 years before AD diagnosis. In the 360 patients who developed AD and who had a follow-up time of 6.6 years or more, past HSV infection (as indicated by the baseline presence of anti-HSV IgG antibodies) increased the risk of developing AD by a factor of 2.25 [33].

Schretlen *et al*. evaluated cognitive performance in a group of patients who had been diagnosed with schizophrenia with an average cohort age of 39 years [34]. Schizophrenia patients who were HSV-1 IgG antibody seropositive performed significantly worse on neuropsychological measures (including psychomotor speed, executive functioning, and explicit verbal memory) than the combined HSV-1 and HSV-2 IgG antibody seronegative control group. Patients who tested seropositive for HSV-1 had decreased grey matter volume in the anterior cingulate and cerebellum seen on morphometric magnetic resonance imaging (MRI) of the brain compared to the HSV-1 seronegative control group [34]. Poor cognitive test performance correlated with decreased grey matter volume in some of the same brain regions that distinguished the patient subgroups defined by HSV-1 status [34]. Several studies have confirmed significant cognitive impairment in HSV-1 IgG seropositive schizophrenia patients compared to HSV-1 IgG seronegative controls with average cohort ages reported as 38 to 42 years old [35–39]. A causal association between exposure to HSV-1 and increased risk for schizophrenia has not been proven [34]; however, HSV-1 exposure in this group of neuropsychiatric patients is associated with cognitive impairment and provides further supportive evidence for the role of HSV-1 in cognitive dysfunction.

Higher levels of HSV-1 humoral immune response appear to play a protective role in the early stages of AD. Analyses performed with voxel-based morphometric brain MRI in AD patients and healthy controls indicate the presence of significant correlations between the preservation of cortical bilateral temporal and orbitofrontal grey matter volumes with higher HSV-1 IgG serum antibody titers [40].

HSV-1 IS HIGHLY PREVALENT IN ELDERLY BRAINS

Polymerase chain reaction (PCR) methods used by Jamieson *et al*. to detect HSV-1 DNA in autopsy brain specimens confirmed that latent HSV-1 is present in a high proportion (70–100%) of sporadic AD and elderly normal brains [4]. HSV-1 was found in brain areas most affected by AD, namely the temporal cortices, frontal cortices, and hippocampus. The Jamieson *et al*. findings have been confirmed in several studies (Table 1) [41]. The virus was found in very low proportions in younger brains [42]. In addition, Mori *et al*. [43] and Rodriguez *et al*. [44] used PCR to identify HSV-1 DNA in AD brains. PCR improves sensitivity in HSV-1 detection when compared to previously applied techniques such as *in situ* hybridization [4]. Some PCR studies had lower detection rates than others, perhaps due to a lower prevalence of HSV-1 infection in Japan [45] or age not having been taken into account. For unknown reasons, Hemling et al. [46] and Marquis et al. [47] detected HSV-1 DNA in a very low proportion of brains.

Intrathecal HSV-1 IgG was found in 52% of an AD cohort and 69% of the age-matched normal group using enzyme-linked immunosorbent assay (ELISA)

testing [48]. This data confirms the aforementioned PCR finding that HSV-1 DNA sequences are present in many elderly brains as a whole functional HSV-1 genome and provides evidence that the virus replicates in the brain [48].

HSV-1 IN THE BRAIN OF *APOE*-ε4 ALLELE CARRIERS INCREASES THE RISK FOR AD

Additional evidence for HSV-1 in AD involves the type-4 allele of the apolipoprotein E gene, known as *APOE*-ε4 or APOE4. A significantly increased risk for sporadic AD is associated with the presence of both HSV-1 in brain and carriage of the *APOE*-ε4 allele [49]. As shown in a study of AD postmortem brains by Itzhaki *et al*. [49] and confirmed by Lin *et al*. [50], neither HSV-1 nor the *APOE*-ε4 allele alone was found to be a risk factor for AD. However, the combination of HSV-1 with the *APOE*-ε4 allele increased the risk for AD by a factor of 12 [50]. HSV-1 in the brains of *APOE*-ε4 allele carriers accounted for over half of AD patients in the study (Table 2) [49]. The proportion of HSV-1 positive elderly controls was similar to that of HSV-1 positive AD patients, indicating that the AD brain is not predisposed to HSV-1 infection. Few HSV-1 positive elderly controls were positive for the *APOE*-ε4 allele, indicating that *APOE*-ε4 allele carriers are not predisposed to HSV-1 infection [49]. Itzhaki's results were later confirmed by Itabashi and colleagues [45].

Table 1
Studies that have detected HSV-1 DNA using PCR in brain tissue from patients with AD and controls (non-neurological cases)

Study	Primers used for PCR	Area of brain sample	HSV-1 DNA-positive individuals	
			AD *n* (%)	Controls *n* (%)
Jamieson *et al*. [4]	TK	Temp, frontal cortex, hippocampus	8 (100)	6 (100)
Jamieson *et al*. [42]	TK	Temp, frontal cortex, hippocampus	21 (67)	15 (60)
Baringer and Pisani [271]	Various	Various	NR	40 (35)
Gordon *et al*. [272]	Various	Hippocampus and frontal cortex		30 (27)
Itabashi *et al*. [45]	gD	Temporal and frontal cortex	46 (30)	23 (22)
Itzhaki *et al*. [49]	TK	Frontal and temporal cortex	46 (67)	44 (64)
Lin *et al*. [50]	TK	Frontal and temporal cortex	61 (74)	48 (63)
Bertrand *et al*. [273]	gD	Various	98 (75)	57 (72)
Cheon *et al*. [274]	gD	Frontal cortex	10 (100)	10 (100)

HSV-1, herpes simples virus type 1; AD, Alzheimer's disease; PCR, polymerase chain reaction; gD, glycoprotein D protein; TK, thymidine kinase; NR, not Reported. Table adapted from Itzhaki R (2004) Herpes simplex virus type 1, apolipoprotein E and Alzheimer' disease. *Herpes* **11**(Suppl 2), 77A-82A. [41] Reprinted with permission from Ruth Itzhaki.

Table 2
APOE genotypes of Alzheimer's disease patients and aged non-Alzheimer's disease patients positive or negative for Herpes Simplex Virus Type 1 in all brain regions

	Overall data for all brain regions					
	Non-AD ($n=44$)			AD ($n=46$)		
	HSV1+	HSV1–	Total	HSV1+	HSV1–	Total
Genotype						
ε2/ ε2	0	0	0	0	0	0
ε2/ ε3	1	3	4	2	1	3
ε2/ ε4	0	0	0	0	0	0
ε3/ ε3	25	11	36	5	7	12
ε3/ ε4	2	2	4	20	2	22
ε4/ ε4	0	0	0	9	0	9
Allele number						
ε2	1	3	4	2	1	3
ε3	53	27	80	32	17	49
ε4	2	2	4	38	2	40
APOE- ε4	3.6%	6.3%	4.5%	52.8%	10.0%	43.4%

HSV-1 in brains of *APOE*-ε4 allele carriers accounts for over 50% of AD brains with testing done postmortem. Table from Itzhaki RF, Lin WR, Shang D, Wilcock GK, Faragher B, Jamieson GA (1997) Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* **349**, 241-244 [49]. Copyright 1997. Reprinted with permission from Elsevier and Ruth Itzhaki.

APOLIPOPROTEIN E INFLUENCES HSV-1 VIRAL LOAD IN ANIMAL BRAIN STUDIES

Apolipoprotein E dosage and the presence of *APOE-ε4* determine latent HSV-1 DNA concentrations in the mouse brain [51]. Burgos and colleagues inoculated mice with HSV-1 and measured brain viral DNA concentrations. Thirty-seven days after infection, the HSV-1 brain DNA concentrations for APOE+/+ wild-type mice were 13.7 times greater than those of APOE–/– knockout mice. HSV-1 brain DNA concentrations for human APOE4 transgenic mice were 13.6 times greater than those of APOE3 mice. Apolipoprotein E4 appeared to facilitate HSV-1 latency in the brain much more than apolipoprotein E3, and APOE dosage correlated directly with the concentration of HSV-1 in the brain [51]. Guzman-Sanchez and collaborators later confirmed that apolipoprotein interacts with HSV-1 in animal models to increase viral load in the brain. 2-month-old wild-type and APOE knock-out mice were infected with HSV-1 and followed for 16 months. Viral load was found to increase with age. Viral load in the brains of aged APOE+/+ wild-type female mice was 43 times that seen in knock-out APOE–/– male mice. Although no neuropathological or brain MRI morphological differences were detected between 18-month-old infected mice when compared to controls, the central nervous system (CNS) HSV-1 infected mice showed associated memory deficit and reduction in metabolic indicators of CNS health [52]. These animal studies which associate APOE4 with increased HSV-1 viral load in the brain may relate to Itzhaki's human postmortem study, indicating that the combined presence of HSV-1 in brain and carriage of the *APOE-ε4* allele are involved in the pathogenesis of AD [49].

GENOME-WIDE ASSOCIATION STUDIES

Two genome-wide association studies identified APOE, complement receptor 1 (CR1), clusterin (CLU), and phosphatidylinositol binding clathrin assembly protein (PICALM) as major susceptibility genes in AD [53]. These susceptibility genes are associated with the HSV life cycle, and relate either directly or indirectly to cellular entry, intracellular transport, nuclear egress, AβPP processing, and Aβ processing [53].

AD AMYLOID PLAQUES CONTAIN HSV-1 DNA

HSV-1 coexists with Aβ in AD amyloid plaques [3]. Using *in situ* PCR to detect HSV-1 DNA and immunohistochemistry or thioflavin S staining to detect amyloid plaques, Wozniak and coworkers discovered a striking co-localization of HSV-1 DNA and Aβ within senile plaques in postmortem brains (Fig. 1) [3]. In AD brains, 90% of the plaques contained HSV-1 DNA and 72% of the total brain HSV-1 DNA was associated with plaques. The HSV-1 DNA associated with plaques was much lower in aged normal brains than in AD brains ($p < 0.001$) [3]. The co-localization of HSV-1 DNA and Aβ within amyloid plaques in the AD brain places HSV-1 in direct juxtaposition with a highly significant AD biomarker and suggests a significant role for HSV-1 in AD pathogenesis.

Fig. 1. Co-localization of HSV-1 DNA and amyloid-β in AD plaques. A strong co-localization showing HSV-1 DNA (brown staining using PCR) and amyloid plaque (blue staining using immunohistochemistry) in a postmortem AD brain (G). Greater than 90% AD plaques contained viral DNA. Scale bar = 50 μm. Figure from Wozniak, MA, Mee AP, Itzhaki RF (2009) Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. *J Pathol* **217**, 131-138 [3]. Copyright 2008. Reprinted with permission from John Wiley and Sons, Inc. and Ruth Itzhaki.

IN VITRO AND ANIMAL STUDIES: HSV-1 INFECTION INDUCES ELEVATED LEVELS OF Aβ AND P-TAU

Human cultured neuroblastoma cells infected with HSV-1 *in vitro* produce $A\beta_{42}$ and $A\beta_{40}$, and increased amounts of the enzymes β-site AβPP-cleaving enzyme (BACE-1) and nicastrin (a component of the γ-secretase enzyme) [10]. Both enzymes are involved in cleavage of the AβPP to produce Aβ. Rapid reduction of AβPP and a dramatic increase in $A\beta_{42}$ and $A\beta_{40}$ is seen in HSV-1-infected neuronal cell cultures [10]. Rat cortical neurons challenged with HSV-1 demonstrate hyperexcitability, membrane depolarization, and increased intracellular calcium levels with enhanced calcium dependent-AβPP phosphorylation and intracellular accumulation of $A\beta_{42}$ [54]. Animal models

also support HSV-1 as causative in the formation of Aβ. Mouse brain infected with HSV-1 produced marked increases in $A\beta_{42}$ five days post-intranasal infection when compared to uninfected controls [10]. These studies indicate that HSV-1 exposure to neuronal cells results in cellular production of Aβ.

HSV-1 is able to induce tau phosphorylation, thus linking HSV-1 to the formation of another abnormal protein found in AD brains. Neuroblastoma cells infected with HSV-1 produce hyperphosphorylated tau protein and increased amounts of the enzymes that phosphorylate tau protein including glycogen synthase kinase-3β (GSK-3β) and protein kinase A [11]. Alvarez *et al*. demonstrated accumulation of hyperphosphylated tau protein within the nucleus of HSV-1 infected neuroblastoma cells [55]. Zambrano *et al*. showed that HSV-1 infection of murine neuronal cultures results in tau hyperphosphorylation and alterations in the microtubule dynamics of the neuronal cytoskeleton [56]. The ability of HSV-1 to induce phosphorylation of tau proteins in neuronal cells is significant because p-tau proteins contribute to the formation of NFTs in AD brains [2].

ADDITIONAL MOLECULAR EVIDENCE AND CELLULAR MECHANISMS RELATING HSV-1 TO AD

Additional studies show a structural link between HSV-1 and Aβ. $A\beta_{34\text{-}42}$ is 67% identical to the HSV-1 envelope protein glycoprotein B (gB) peptide sequence, indicating peptide homology [57]. Synthetic peptides derived from the HSV-1 gB fragment self-assemble into thioflavin-positive fibrils and form β-pleated sheets that are ultrastucturally indistinguishable from Aβ [57]. The gB fragment accelerates *in vitro* formation of Aβ fibrils that are toxic to primary cortical neurons at a dose comparable to Aβ [57].

HSV-1 travels inside the neuronal cytoplasm in association with AβPP [58]. In squid axons, HSV-1 travels with AβPP during fast anterograde transport from the nerve cell body down the axon [59]. The virus interferes with AβPP processing in HSV-1-infected neuronal cells, reducing the level of AβPP and increasing the level of a 55 kDa C-terminal AβPP fragment containing Aβ [60]. De Chiara *et al*. found that HSV-1 infection of neuroblastoma cells and rat cortical neurons induces multiple cleavages of AβPP with resultant neurotoxic intra and extracellular AβPP fragments that comprise portions of Aβ. Components of the amyloidogenic AβPP processing pathway, including host cell β-secretase, γ-secretase, and caspase-3 like enzymes, were shown to be involved in the AβPP cleavage process. These findings suggest that repeated HSV-1 reactivation in the presence of other risk factors may play a co-factorial role in the development of AD [61]. Cheng and colleagues evaluated HSV-1 interactions with AβPP using immune-fluorescence, immunogold electron microscopy, and live cell confocal imaging to visualize newly synthesized viral particles inside epithelial cells as they traveled to the cell surface. Cytoplasmic HSV-1 particles labeled with green fluorescent protein co-localized and traveled with AβPP inside living cells. Most intracellular HSV-1 particles interacted frequently with AβPP, which facilitated viral transport while interfering with normal AβPP transport and distribution. Intracellular HSV-1 interactions with AβPP provide a mechanistic basis for the association between HSV-1 seropositivity and AD [62].

Santana *et al*. demonstrated that HSV-1 infection of neuroblastoma cells induced significant intracellular accumulation of Aβ in autophagosomes and a marked decrease in Aβ secretion. Aβ failed to fuse with lysosomes in HSV-1-infected neuroblastoma cells, indicating the impaired degradation of Aβ localized in autophagic vesicles [63]. HSV-1 decreases autophagy using HSV-1 infected cell polypeptide 34.5 (ICP 34.5) which blocks the protein kinase R (PKR) and eukaryotic initiating factor 2α (eIF2α) signaling pathways [64]. This action inhibits HSV-1 degradation by interfering with autophagy of the virus [64, 65]. Itzhaki suggests that this may lead to a decrease in Aβ clearance and an accumulation of senile plaques in AD [66].

HSV-1 can both block and induce neuronal apoptosis. HSV-1 protein ICP34.5 dephosphorylates eIF2α to block both the shutdown of host cell protein synthesis and apoptosis [66, 67]. HSV-1 infection of murine neuronal cultures results in marked neurite damage and neuronal apoptosis [56].

HSV-1 INDUCES AD-LIKE INFLAMMATION AND OXIDATIVE STRESS

Elevated levels of pro-inflammatory cytokines are consistently found in the brains of AD patients [19, 20]. Infection by HSV-1 induces expression of cytokines and pro-inflammatory molecules, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, IL-8, macrophage inflammatory protein 1-α (MIP-1 α), chemokine (C-C motif) ligand 5 (CCL5), and chemokine CXCL 10 in human microglial cells

[17]. Persistent cytokine expression occurs in mouse trigeminal ganglion infected with HSV-1, including IL-2, IL-6, TNF-α, interferon- γ (IFN- γ), IL-10, and CCL5 [18]. The direct effects of HSV-1 on neurons and the host inflammatory response to infection can lead to oxidative damage due to increased formation of reactive oxygen and reactive nitrogen species [68].

Interactions between HSV-1 and oxidative stress promote neurodegenerative processes found in AD. In HSV-1 infected human neuroblastoma cells, experimentally induced oxidative stress was found to significantly enhance the accumulation of intracellular Aβ, inhibit Aβ secretion, and appeared to be mediated by HSV-1 infection [69]. Oxidative stress also potentiated the accumulation of autophagic compartments within the cell [69]. HSV-1 interactions with oxidative stress are significant because oxidative damage is thought to occur early in the pathogenesis of AD [70].

HSV-1 REACTIVATION IN THE BRAIN

Itzhaki points out the lack of methodology for detecting the hypothesized sub-clinical limited reactivation of HSV-1 in localized areas of the brain in AD patients [32]. This contrasts with clinically apparent acute HSV encephalitis, where detection of HSV-1 DNA in cerebrospinal fluid (CSF) is commonly used for diagnosis [32]. Mild forms of HSV-1 encephalitis in humans have been reported [71, 72]. These patients usually have less severe symptomatology and good prognoses when compared to patients with severe diffuse HSV-1 meningoencephalitis. Klapper *et al*. suggest that sub-acute HSV-1 encephalitis may be a more common and often missed sub-clinical presentation of the disease [71].

Peter and collaborators reviewed 3,200 randomly selected CSF specimens submitted for HSV testing and found a total of 62 HSV positive specimens. HSV-2 was detected more often than HSV-1 (36:26). However, the HSV-2: HSV-1 ratio reversed in the patients over age 60 with HSV-1 being more prominent (3:13). Female patients who were positive for HSV-1 predominated in the over-70 age group (10 female and 1 male with 90% of females positive for HSV-1). This study shows predominance in the reactivation of HSV-1 rather than HSV-2 in older females, a group known for having a higher incidence of AD [73].

Saldanha *et al*. found that HSV-1 reactivates in the brains of immunosuppressed patients. HSV-1 DNA was detected by *in situ* hybridization in post-mortem frontal and temporal lobe human brain samples from immunosuppressed leukemia patients who were seropositive for HSV-1. HSV-1 DNA was not found in HSV-1 seronegative patients or in those who had not been immunosuppressed [74].

The reactivation rate of HSV-1 in the human brain is not known; however, animal and human studies involving the CNS and peripheral nervous system suggest that periodic sub-clinical reactivation may occur with subsequent immune response and neurodegeneration. Kaufman *et al*. measured the rate of asymptomatic HSV-1 reactivation and shedding in human tears from normal adults without signs of ocular herpetic disease. 74% of the 50 subjects were positive for HSV IgG by ELISA. 49/50 (98%) of subjects shed HSV-1 DNA at least one time during the course of the 30-day study [75].

Margolis and colleagues described spontaneous molecular reactivation of HSV-1 with viral protein expression, positive HSV-1 antigen staining, and infectious virus found in 6% of "latently" infected murine sensory ganglia 37 days post ocular infection [76]. Using *in situ* hybridization and immunohistochemistry, Feldman *et al*. found that HSV-1 spontaneous reactivation occurred in one neuron per 10 HSV-1 latently infected mouse trigeminal ganglia tested [77]. These neurons were surrounded by focal white cell infiltrate, indicating an inflammatory response. The authors estimate that this is equivalent to one neuron expressing high-level productive cycle viral genes in each ganglion every 10 days [77].

Asymptomatic reactivation of HSV-1 occurs *in vivo* in the CNS of mice and is associated with production of markers of neurodegeneration found in AD [78]. HSV-1 reactivation from the asymptomatic latent phase, was demonstrated by detection of viral ICP4 protein in the trigeminal ganglion and cerebral cortex of mice 60 days post-infection. Reactivation was accompanied by upregulation of both markers of neuroinflammation [(toll-like receptor (TLR)-4, interferon α/β, and phosphorylated interferon regulatory factor 3 (p-IRF3)] and early neurodegeneration (phospho-tau and caspase-3 cleaved tau proteins) [78]. These findings support the hypothesis that recurrent HSV-1 CNS reactivation could result in AD associated neurodegenerative processes.

PROPOSED MECHANISM OF HSV-1 PATHOGENESIS IN AD

The above data and studies support the hypothesis that HSV-1 in combination with *APOE-ε*4 allele carriage is a major cause of sporadic AD. As proposed

by Itzhaki, this highly prevalent virus reactivates and enters the brain in older age by way of the peripheral nervous system or the olfactory route. HSV-1 becomes latent in the brain, but periodically reactivates in association with systemic infection, immunosuppression, or other stressors. The reactivated virus causes limited local damage via direct viral action and through inflammatory and oxidative effects. This leads to deposition of Aβ and abnormal phosphorylation of tau, which eventually forms amyloid plaques and NFTs. Defective autophagy due to aging and viral ICP34.5 action prevents degradation of HSV-1 and reduces the degradation of Aβ and phosphorylated tau protein. This results in decreased clearance of these proteins [66, 79, 80].

CYTOMEGALOVIRUS AND OTHER *HERPESVIRDAE*

Cytomegalovirus

CMV is a β-herpes virus prevalent in humans causing persistent lifelong asymptomatic infection in the immunocompetent host. Primary infection usually occurs early in life and is asymptomatic but occasionally causes a self-limiting mononucleosis-like syndrome [81]. CMV seropositivity in the human population ranges from 20%–100% depending on socioeconomic status and age [82]. The virus resides in the myeloid cell compartment, remaining latent in monocytes [83], but has tropism for numerous cell types such as endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, neuronal cells, hepatocytes, trophoblasts, macrophages, and dendritic cells [81]. As with other members of the *Herpesviridae* family, CMV may reactivate under stress conditions or other stimuli. Other diseases associated with CMV infection in normal hosts include Guillain-Barre syndrome, meningoencephalitis, hemolytic anemia, and thrombocytopenia [81]. CNS infection by CMV in immunocompetent patients is rare. Most CMV brain infections occur in those who are immunocompromised, such as HIV-infected patients, transplant recipients, and infants with congenital CMV disease contracted *in utero* [82].

Epidemiological studies: CMV humoral response, cognitive decline, and AD

Several studies have shown an association between CMV infection and increased risk of both cognitive impairment and development of AD. Aiello *et al*. found that individuals with higher levels of IgG antibody to CMV at baseline experienced a more rapid rate of cognitive decline over a 4-year study period than those with lower levels [84]. Strandberg *et al*. [26] and Katan *et al*. [27] found that CMV was one of the viruses from the *Herpesviridae* family associated with cognitive decline as discussed in the HSV-1 section above. Carbone *et al*. studied a group of elderly patients and found baseline CMV IgG antibody levels to be significantly increased in patients who developed clinical AD over a 5-year follow-up period compared to patients who remained cognitively healthy [85]. Barnes *et al*. followed 849 participants and found that baseline CMV seropositivity doubled the risk of developing clinical AD over a 5-year follow-up period and noted a faster rate of decline in global cognition [86].

Tarter *et al*. studied cognitive impairment in various age groups in relation to CMV and HSV-1 seropositivity. Among children (ages 6–16 years), HSV-1 seropositivity was associated with lower reading and spatial reasoning test scores. Both HSV-1 and CMV seropositivity in middle–aged adults (ages 20–59 years) was associated with impaired coding speed. CMV seropositivity was also associated with impaired middle-aged learning and recall. Among older adults, HSV-1 seropositivity was associated with immediate memory impairment. The data indicated that HSV-1 may have a life course effect on cognition across all age groups, while CMV appeared to adversely affect cognition specifically in the middle aged. The authors suggest that individuals who acquire infection with these *Herpesviridae* earlier in life with more reactivations and subsequent immune activation may be at greater risk for developing social disparities in cognition, educational attainment, and social mobility across the life course [87].

Prevalence of CMV in the AD Brain

Data does not indicate a definitive direct infiltrative CNS role for CMV in AD. Using PCR, Lin *et al*. found CMV present in 16/45 (35.6%) of postmortem AD brains compared with 10/29 (34.5%) of non-AD controls, which was not statistically significant [88]. The authors point out that these values may be artificially high due to the possibility of CMV residing in lymphocytes within blood vessels rather than brain cells. In a more recent 2013 study, 93 AD brains were tested for CMV DNA using nested PCR and all samples were negative for CMV [85]. In contrast, Lin *et al*. found CMV in a very high proportion of postmortem vascular dementia brains [89]. CMV was found in 14/15 (93%) of brains from subjects who had been diagnosed with vascular dementia and was present in only 10/29

(34%) of age-matched normal controls. The results were statistically significant and suggest a possible role for CMV in vascular dementia [89].

CMV and immunosenescence: Impairment of the elderly immune system

CMV appears to be a strong causative factor in the development of immunosenescence by adversely affecting T cell immunity with resultant immune dysregulation and impairment in the elderly [90]. CMV has been implicated in T cell oligoclonal expansions, altered phenotypes and function of CMV specific CD8+ T cells, and decrease in the naïve and early memory T cell pool seen in the elderly [90]. Koch *et al.* reviews evidence for CMV involvement in immunosenescence and suggests that the long-term attempt of the T-cell immune system to keep CMV from spreading results in reduction of the naïve T-cell pool, leading to deficits in the immunological response to new antigens in the aged [90]. Clonal expansions of CD8+ T cells directed against another Herpes virus, EBV, are also seen in the aged population [91].

Increased reactivation of CMV and other herpesviridae in the elderly

Using molecular and serological techniques, Stowe *et al.* found significant increases in reactivation of CMV and EBV in elderly subjects compared to younger subjects. Increases in CMV DNA in urine and Epstein Barr viral load in peripheral blood were demonstrated. In addition, elevated levels of CD8+ T cells directed against CMV and EBV were found in the elderly group. The authors concluded that the aged immune system is no longer able to control EBV and CMV reactivation, resulting in chronic infection and age-related clonal expansions of CD8+ T cells directed against EBV and CMV [91].

Evidence suggests that CMV infection may adversely influence the immune response, allowing for increased HSV-1 reactivation. Stowe *et al.* measured serum CMV and HSV-1 antibody levels in 1,454 multiethnic subjects. Higher HSV-1 IgG serum antibody levels, which presumably reflect higher rates of HSV reactivation, were more common in CMV seropositive subjects. Elevated antibody titers to latent HSV-l were significantly associated with both CMV seropositivity and high CMV antibody levels. Increases in HSV-1 antibodies by age occurred in CMV seropositive individuals but not CMV seronegative subjects. Among CMV seropositive subjects, increases in HSV-1 antibodies by age were only found in individuals with low CMV antibody levels, as those with high CMV antibodies already exhibited elevated HSV-1 antibodies. The results suggest chronic CMV infection is able to accelerate immunosenescence, leading to immune dysregulation with increased HSV-1 reactivation [92].

CMV is associated with elevated IFN-γ that associates with AD

CMV-specific CD8+ T cells have been shown to produce increased amounts of pro-inflammatory IFN-γ and very low levels of anti-inflammatory cytokines IL-2 and IL-4 with a potential shift to a pro-inflammatory cytokine profile in the elderly [93]. Westman *et al.* measured the cytokine response of peripheral blood mononuclear cells (PBMCs) from CMV seropositive and seronegative AD patients. PBMCs from CMV seropositive AD patients challenged by CMV antigens produced increased amounts of IFN-γ compared with CMV seronegative AD patients and CMV seropositive non-demented controls [94]. The authors suggest CMV acts as an inflammatory promoter in AD immunology.

In the Rush AD Center Religious Orders Study, Lurain and colleagues studied a clinical-pathological community cohort by evaluating CMV serum antibody levels, CSF IFN-γ levels, cryopreserved lymphocytes, and brain pathology from deceased and autopsied subjects [82]. CMV-specific serum IgG antibody levels were significantly associated with NFTs. CSF IFN-γ was detected in greater than 80% of CMV seropositive but not in CMV seronegative subjects. In the CMV seropositive subjects, CSF IFN-γ levels were associated with NFTs. Therefore, this study showed an association between CMV seropositivity and detection of IFN-γ in CSF, which in turn was associated with AD pathology in the form of NFTs. In addition, the percentage of senescent T cells (CD28- CD57+) from the peripheral circulation was significantly higher for CMV-seropositive as compared to CMV-seronegative subjects [82]. This study did not prove CMV presence in the brain of AD patients as CMV intrathecal antibody levels were not measured, and thus viral replication of CMV within the brain was not substantiated [95]. However, results from the Lurain *et al.* study support an association between CMV infection and the development of AD with CMV-induced inflammation as one potential mechanism for this association [96].

Human herpesvirus 6

HHV-6 is a neurotropic virus and exists in 2 forms: type A and type B. The HHV-6A variant is considered more neurotropic than type B [97]. HHV-6B primary infection is the cause of the common childhood

illness exanthem subitum, which is also known as roseola infantum or sixth disease. This illness affects infants and typically presents with self-limiting fever followed by a maculopapular rash. Febrile seizures occur in 10–15% of cases, and severe CNS complications have been reported in rare cases. The virus is highly seroprevalent, affecting nearly 100% of the population by age 3 [98]. HHV-6 can cause meningoencephalitis, and has been associated with multiple sclerosis, seizures, and temporal lobe epilepsy [99]. HHV-6 establishes latency in the brain and can reactivate under conditions of immunosuppression [97].

HHV-6 has been found in the brains of AD patients in various studies using PCR; however, increased incidence in AD patients versus controls has not been shown with consistent statistical significance. Lin and collaborators studied 50 postmortem AD brains and found HHV-6 in 72% of frontal and temporal cortex samples versus 40% of age-matched normal brain samples, which was statistically significant [88]. In the HHV-6 positive brains, 59% (17/29) had type B alone, 3% had type A alone, and 38% (11/29) had both types. No additional increased risk for AD was found in *APOE*-ε4 carriers who were HHV-6 positive. The authors reasoned that HHV-6 might enhance the damage caused by HSV-1 and *APOE*-ε4 in AD. However, it was not possible to exclude HHV-6 as an opportunistic secondary infection, or the possibility that HHV-6 DNA is present within leucocytes within the brain vasculature [88]. Hemling *et al*. examined autopsy brain samples from hippocampus, temporal cortex, frontal cortex, and anterior cingulate gyrus, and found HHV-6 in 88% of AD and 87.5% of normal controls, indicating no significant difference between the two groups. However, the number of specimens from the different brain regions tested was not specified [46]. Carbone and colleagues found HHV-6 in 17.3 % of frontal cortex samples from postmortem AD patients using qPCR with no *APOE*-ε4 carrier association found. The same group found that baseline HHV-6 DNA positivity in peripheral blood leukocytes (PBLs) was significantly associated with cognitive decline and development of AD at 5-year follow-up [85].

Epstein barr virus

EBV is a Herpes virus that infects 95% of humans early in life resulting in lifelong latent asymptomatic infection residing in B-lymphocytes [100]. Primary infection of the oropharynx often occurs during childhood and is generally asymptomatic, although the virus does cause acute infectious mononucleosis in a minority of immune competent subjects. Intermittent reactivation of the virus occurs throughout life within B cells, involving a lytic cycle at mucosal sites with low levels of asymptomatic viral shedding [101]. EBV is causatively linked to Hodgkin lymphoma, Burkitt lymphoma, and nasopharyngeal carcinoma [102, 103]. EBV is also associated with neurological diseases including encephalitis, myelitis, mononeuritis [104,105], and multiple sclerosis [106].

Although EBV-related AD data is limited, the virus may be a risk factor for development AD. Carbone *et al*. measured EBV DNA in PBLs and postmortem brain samples from a group of AD subjects and non-AD controls. 45% of PBLs were EBV DNA positive in AD patients compared to 31% of controls, which was statistically significant. Using qPCR, only 6% of AD brains were EBV DNA positive with all of these subjects found to be *APOE*-ε4 positive. The same researchers found that baseline EBV DNA positive PBLs and serum IgG levels for EBV antigens were significantly increased in a group of elderly individuals who developed AD during a subsequent 5-year follow-up period [85]. Thus, positive IgG levels for EBV and peripheral viral infection involving PBLs with either EBV or HHV-6 have been associated with increased risk for AD even though significant infiltrative CNS presence has not been demonstrated for EBV and has been equivocal for HHV-6.

Bacterial pathogens

Chlamydophila pneumoniae

C. pneumoniae is an obligate intracellular respiratory pathogen that can persist as a chronic infection in monocytes, macrophages, and other cell types for long periods of time. Serum antibody prevalence reaches 70% to 80% by 60 to 70 years of age [107]. Evidence indicates that *C. pneumoniae* crosses the blood-brain barrier (BBB) after infection of the respiratory mucosa, with subsequent hematogenous and lymphatic dissemination within infected monocytes [108, 109]. *C. pneumoniae* is also thought to enter the CNS via the olfactory route [80]. *C. pneumoniae* can evade the mechanisms of bactericidal and oxidative stress, activate endothelial cells with production of adhesion molecules, and induce cytokine overproduction [107].

C. pneumoniae has a biphasic life cycle. The elementary body is spore-like, infectious, metabolically inactive, and attaches to and enters the host cell. Elementary bodies then differentiate into reticulate bodies, which are the reproductive forms. The reticulate bodies undergo binary fission, differentiate back into

elementary bodies, and exit the cell either by cytolysis with apoptosis or by exocytosis, leaving the cell intact [107, 110].

C. pneumoniae interferes with the normal apoptotic signaling pathways and can both inhibit and induce cellular apoptosis. The bacterium can evade the host cell's defense mechanisms, and exist as an acute infection or a chronic persistent infection [107, 111].

Under certain conditions, *C. pneumoniae* can enter into a chronic persistent phase characterized by aberrant reticulate bodies and other pleomorphic forms [112]. Metabolic activity is reduced and the organism is viable but non-cultivable, resulting in a chronic infection of the host cell [110, 112]. This persistent phase has been associated with several chronic diseases including asthma and chronic obstructive pulmonary disease [112].

Chlamydophila pneumoniae vascular infection and dissemination into the brain

Vascular infections with *C. pneumoniae* are associated with atheromasic plaques and may be an important factor in the development of brain infection with this pathogen. Using PCR and IHC techniques, Rassu *et al.* detected the presence of *C. pneumoniae* in atheromasic plaques sampled from five vascular sites in 18 autopsy cases (basilar artery, coronary artery, thoracic aorta, abdominal aorta, and renal arteries). The study showed 100% patient positivity with *C. pneumoniae* present at 2–5 sites for each case tested [113]. Di Pietro *et al.* investigated 19 postmortem cases with past chlamydial vascular infection using immunohistochemistry, PCR, *in situ* PCR and *in situ* reverse transcription PCR. *C. pneumoniae* was detected in the brain tissue of 16 out of 19 subjects (84.2%) who also had *C. pneumoniae* vascular infection. The organism was not detected in the brains of control subjects who were negative for *C. pneumoniae* vascular infection ($p = 0.0002$) [114]. These results provide evidence that a *C. pneumoniae* vascular infection can disseminate to the brain.

Prevalence of Chlamydophila pneumoniae in the AD brain

Balin *et al.* used PCR to identify *C. pneumoniae* in 17/19 (90%) of AD postmortem brain samples, and in only 1/19 (5%) of control brain samples, suggesting that infection with the organism is a risk factor for AD [6]. The results were confirmed using multiple methodologies. Electron microscopy and immunoelectron-microscopy studies identified chlamydial elementary and reticulate bodies in affected AD brain regions. *C. pneumoniae* was present in areas of the brain showing the typical AD neuropathology. Immunohistochemical tests on AD brains showed *C. pneumoniae* within pericytes, microglia, and astroglia. Reverse transcription (RT)-PCR assays using RNA from affected areas of AD brains confirmed the presence of transcripts from two important *C. pneumoniae* genes not seen in controls. Cultures were strongly positive for *C. pneumoniae* from a subset of affected AD brain tissues and negative in controls. *C. pneumoniae* was present, viable, and transcription-

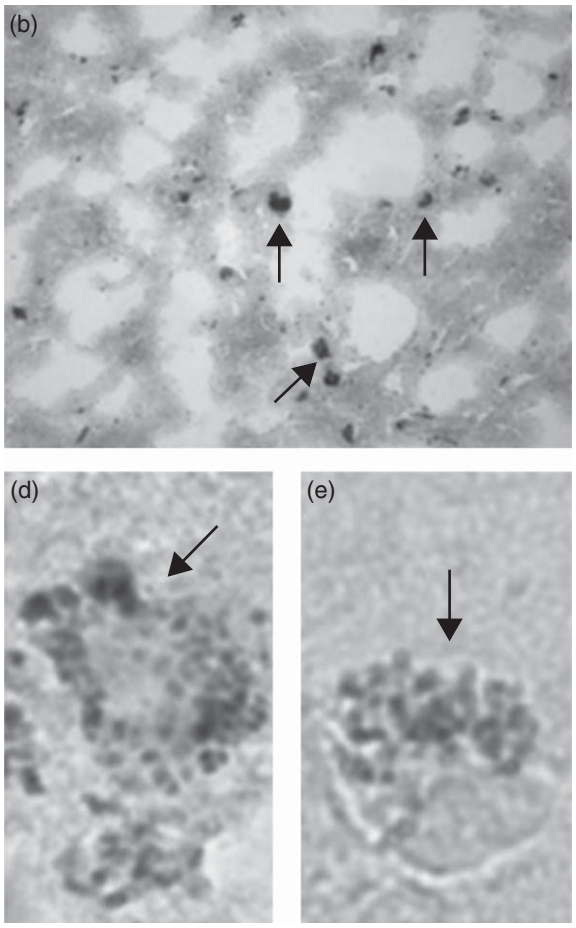

Fig. 2. Images demonstrating *Chlamydophila pneumoniae* in AD brain tissue by *in situ* hybridization. Figure (b) demonstrates *C. pneumoniae* from the hippocampus of an AD brain by *in situ* hybridization. Figures (d) and (e) show photographic enlargement of cells harboring *C. pneumoniae* inclusions identified in AD brain tissue. Arrows indicate the signal for C. *pneumoniae*. Image (b) was obtained using a x40 objective. Figure from Gérard HC, Dreses-Werringloer U, Wildt KS, Deka S, Oszust C, Balin BJ, Frey WH 2nd, Bordayo EZ, Whittum Hudson JA, Hudson AP (2006) Chlamydophila (Chlamydia) pneumoniae in the Alzheimer's brain. *FEMS Immunol Med Microbiol* **48**, 355-366 [7]. Copyright 2006. Reprinted with permission from John Wiley and Sons and permission from Brian Balin.

ally active in areas of neuropathology in the AD brain [6]. In addition to the standard morphological forms of the organism, pleomorphic forms of *C. pneumoniae* were later observed on ultrastructural analysis, suggesting an adaptive response and/or persistent state of infection for these organisms in AD [115]. As reviewed by Shima [116] and Balin [117], four studies [118–121] failed to detect significant *C. pneumoniae* in AD brains potentially due to sampling error, variable methodologies, and/or absence of standardized techniques.

Gerard and colleagues found *C. pneumoniae* in 20/25 (80%) of AD postmortem brain samples and 3/27 (11%) of controls (Fig. 2) [7]. Immunohistochemical analyses found that neurons, microglia, and astrocytes all served as host cells for *C. pneumoniae* [7]. Infected cells were seen in close proximity to senile neuritic plaques and NFTs [7]. In situ hybridization analysis in AD postmortem brains indicates an increase in the number of *C. pneumoniae* infected cells in *APOE-ε*4 carriers [123].

A statistically significant increase in CSF levels of *C. pneumoniae* DNA has been found in AD patients [122]. Miklossy found that combined data from studies attempting to isolate *C. pneumoniae* reached statistical significance in AD brains and AD brains plus CSF compared to controls (Table 3) [5].

Table 3
Detection of *Chlamydophila pneumoniae* in Alzheimer's disease

Material	Number	Method	AD	Control	Ref
Brain	38	PCR, EM, IHC, RT-PCR, Cult	17/19	1/19	[6]
Brain	25	PCR, IHC	0/25		[118]
Brain	20	PCR, IHC	0/20		[120]
Brain	20	PCR, Cult	2/15[a]	1/5[a]	[119]
Brain	21	PCR, ISH	21/21	0/1	[123]
Brain	52	PCR, Cult, RT-PCR	20/25	3/27	[7]
CSF	104	PCR, Cult	25/57	5/47	[122]
Total Brain	177	$p = 4.5 \times 10^{-7}$, OR = 8.7 CI = 3.1–29.5	60/125	5/52	
Brain and CSF	281	$p = 9.8 \times 10^{-11}$, OR = 7.8 CI = 3.7–17.8	85/182	10/99	

AD, number of AD cases with positive detection/number of AD cases analyzed: Control, number of control cases with positive detection/number of control cases analyzed; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; RT-PCR, reverse transcriptase-PCR; EM, electron microscopy; Cult, culture; P, exact value of significance following Fisher test; OR, odds ratio; CI, 95% confidence interval values; IHC, immunohistochemistry. [a]Positive in at least one of several samples. Table adapted from Miklossy J (2011) Emerging roles of pathogens in Alzheimer disease. *Expert Rev Mol Med* 13, e30 [5]. Copyright 2011. Reprinted with permission from Cambridge University Press and Judith Miklossy.

Chlamydophila pneumoniae induces inflammation, Aβ plaque formation, and neurodegeneration

Increased levels of cytokines IL1-β, IL-6 and TNF-α were found in supernatants of *C. pneumoniae*-infected murine microglial cells *in vitro* [124]. Neurons exposed to the supernatants displayed a significant increase in apoptosis [124].

C. pneumoniae infection in the brains of BALB/c mice via the intranasal route induces a significant increase in Aβ plaques compared with non-infected mice [12]. Early treatment of infected mice with moxifloxacin decreased the number of Aβ plaques to levels similar to those seen in uninfected control mice. Infected untreated mice had 8-9 times more Aβ plaques than the antibiotic treatment group [117, 125].

Spirochetes

Spirochetes are Gram-negative, helical bacteria that possess endoflagella. Spirochetes cause a number of chronic diseases including syphilis (*Treponema pallidum*), Lyme disease (Borrelia burgdorferi), and periodontal disorders such as gingivitis (oral periodontal Treponema spirochetes such as *T. sokranski* and *T. pectinovarum*) [5]. Spirochetes can invade the brain and form chronic persistent infections. They are known to spread by hematogenous dissemination, through the lymphatic system, and along nerve fibers [126].

Prevalence of spirochetes in AD brain

Spirochetes have been detected using various methodologies with prevalence approaching 90% in AD brains (Fig. 3) [5, 126]. The association was statistically significant in postmortem AD brain studies of all types of spirochetes combined, oral spirochetes, and *Borrelia burgdorferi*. Combined, studies detecting all types of spirochetes and their specific species indicated a prevalence of 68% (90/131) in AD brains compared to 8.45% in controls [5]. The spirochete frequency detected in all studies reviewed by Miklossy was eight times higher in AD brains than in controls [5]. Miklossy's extensive review of research data regarding spirochetes and AD indicate a probable causal relationship between neurospirochetosis and AD based on Koch's and Hill's criteria [126].

Using dark field microscopy, Miklossy identified spirochetes in blood, CSF, and brain in 14/14 AD autopsy cases and in 0/13 non-AD controls [9]. In this study, spirochetes were cultured from the blood of four AD cases. Concurrent silver stained and anti-AβPP-immunostained frozen section AD

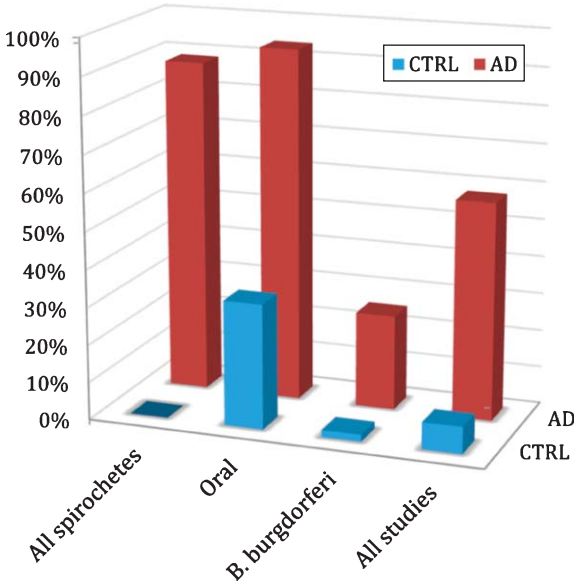

Fig. 3. Association of spirochetes with Alzheimer's disease. The frequency of spirochetes is significantly higher in the brains of Alzheimer's disease patients compared to controls. Graph from Miklossy J (2011) Alzheimer's disease - a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J Neuroinflammation* **8**, 90 [126]. Copyright 2011. Reprinted with permission under the terms of the Creative Commons Attribution License, (http://creativecommons.org/licenses/by/2.0) and permission from Judith Miklossy.

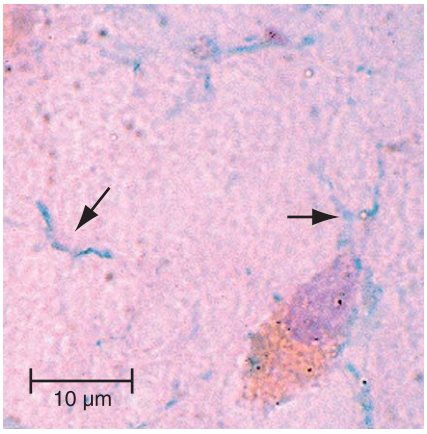

Fig. 4. Image of oral spirochetes in Alzheimer's disease brain. The oral spirochete *T. pectinovorum* stained dark blue (arrows) in a section from the hippocampus from an 84-year-old woman with Alzheimer's disease. The section was incubated with monoclonal antibodies to *T. pectinovorum*, and binding was disclosed using biotinylated anti-mouse antibodies and avidin-peroxidase. The photomicrograph was taken at1000X. Scale bar = 10 µm. Figure from Riviere GR, Riviere KH, Smith KS (2002) Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol Immunol* 17, 113-118 [127]. Copyright 2002. Reprinted with permission from John Wiley and Sons, Inc. and George Riviere.

brain specimens evaluated with electron microscopy revealed spirochetes located in areas of AD pathology. Immunohistochemistry using a specific antibody against *B. burgdorferi* identified spirochetes in senile plaques, neurons, neuropil threads, and in the leptomeningeal and cortical vessel walls in a patient with concurrent Lyme disease and AD [9]. Electron microscopy and atomic force microscopy techniques have been used to identify spirochetes isolated and cultured from postmortem AD brains [8]. PCR and immunohistochemistry identified oral spirochetes in 14/16 AD and 4/18 non-AD postmortem brains (Fig. 4) [127]. DNA identified within neuropil threads in AD brains using the florescent dye DAPI revealed a helically shaped morphology similar to the morphology and distribution in reference spirochete samples [128]. Spirochetes were detected in the brains of 8/8 postmortem AD cases and in the blood samples from five living AD patients [129]. Using immunohistochemical techniques, Borrelia antigens (including the outer surface protein A (OspA) of *B. burgdorferi*) and Borrelia genes were co-localized with Aβ deposits and NFTs in three AD brains from which *B. burgdorferi* was cultured [130]. Bacterial peptidoglycan has been immunolocalized to senile plaques and NFTs in autopsied brain specimens from 54 AD patients [131, 132]. In addition, peptidoglycan and was found co-localized with Aβ in AD brains but not in controls [131]. The synthetic peptide BH (9-10), which corresponds to a β-hairpin segment of the *B. burgdorferi* OspA protein, forms amyloid-like fibrils *in vitro* [133].

B. burgdorferi induces Aβ and p-tau formation, inflammation, and neurodegeneration

In vitro, *B. burgdorferi* invades mammalian neurons and glial cells to cause an AD-like host cell reaction. Aβ deposition is induced *in vitro* by exposure of mammalian neurons, astrocytes, microglial cells, and brain organotypic cell aggregates to *Borrelia burgdorferi sensu strictu* [13]. Histochemical and immunohistochemical analysis showed morphological changes including Aβ plaques with β-pleated sheet conformation and tangles. Intracytoplasmic granules found in astrocytes were similar to the granulovacuolar degeneration seen in AD neurons [13]. Increases in AβPP, Aβ, and hyperphosphorylated tau proteins were detected by western blot in these cells [13]. Nuclear fragmentation in rat astrocyte cells exposed to pleomorphic and cystic forms of *B. burgdorferi* suggests

that the spirochete can cause functional damage and apoptosis [134].

Exposure of rat glial cells to *B. burgdorferi* recombinant lipidated outer surface protein A (L-OspA) induces astrocyte proliferation and apoptosis. Astrocytes produce IL-6 and TNF-α in response to L-OspA [14]. *Ex vivo* stimulation of monkey brain explants with *B. burgdorferi* induces the production of cytokines IL-6, IL-8, IL-1β, cox-2, and the chemokine B lymphocyte chemoattractant (CXCL13) by glial cells, with concomitant glial and neuronal apoptosis [16].

Additional periodontal pathogens

In addition to the oral spirochetes discussed above, Kamer *et al*. found that both the number of positive tests for IgG serum antibodies against periodontal bacteria commonly involved in periodontitis (*A. actinomycetemcomitans, P. gingivalis*, and *T. forsythia*) and plasma TNF-α level were elevated in AD patients compared to normal controls. Both endpoints were independently associated with AD [135]. Results from the NHANES III study involving a large community sample, showed that the extent of periodontal disease, as measured by gingival bleeding, loss of periodontal attachment, and loss of teeth, was associated with significantly decreased cognitive function in early-, mid-, and late-adult life [136]. Cognitive testing included the Symbol Digit substitution and the Serial digit Learning Tests among patients 20–59 years of age and a Story Recall test in participants aged 70 years of age or older. Worse scores on all three measures of oral health status were significantly associated with poorer performance on all three measures of cognitive function after adjustment for age. Level of education was found to be an important confounding factor. The authors concluded that poor oral health is associated with impaired cognitive function throughout adult life [136]. A separate study from NHANES III showed an association between high serum antibody levels against *P. gingivalis* and lower cognitive function with delayed verbal recall and impaired subtraction in subjects greater than 60 years of age [137]. Thus, exposure to oral pathogens is associated with systemic inflammation, cognitive decline, and AD.

Helicobacter pylori

H. pylori is a curved spiral Gram-negative bacterium which colonizes the gastric mucosa in more than 50% of humans worldwide. The bacterium causes gastric disorders including functional dyspepsia, gastritis, peptic ulcer disease, and gastric cancer [138, 139]. *H. pylori* infection is associated with extra-digestive disorders including idiopathic thrombocytopenic purpura, vitamin B12 deficiency, and iron deficiency anemia [140]. The bacterium is also associated with vascular disorders such as atherosclerosis, ischemic stroke, and coronary artery disease [141].

H. pylori gastric infections may be diagnosed with non-invasive procedures including urea breath test, serology, or whole blood antibody testing depending on clinical circumstances [142]. Diagnostic tests for *H. pylori*, which require upper endoscopy with biopsy sampling of the gastric mucosa, include rapid urease test, histology, bacterial culture, and polymerase chain reaction technique [143].

Epidemiological studies: H. pylori infection, cognitive decline, and AD

Epidemiological studies support an association between *H. pylori* infection and both MCI and AD. Kountouras *et al*. studied sixty-three patients with amnestic MCI who underwent upper gastrointestinal endoscopy with histological and serological testing for *H. pylori* infection. Significantly increased *H. pylori* gastric infection, higher mean serum anti-*H pylori* IgG concentrations, and higher plasma total homocysteine titers were found in MCI patients compared to non-MCI anemic controls [144]. In another study, Kountouras and colleagues found a significantly higher rate of histologically proven *H. pylori* gastric infection among 50 AD patients compared to thirty non-AD anemic controls [145]. A longitudinal study by Roubaud-Baudron *et al*. followed 603 subjects who were initially free of dementia and 65 years or older. Baseline seropositivity for *H. pylori* IgG antibody was associated with a 1.46 times increased risk for the development of dementia over the 20 year follow-up period compared to non-infected controls [146]. In a prospective non-randomized study, Kountouras *et al*. found that AD patients had significantly higher levels of anti-*H. pylori*-specific IgG antibodies in CSF and serum than age-matched cognitively normal controls. The severity of AD, as indicated by lower MMSE scores, correlated with higher levels of anti-*H. pylori* IgG antibodies in the CSF of these patients. The authors concluded that the data appears to link *H. pylori* infection to the pathophysiology of AD. They could not exclude the passage of *H. pylori* IgG and antibodies through an AD-related dysfunctional blood-CSF barrier to explain their findings [147].

In vitro and animal models: H. pylori induces formation of $A\beta_{42}$ and P-tau

Mouse neuroblastoma N2a cells transfected with human AβPP are found to overexpress AβPP. Incubation of *H. pylori* filtrate with these cells resulted in increased production of presenilin-2 (a component of the gamma secretase enzyme complex) and $A\beta_{42}$. In the same study, intraperitoneal injection of *H. pylori* filtrate resulted in spatial learning and memory deficits in rats, abnormal hippocampal dendritic spine maturation, and increased presenilin-2 and $A\beta_{42}$ in rat brain hippocampus and cortex [148].

H. pylori filtrate induced significant tau hyperphosphorylation at several AD-related tau phosphorylation sites in mouse neuroblastoma N2a cells through activation of glycogen synthase kinase-3β. In the same study, intraperitoneal injection of *H. pylori* filtrate in rats resulted in significant tau hyperphosphorylation in hippocampal areas of rat brain. Microglial activation and elevated brain/plasma cytokine levels were not found. The authors concluded that soluble exotoxins of *H. pylori* may induce tau hyperphosphorylation and that *H. pylori* eradication may be beneficial in the prevention of tauopathy [149]. These studies provide evidence which links *H. pylori* infection with AD-like Aβ and p-tau pathology.

Potential H. pylori pathogenic mechanisms in AD

Evidence for direct brain infiltration by *H. pylori* is lacking, and exactly how a gastrointestinal infection like *H. pylori* might influence neurodegeneration in AD is unknown. However, gastric inflammation has been found in patients infected by *H. pylori*, with increased production of IL-1, IL-6, IL-12, IL-18, TNF-α, and IFN-γ [150]. Lagunes-Servin *et al*. found that *H. pylori* gastric mucosa infection in children was associated with upregulation of toll-like receptors TLR2, TLR4, TLR5, and TLR9, and overproduction of cytokines, including TNF-α, IL-10, and IL-8 [151]. These findings are potentially significant because increased levels of pro-inflammatory cytokines and TLR-induced cell signaling cascades are implicated in AD pathogenesis [19, 20].

As reviewed by Kountouras and collaborators, additional proposed mechanisms for *H. pylori* related AD pathogenesis include: i) influences on neuronal apoptosis through molecular mimicry, in which homologous *H. pylori* epitopes induce humoral and cellular immune responses, which then cross-react with components of nerves; ii) molecular mimicry between *H. pylori* and endothelial antigens; iii) mononuclear cell production of a tissue factor-like pro-coagulant that converts fibrinogen into fibrin; iv) production of reactive oxygen species and circulating lipid peroxidases; v) platelet activation and aggregation; and vi) reduced levels of vitamin B12 and folate secondary to chronic atrophic gastritis, resulting in elevated serum homocysteine levels and subsequent endothelial damage and neurodegeneration [144, 152].

NEUROINFLAMMATION, PATHOGENS, AND NEURODEGENERATION

The innate immune system: Glial cells and the vicious cycle of inflammation

Gao and Hong [1] and Griffin [153] have advanced the hypothesis that uncontrolled inflammation drives neurodegenerative disease (Fig. 5) [1]. They propose that CNS pathological processes are initiated by environmental insults interacting with genetic susceptibility. Interactions between damaged neurons and deregulated, over activated microglia create a vicious self-propagating cycle causing uncontrolled long-term inflammation and progression of chronic neurodegenerative disease [1, 153].

Chronic overexpression of IL-1β is found in AD brains [20] and has been induced by pathogens *in vitro* [17,124] and *ex vivo* [16]. IL-1β has been shown to increase neuronal AβPP production [153, 154], apolipoprotein E levels, and astrocyte-mediated S100β protein levels [153]. BACE-1 levels in neurons are increased by cytokines [155], oxidative stress molecules [155], and pathogens such as HSV-1 [10]. Along with γ-secretase, BACE-1 catalyzes the conversion of AβPP to Aβ, resulting in elevated levels of toxic forms of Aβ [155]. Aβ activates microglial RAGE receptors, which appear to mediate the proinflammatory response to Aβ. Fibrillar Aβ activates microglia cell surface protein CD36 and scavenger receptors A and B (SR-A and SR-B). Activation of these receptors induces production of reactive oxygen species by microglia [156].

The cycle is further accelerated by neuronal injury and neuron cell membrane breakdown products, cytosolic compounds, and glutamate excess, which further activates microglia [1]. There is loss of homeostasis from a tightly controlled and regulated process where anti-inflammatory cytokines are utilized for tissue repair and recovery of function. The resultant uncontrolled inflammation and amplified cytokine cycle induces neuronal injury, apoptosis, and chronic disease progression [1, 153, 157].

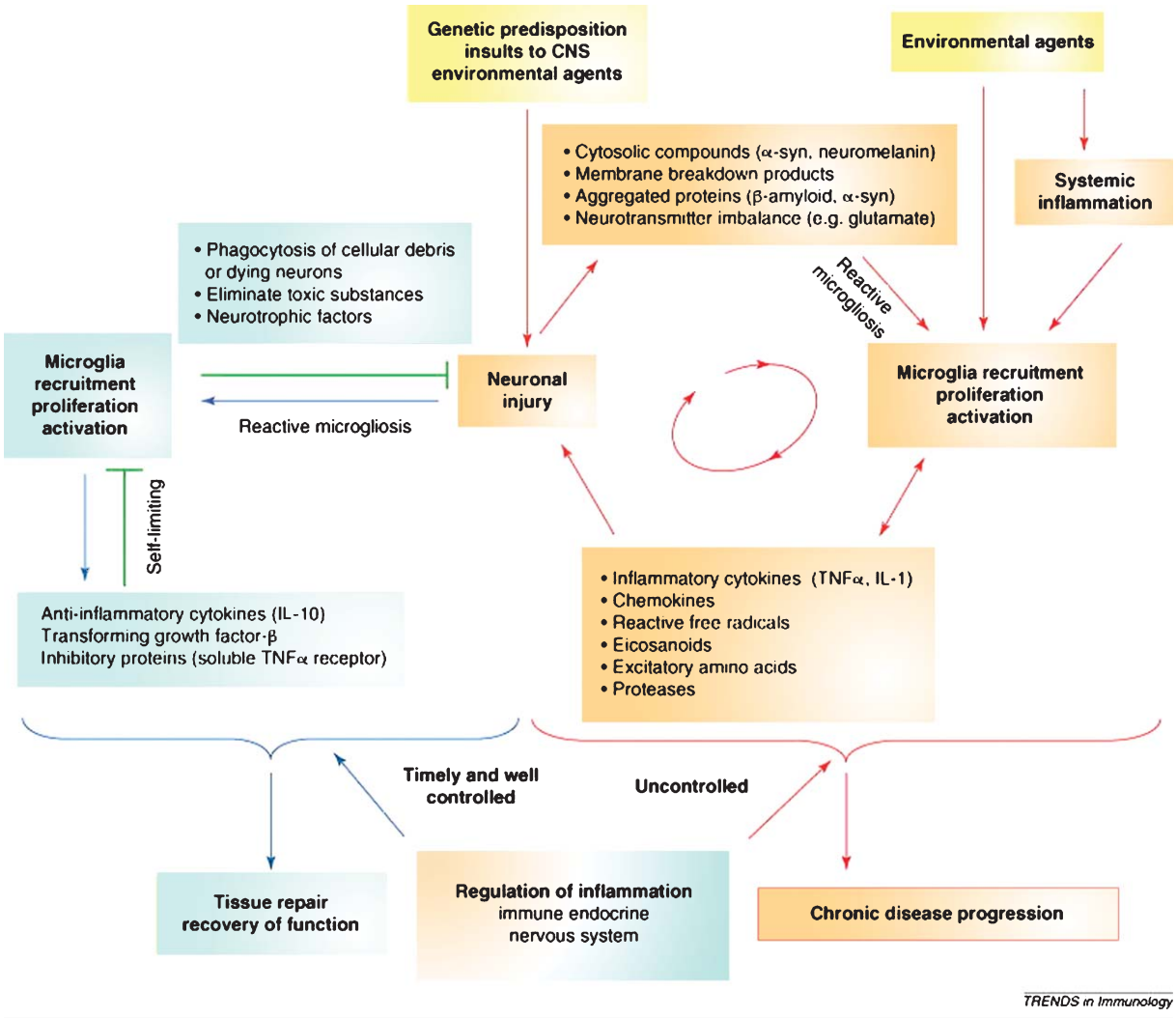

Fig. 5. The vicious cycle of neurodegeneration. Neuroinflammation when controlled is reparative and self-limiting but when uncontrolled forms a vicious cycle and leads to chronic neurodegeneration. Figure from Gao HM, Hong JS (2008) Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. *Trends Immunol* **29**, 357-365 [1]. Copyright 2008. Reprinted with permission from Elsevier and John Hong.

Microglia function as innate immune cells in the brain [156]. Pattern recognition receptors located on the microglia cell surface identify pathogen associated molecular patterns on viruses and bacteria, leading to microglial production of pro-inflammatory molecules [5]. Pathogen associated molecular patterns include lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, flagellin, bacterial lipoprotein, and nucleic acid structures such as bacterial DNA or viral RNA [5]. Examples of pattern recognition receptors located on the microglia cell surface include TLRs1-9, scavenger receptors (SRA, SRB, Macrophage receptor with collagenous domain (MARCO), CD36) and receptors for advanced glycogen end products (RAGE) [156].

Additional receptors include Major histocompatibility complex II (MHCII), cytokine receptors (CD40) and chemokine receptors (CCR3, CCR5) [20]. The NADPH oxidase receptor is a membrane-bound enzyme that catalyzes the production of superoxide from oxygen. This receptor is activated in the AD brain, and is associated with neurodegeneration [156]. Many of these receptors are upregulated in regions of typical AD brain pathology [19]. Increases in the levels of pattern recognition receptors in animal

models and cell cultures are associated with neurodegeneration [19].

Microglia produce pro-inflammatory molecules via intracellular signaling pathways in cell cultures and animal models. For example, pathogens activate microglia TLRs leading to activation of nuclear factor κB, the mitogen activated protein kinases, and jun kinase. Pathogens can also induce activation of a second microglial pathway involving interferon regulatory factor-3 [5]. These pathways lead to the induction of inflammatory genes that produce cytokines and other pro-inflammatory compounds [5].

Microglia and astroglia are consistently found surrounding amyloid plaques in AD brains [157]. Amyloid deposition causes a microglial-mediated inflammatory response [19]. Pro-inflammatory molecules have been shown to be involved in pathways of neuronal apoptosis [20]. Aβ stimulated microglia secrete TNF-α and glutamate *in vitro*, resulting in simultaneous activation of neuronal TNF-α and N-methyl-D-aspartate (NMDA) receptors and subsequent neuronal apoptosis [158].

Pro-inflammatory compounds produced by glial cells and upregulated in AD brains include cytokines (IL-1α, IL-1β, IL-6, TNF-α), chemokines including macrophage inflammatory protein-1β and monocyte chemotactic protein-1, prostaglandins (cox 2), growth factors such as macrophage colony stimulating factor, and complement components (C1q, and C1 to C9) [19, 20]. Additional neurotoxic compounds produced by activated microglia include superoxide, hydrogen peroxide, and nitrous oxide [156]. Oxidative stress (lipid oxidation, protein oxidation, DNA oxidation, and glycol-oxidation) contributes to neurodegeneration in AD [159]. Associated pathological processes include endoplasmic reticulum stress associated with change in endoplasmic reticulum calcium homeostasis [160, 161], release of free electrons from dysfunctional mitochondria [162], and formation of reactive oxygen species [163].

Infection with either HSV-1 [10] or *C. pneumoniae* [12] induces $A\beta_{42}$ deposits and plaques, and *H. pylori* filtrate [148] results in elevated levels of $A\beta_{42}$ in animal brain models. *In vitro* infection by HSV-1 [10], *B. burgdorferi* [13], and *H. pylori* filtrate [148] induces Aβ deposition in mammalian neuronal or neuronal/glial cell models.

Aβ has been shown *in vitro* to be an anti-microbial peptide against eight specific microorganisms, including *Escherichia coli, Streptococcus pneumonia*, and *Candida albicans*. AD whole brain homogenates have significantly higher antimicrobial activity compared to age matched non-AD control samples [164]. $A\beta_{42}$ has shown anti-microbial peptide properties by attenuating HSV-1–induced miRNA-146a levels in human neuronal-glial cell cultures and significantly reducing pathological HSV-1 effects on cultured brain cells [165]. Aβ production may be part of the CNS immune response to infection with eventual harmful effects to neurons due to overproduction of Aβ [166].

Evidence supporting the role of the adaptive immune system in AD

Lynch has proposed that BBB permeability, which is increased in AD, together with the creation of a chemotactic gradient, leads to infiltration of IFN-γ-producing T cells into the AD brain [167]. T cell production of IFN-γ induces classical microglia activation, which leads to inflammatory cytokine and chemokine production. This in turn results in increased AβPP processing, Aβ accumulation, further BBB permeability, and infiltration of more T cells, leading to a continuous cycle of neurodegeneration (Fig. 6) [167].

Resident cells in the brain produce only limited IFN-γ [167]. Under normal conditions, T cell entry into the CNS is limited and thought to be related to T cell immuno-surveillance [168, 169]. Significant infiltration of immune cells does occur in neuroinflammatory conditions [170] and T cells have been localized in the brains of AD patients [171–178]. Breakdown of the BBB (see BBB section below), increased expression of T cell attractant chemokines such as interferon-γ-inducible protein 10 (IP-10), and corresponding chemokine receptors such as CXCR3 on neuronal cells have been found in AD brains [179] and may contribute to infiltration of T cells into the AD brain [167].

T cells can interact with microglia to modulate their function, as demonstrated by *in vitro* co-culture experiments [167]. Resting microglia cells from BALB/c mice developed features of antigen presenting cells, including strongly upregulated surface expression of MHCII, CD40, and CD54 when co-cultured with type 1 T helper cells (Th1) [180]. Conversely, mouse microglia induce Th1 cells to release IFN-γ [180]. Supernatants produced by allo-antigen and myelin basic protein-specific human pro-inflammatory Th1 T-cell lines augmented expression of cell surface molecules MHC class II, CD80, CD86, CD40, and CD54, enhanced the functional antigen-presenting cell capacity in a mixed lymphocyte reaction, and increased cytokine/chemokine secretion (TNF-α, IL-6, and CXCL10/IP-10) by CNS-derived human microglia [181]. Co-culture of Aβ-specific Th1 or Th17 cells and microglia induced pro-inflammatory cytokine production (IL1-β, TNF-α, and IL-6) and

antigen presenting cell capacity of microglia in a murine model [182]. IFN-γ activates murine microglial cells and results in microglial production of TNF-α and inducible nitric oxide synthase (i-NOS) *in vitro* [183].

Browne and colleagues investigated the role of Aβ-specific T cells on Aβ accumulation in transgenic mice that overexpress AβPP and presenilin 1 (AβPP/PS1 mouse model), and found significant infiltration of T cells in these brains. Aβ-specific CD4+ T cells were generated by immunization with Aβ and a TLR agonist and polarized *in vitro* to Th1-, Th2-, or IL-17-producing CD4+ T cells. A proportion of these T cells secreted IFN-γ or IL-17. These Aβ-specific T cells were then adoptively transferred to AβPP/PS1 mice at 6 to 7 months of age. At 5 weeks, Th1 cells, but not Th2 or IL-17-producing CD4+ T cells had infiltrated into these brains. Additionally, there was increased microglial activation and CNS Aβ deposition. All of these findings were associated with impaired cognitive function. Treatment of the AβPP/PS1 mice with an anti-IFN-γ antibody attenuated the Th1 cell effects. The authors suggest that the release of IFN-γ from infiltrating Th1 cells significantly accelerates markers of diseases in an animal model of AD [184]. Murphy *et al*. demonstrated that murine Th17 and CD4+ lymphocytes, which produce IL-17 and IFN-γ, induce microglial production of IL-1β, IL-6, and TNF-α in experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis [185]. The combination of IFN-γ and TNF-α has been shown to induce the production of Aβ peptides and inhibit the secretion of soluble AβPP by human neuronal and extraneuronal cells *in vitro* [186].

These findings lend support to the hypothesis that T lymphocytes activated peripherally may cross the BBB and secrete IFN-γ and other cytokines, interact with microglia, and influence the neurodegenerative processes involved in AD. Thus, T cells may be an important link between the systemic adaptive immune system and the innate immune system in the AD brain.

Infection-induced acute or chronic inflammation exacerbates tau pathology in vivo

Sy *et al*. demonstrated that acute inflammation induced by viral infection or chronic inflammation induced by bacterial LPS resulted in AD-like pathology in animal brains using the triple transgenic AD mouse model (3xTg-AD). Aged 3xTg-AD and non-Tg mice infected with a single dose of mouse hepatitis virus (MHV) by injection into the hippocampus developed similar acute neuroinflammatory responses with increased infiltration into the brain by macrophages, CD4+ T cells, CD8+ T cells, and activation of microglia. After viral clearance at 2 and 4 weeks post-infection, MHV-infected 3xTg-AD mice showed

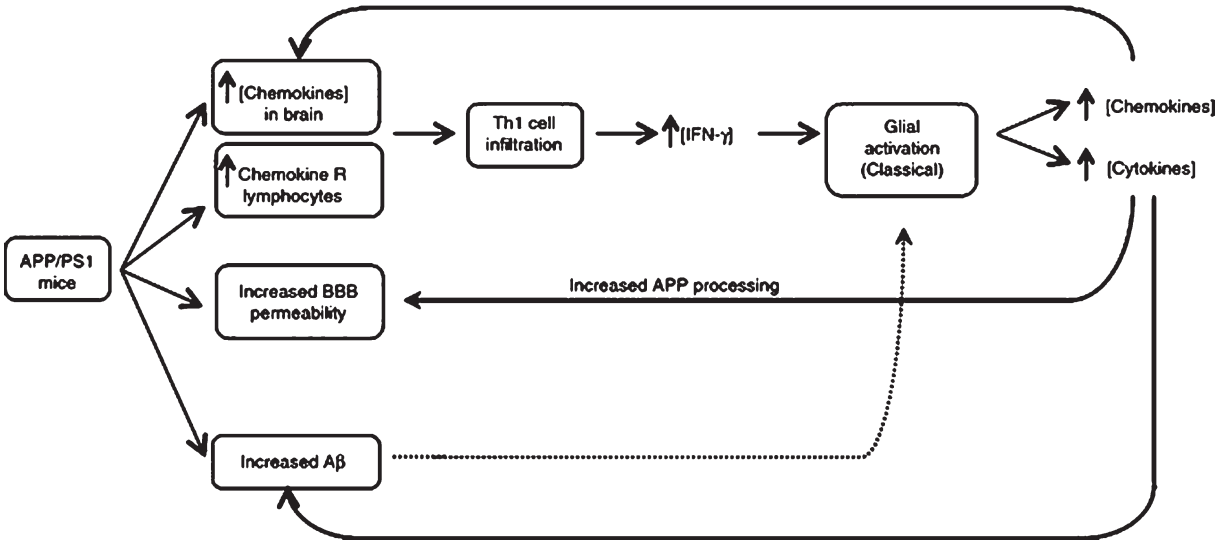

Fig. 6. The Lynch Hypothesis: T-cell lymphocytes infiltrate the brain and secrete IFN-γ which induces microglia activation and contributes to neurodegeneration in AD. Proposed sequence of events leading to amyloid pathology and microglial activation in AD. T lymphocytes cells activated peripherally cross the BBB and secrete IFN-γ and other cytokines, interact with microglia, and influence the neurodegenerative processes involved in AD. Figure from Lynch MA (2014) The impact of neuroimmune changes on development of amyloid pathology; relevance to Alzheimer's disease. *Immunology* 141, 292-301 [167]. Copyright 2014. Reprinted with permission from John Wiley and Sons, Inc. and Marina Lynch.

a marked increase in phosphorylated tau protein levels in the brain. In addition, increased activation of GSK-3β, one of the enzymes that phosphorylates tau protein, was found. This effect was not seen in MHV infected non-Tg mice. Sustained brain inflammation was induced in 3xTg-AD aged mice by intraperitoneal injection of lipopolysaccharide, which is an outer membrane Gram-negative bacterial endotoxin that simulates bacterial infection. Injection of LPS twice weekly for 6 weeks resulted in significant elevation of phosphorylated tau protein levels, increased GSK-3β activity, and cognitive decline compared with saline injected 3xTg-AD aged control mice. Based on these findings, the authors suggest that certain microbial infections may act as comorbid factors in the pathogenesis of AD by inducing inflammation, increasing levels of phosphorylated tau proteins, and exacerbating cognitive decline [187].

Infectious burden is associated with systemic inflammation and serum Aβ levels in AD

Bu and co-workers studied IB, serum cytokine, and Aβ levels, and cognition in 128 AD patients and 135 healthy controls. IB consisted of serum antibody levels to CMV, HSV-1, *B. burgdorferi*, *C. pneumoniae*, and *H. pylori*. Total IB, bacterial burden, and viral burden were independently associated with AD after adjusting for age, gender, education, *APOE* genotype, and other comorbidities. They found a significant association between AD and an increasing number of pathogens to which an individual had been exposed, with an OR of 3.988 in patients seropositive for 4-5 pathogens compared with those seropositive for 0–2 pathogens. Furthermore, higher IB was associated with higher serum Aβ levels in both AD and healthy controls with seropositivity to 3 or more pathogens. There were significantly higher serum levels of IFN- γ, TNF-a, IL-1β, and IL-6 in participants seropositive for 4-5 pathogens than those seropositive for 0–2 pathogens when including all cases. AD patients seropositive to 4-5 pathogens exhibited significantly higher levels of IFN-γ, TNF-α, and IL-6 when compared with AD patients seropositive to 0–2 pathogens. The authors suggest that higher levels of IB may promote the development of AD by infection-induced inflammation and elevated Aβ levels [188].

APOLIPOPROTEIN E OVERVIEW

Apolipoprotein E (ApoE) is a 299 amino acid protein component of lipoproteins. The primary metabolic role for ApoE is to shuttle and distribute lipids from one tissue or cell type to another and to regulate lipid metabolism [189]. Various ApoE isoforms appear to play a role in disease susceptibility and outcome of certain infections, with evidence also supporting involvement in immune regulation [189]. The liver synthesizes the majority of ApoE; however, 20–40% of ApoE is produced by extra-hepatic cells including glial cells [190] and neurons [191].

ApoE associates with lipoproteins including VLDL, LDL, and HDL during systemic transport of triglycerides and cholesterol and is a primary carrier of lipids across the BBB into the brain [190]. When carrying lipids, ApoE binds to members of the low density lipoprotein receptor (LDLR) family [191]. A secondary proposed ApoE binding site involves the heparan sulphate proteoglycan (HSPG)/LDL-C receptor-related protein pathway [192].

The human *APOE* gene is located on chromosome 19 as a single gene locus with three major allele isoforms designated ε4, ε3, and ε2 [189]. *APOE* allele frequencies vary between ethnicities, with the *APOE*-ε4 allele variant occurring at a frequency of 5–30%, the *APOE*-ε3 allele variant at 50–90%, and the *APOE*-ε2 allele variant at 0–15% [190]. The corresponding products of these alleles are the ApoE isoforms named ApoE4, ApoE3, and ApoE2. The 6 resultant ApoE phenotypes include 3 homozygous phenotypes (E4/4, E3/3, and E2/2) and 3 heterozygous phenotypes (E4/3, E4/2, and E3/2) [190].

The APOE-ε4 allele impacts outcomes in certain infections

As discussed above, Itzhaki demonstrated increased risk of AD by a factor or 12 in *APOE*-ε4 carriers who have HSV-1 in the brain [49]. Patients with the *APOE*-ε4 allele had a higher rate of oral herpetic lesions compared to non-*APOE*-ε4 allele carriers with a relative risk of 4.64 [193]. Transgenic APOE4 mice infected with HSV-1 demonstrate higher CNS viral loads compared with APOE3 [51]. HSV-1 binds to HSPG located on the target cell membrane to facilitate entry into the cell [194]. Itzhaki *et al*. suggest that ApoE4 may compete with HSV-1 for binding to cell surface HSPG receptors, and that ApoE4 is less competitive than ApoE3 or ApoE2, allowing more virus particles to gain entry into the cell [191].

C. pneumoniae elementary bodies bind to human astrocytoma cells possessing the *APOE*-ε4 allele at levels 3-fold higher than non-*APOE*-ε4 allele bearing cells. A separate line of astrocytoma cells transfected

with plasmids expressing the ε4 coding sequence had 3-fold more *C. pneumoniae* elementary body attachment than astrocytoma cells encoding ApoE3. These findings demonstrate that expression of ApoE4 enhances attachment of *C. pneumoniae* elementary bodies to target host cells, which may enhance infectivity [195].

APOE-ε4 allele carriage is associated with a reduced risk of acquiring chronic hepatitis C virus (HCV) [190, 196]. Carriage is also protective against severe liver disease caused by HCV [197]. The exact mechanism for this protective effect has yet to be elucidated; however, HCV associates with serum lipoproteins including apoE to enter cells via the LDLR [198]. The expression of LDLR on the cell surface of hepatocytes is inversely related to the concentration of LDL in serum [199]. One hypothesis suggests that a higher serum LDL concentration in *APOE*-ε4 carriers leads to a lower LDLR expression, which potentially decreases virus entry and spread between hepatocytes [197].

Corder and colleagues phenotyped sera from HIV patients for ApoE and found that patients who possessed a single copy of the ApoE4 isoform had significantly higher rates of dementia and peripheral neuropathy compared to those who were ApoE4 negative [200]. Burt *et al*. demonstrated that HIV positive patients with the *APOE*-ε4/ε4 genotype have both accelerated disease progression and progression to death compared to *APOE*-ε3/*APOE*-ε3 carriers. An association between *APOE*-ε4/ε4 genotype and HIV-associated dementia was not confirmed in this study. However, using an *in vitro* cell model with specialized HeLa cells, the authors found that the HIV infection rate was significantly higher in the presence of ApoE4 compared with ApoE3 [201].

The target cells of HIV include CD4+ T-cells, macrophages, and microglia cells. HIV initiates entry into the cell by attaching to the HSPG receptor on the target cell membrane [190]. HIV envelope glycoproteins then bind to the CD4 receptor. Fusion to the cell membrane requires cholesterol in HIV particles and lipid rafts, which are cell membrane micro domains enriched in certain lipids, cholesterol and proteins [190]. The mechanism by which ApoE isoforms impact HIV disease outcomes is not known. Research has focused on the differential effects of ApoE isoforms on HIV particle binding activity and uptake involving the LDL-R and the HSPG receptors [190]. One proposed mechanism is that ApoE4 may be less competitive compared with HIV at the target cell HSPG receptor than ApoE3 or ApoE2 resulting in increased HIV cell entry [190]. An additional hypothesis is that ApoE4 on the HIV viral envelope promotes HIV binding activity at the low-density lipoprotein receptor of the target cell [190].

APOE-ε4 allele is associated with enhanced human innate immune responses

Gale *et al*. demonstrated that carriage of the *APOE*-ε4 allele is associated with enhanced *in vivo* innate immune responses in human subjects [202]. Whole blood from healthy genotyped *APOE*-ε3/*APOE*-ε4 volunteers exposed *ex vivo* to TLR2, TLR4, or TLR5 ligands induced significantly higher levels of TNF-α than blood from *APOE*-ε3/*APOE*-ε3 carriers. Blood from *APOE*-ε3/*APOE*-ε4 carriers also induced significantly higher levels of IL1-β, IL-6, IFN-γ, and additional cytokines and chemokines after exposure to TLR2 or TLR4 when compared with blood from *APOE*ε3/*APOE*ε3 carriers. No difference was seen between the two ApoE phenotypes regarding production of anti-inflammatory compounds IL-4 and IL-1 receptor antagonist. Thus, ApoE4 is associated with a broad pro-inflammatory response to TLR cascades. Human *APOE*-ε3/*APOE*-ε4 subjects exposed to an intravenous LPS challenge demonstrated enhanced immune responses with significantly higher plasma levels of TNF-α and greater sustained body temperatures compared with *APOE*-ε3/*APOE*-ε3 subjects [202].

Differences in monocyte lipid rafts have been found in *APOE*-ε3/*APOE*-ε4 human peripheral blood monocytes compared with *APOE*-ε3/*APOE*-ε3 monocytes [202]. Lipid rafts within the cell membrane organize cellular signaling events [203]. Several TLR cascades are initiated in lipid rafts, which and are enhanced by cholesterol loading [202]. ApoE4 is less efficient at inducing cholesterol efflux and removing cholesterol in mouse macrophages [204]. Gale *et al*. used a fluoro-tagged cholera toxin B subunit lipid raft probe to study *in vitro* CD14 monocytes. Augmented lipid raft assembly in *APOE*-ε3/*APOE*-ε4 monocytes compared to *APOE*-ε3/*APOE*-ε3 monocytes was demonstrated. The authors suggest that ApoE4 may enhance cholesterol loading in monocyte lipid raft structures due to decrease in cholesterol efflux, which in turn enhances TLR responses with pro-inflammatory consequences [202].

The *APOE*-ε4 genotype with resultant ApoE4 phenotype appears to influence disease outcome for several infections and elevate the pro-inflammatory innate immune response. Evidence supporting ApoE4 associated enhanced infectivity and brain infiltration would explain the increased risk of AD in patients who

are co-factor positive for HSV-1 in the brain and the *APOE-ε4* allele [50]. Increased binding of *C. pneumoniae* elementary bodies to *APOE-ε4* target host cells suggests increased infectivity as a mechanism to explain elevated numbers of *C. pneumoniae* infected cells in *APOE-ε4* allele carrier AD brains [123]. An increased ApoE4 associated pro-inflammatory response may contribute to AD pathogenesis by both increasing BBB permeability and elevating levels of pro-inflammatory cytokines.

THE BLOOD-BRAIN BARRIER AND THE AD PATHOGEN HYPOTHESIS: BBB IMPAIRMENT IN AD

Breakdown of the BBB with increased permeability is involved in the pathophysiology of AD [205, 206]. Postmortem studies have shown BBB damage in AD with accumulation of blood-derived proteins including albumin, fibrinogen, thrombin, and immunoglobulins in the hippocampus and cortex [207]. BBB breakdown appears to be an early event in the aging human brain that begins in the hippocampus and may contribute to cognitive impairment [207]. Montagne and collaborators used an advanced dynamic contrast enhanced MRI protocol with high spatial and temporal resolutions to quantify regional BBB permeability in the living human brain. In doing so, they demonstrated an age-dependent BBB breakdown in the hippocampus, an area of the brain affected early in AD. The BBB breakdown in the hippocampus and its CA1 and dentate gyrus subdivisions worsened with MCI that correlated with injury to BBB-associated pericytes, as shown by the CSF analysis [207]. Evidence suggests that pro-inflammatory cytokines, Aβ, and APOE4 genetics are contributing factors in the breakdown of the BBB [212–214], with data indicating pathogen association and interactions with each of these factors as previously discussed.

Composition of the blood-brain barrier

The BBB is located at the level of the cerebral microvasculature and serves as the largest interface for blood-brain exchange [208]. The BBB forms a physical, enzymatic, and transport barrier between the vasculature and the brain parenchyma [209]. Brain microvascular endothelial cells (BMECs) line cerebral blood vessels to form a barrier on the endothelial side of the vessel. Pericytes and astrocyte end feet form a continuous barrier in association with the basement membrane on the adluminal vessel surface [210]. BMECs form intercellular contacts via two types of junctions known as adherens junctions (AJs) and tight junctions (TJs). TJs in BMECs are composed of the membrane proteins occludin, claudins, junctional adhesion molecules, and cell-selective adhesion molecules, which are linked to the actin cytoskeleton by cytoplasmic proteins ZO-1, ZO-2, ZO-3, and cingulin [210]. AJs contain cadherins bound to actin microfilaments by a submembranous zone of catenins [211]. These junctions promote high transendothelial electrical resistance that restricts paracellular permeability [210]. This in turn blocks the transport of a wide range of molecules and regulates the passage of ions, macromolecules, and polar molecules from the systemic circulation [210]. Endothelial cells of the BBB lack fenestrations and have a reduced number of pinocytotic vesicles, which restricts transcellular flux [208]. However, some molecules and solutes are transported across the BBB by various mechanisms including transporters, receptor and/or adsorption-mediated transcytosis, and passive diffusion [208].

The blood-brain barrier: Pathogens and neuroinflammation

Certain pathogens may cross the BBB either transcellularly, paracellularly, or by means of infected phagocytes by a Trojan horse mechanism [215]. *N. meningitidis* exemplifies a bacterium which crosses the BBB transcellularly [215]. Evidence suggests that *B. burgdorferi* may cross the BBB paracellularly [215], whereas *C. pneumoniae* may cross by way of the "Trojgan horse" mechanism, intracellulary within monocytes or macrophages [108, 109]. Other pathogens including HSV-1 cross the BBB via the olfactory nerve and/or trigeminal nerve [5, 208]. In addition to pathogens that cross the BBB directly, blood-born cytokines including IL-1α, IL1-β, IL-6, TNF-α, and others can cross BBB via transport systems and act directly on brain tissue as demonstrated in animal models [209, 216].

CNS infections and other diseases associated with elevated pro-inflammatory cytokine levels increase the permeability of the BBB [214]. Cytokines such as IL-1 act on brain endothelial cells to increase the expression of endothelial adhesion molecules and chemokines, including CC chemokine ligand-2 (CCL2), selectins, and intracellular adhesion molecule-1 (ICAM-1), which contribute to BBB permeability [217]. Pro-inflammatory cytokines also increase expression of matrix metalloproteinases (MMPs), which degrade extracellular matrix components in the endothelial

basement membrane and tight junctions, resulting in increased BBB permeability [218, 219]. Labus *et al*. found that IL-1β induced an inflammatory response and breakdown of the endothelial layer in an *in vitro* BBB model. IL-1β induced endothelial cells expression of adhesion molecule ICAM-1, IL-6, IL-8, and TNF- α. IL-1β also reduced the expression of tight junction protein ZO-1. Increases in both paracellular permeability and leukocytes crossing the cell layer of the BBB model were demonstrated [214].

Aβ and the blood-brain barrier

Evidence indicates that Aβ and BBB disruption interact to mutually promote their effects on AD pathogenesis [213]. Accumulation of Aβ in the brain may contribute to the breakdown of the BBB. Conversely, dysfunction of the BBB may result in Aβ accumulation due to BBB leakage or decreased clearance [213]. P-glycoprotein, an efflux pump highly expressed on the luminal surface of brain capillary endothelial cells of the BBB, has been shown to transport Aβ from the brain to the blood [220, 221]. Decreased function of this transporter has been reported in AD brains [221]. $A\beta_{42}$ is able to modify the expression of TJs and alter the functionality of an epithelial BBB *in vitro* model [222]. In addition, Aβ accumulation has been shown to cause BBB dysfunction by inducing endothelial cell toxicity both *in vitro* and *in vivo* in animal studies, human studies, and human postmortem studies [223]. Soluble Aβ also stimulates BBB endothelial cells to increase monocyte adhesion, which has been hypothesized to increase monocyte permeability across the BBB leading to monocyte transmigration into the brain parenchyma [223].

APOE-ε4 and the blood-brain barrier

Animal models support a role for genetic susceptibility in the breakdown of the BBB. Using *APOE* transgenic mice, Bell *et al*. found that expression of ApoE4 and lack of murine ApoE lead to BBB breakdown by activating a pro-inflammatory cyclophilin A (CypA)-nuclear factor-κB-MMP-9 pathway in pericytes through a lipoprotein receptor. This was followed by neuronal uptake of neurotoxic proteins and reductions in microvascular and cerebral blood flow. Breakdown in the BBB preceded neuronal dysfunction and the initiation of neurodegenerative changes [224].

In a postmortem study, Halliday and colleagues demonstrated accelerated pericyte degeneration in AD *APOE-ε4* carriers > AD *APOE-ε3* carriers > non-AD

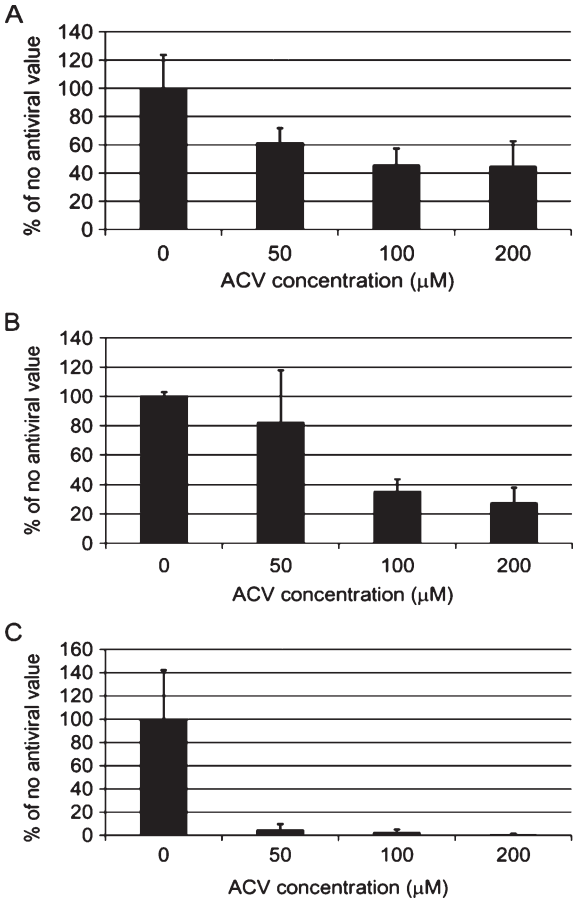

Fig. 7. Quantification of HSV-1 proteins (A), amyloid-β (B), and abnormal tau phosphorylation (C) in HSV-1-infected cells after acyclovir treatment. HSV-1 infected vero cell cultures treated with 0 μM, 50 μM, 100 μM, or 200 μM acyclovir (ACV). ACV significantly inhibited replication of HSV-1 as shown by a decrease in HSV-1 proteins (A). Aβ in cell cultures was reduced by 70% at a 200 μM concentration of acyclovir (B). Abnormal tau phosphorylation was reduced nearly 100% at a 200 μM concentration of acyclovir (C). $p < 0.0001$ compared to controls at all ACV concentrations for A and C and at 100 μM and 200 μM ACV concentrations for B. Graph from Wozniak MA *et al*. (2011) Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with Herpes simplex virus type 1. *PLoS One* **6**, e25152 [166]. Copyright 2011. Reprinted under the terms of the Creative Commons Attribution License, (http://creativecommons.org/licenses/by/2.0) and permission from Ruth Itzhaki.

controls, which correlated with the magnitude of BBB breakdown as measured by permeability to immunoglobulin G and fibrin. Accumulation of CypA and MMP-9 in pericytes and endothelial cells was greater in AD subjects who were *APOE-ε4* carriers than those who were *APOE-ε3* carriers. Levels of the ApoE lipoprotein receptor, low-density lipoprotein receptor-related protein-1 (LRP1), were reduced in AD

APOE-ε4 and *APOE*-ε3 carriers. This data suggests that possession of *APOE*-ε4 leads to accelerated pericyte loss and enhanced activation of LRP1-dependent CypA-MMP-9 BBB-degrading pathway in pericytes and endothelial cells. These processes mediate BBB damage, which is more severe in AD *APOE*-ε4 carriers than AD *APOE*-ε3 carriers [212].

POTENTIAL ANTIMICROBIAL TREATMENTS FOR AD CLINICAL TRIALS

Treatment of HSV-1: Acyclovir and valacyclovir

Acyclovir decreases HSV-1-induced Aβ accumulation in cultured neuroblastoma cells [166]. Aβ in cell cultures was reduced by 70% at a 200 μM concentration of acyclovir. In addition, acyclovir inhibits HSV-1-induced abnormal tau phosphorylation *in vitro*. Abnormal tau phosphorylation was reduced nearly 100% at a 200 μM concentration of acyclovir (Fig. 7) [166]. Acyclovir decreased Aβ by reducing cellular viral spreading and decreased tau phosphorylation by interfering with viral replication [166]. Penciclovir and foscarnet, which also inhibit viral DNA replication, were shown to reduce phosphorylated tau and Aβ, with foscarnet being less effective than acyclovir and penciclovir [166].

Acyclovir is a nucleoside analogue that interferes with HSV-1 replication and reactivation. Viral thymidine kinase is required to convert acyclovir into acyclo-guanosine monophosphate. Subsequently, the monophosphate form is converted to the active triphosphate form by cellular kinases. As a substrate, acyclo-guanosine triphosphate is incorporated into viral DNA, resulting in premature chain termination. Acyclovir is able to cross the BBB [225]. The drug would directly target a potential cause of AD, act on infected cells only, and would not affect the normal metabolism of infected neurons [166].

Acyclovir is FDA approved and widely used for the treatment of HSV infections. The side effect profile is mild; however it would be necessary to monitor renal function [225]. Rarely, treatment is complicated by reversible neuropsychiatric symptoms occurring more frequently in patients with pre-existing renal impairment [225].

Valacylovir is the biodrug of acyclovir. Following oral administration, valacyclovir is rapidly hydrolyzed to acyclovir via first-pass intestinal and hepatic metabolism [225]. Valacyclovir has better oral absorption than acyclovir and is able to cross the BBB as acyclovir following hydrolysis [226]. Oral valacyclovir has been used to successfully treat herpes simplex encephalitis [227]. In a multiple sclerosis trial evaluating HHV-6, valacyclovir at a dose of 3 grams per day for 2 years was shown to be safe with no patient discontinuation due to side effects or toxicity [228]. Both acyclovir and valacyclovir have been found safe during long-term use in patients being treated for HSV suppression [229]. Furthermore, resistance rates are low (<0.5%) among immunocompetent patients [229].

Prasad *et al*. conducted a clinical trial involving 24 HSV-1 IgG seropositive schizophrenia patients who were treated with valacyclovir 1.5 grams twice daily for 18 weeks. The treatment group demonstrated significantly improved cognition, specifically in verbal memory, working memory, and visual object learning, compared with the HSV-1 IgG seronegative schizophrenia control group. Both treatment and control groups were treated with anti-psychotic medication. While psychotic symptoms did not improve with valacyclovir, the study did show that valacyclovir improved cognition in a select group of HSV-1 exposed neuropsychiatric patients [230].

Intravenous immunoglobulin (IVIG)

Natural antibodies to Aβ in human IVIG promote microglial recognition and removal of natively formed human Aβ deposits *ex vivo* in an AβPP/PS1 mouse model of AD [231]. IVIG administered peripherally *in vivo* crossed the mouse BBB, reaching highest concentrations in the hippocampus. The antibodies bound selectively to Aβ deposits and co-localized with microglia [231]. Preclinical and small clinical studies treating early stage AD patients with IVIG showed decreases in CSF Aβ levels and increases in serum Aβ levels compared to baseline, with reductions in cognitive decline compared to controls [232, 233]. However, the Gamma Globulin Alzheimer's Partnership (GAP) study, a large double blind IVIG clinical trial treating mild to moderate AD patients, did not meet primary outcome objectives regarding cognitive function and activities of daily living. Biomarker studies did indicate dose dependent increases in plasma and CSF immunoglobulins and decreases in plasma $A\beta_{42}$ levels [234].

IVIG has antiviral activity against HSV-1 and can neutralize extracellular virus [235]. Additionally, IVIG in conjunction with lymphocytes is able to destroy cells infected with HSV-1 [235–237]. IVIG (Privigen) treated HSV-1 infected Vero cells demonstrated statistically significant reductions of Aβ, phosphorylated

tau, and HSV-1 proteins [238]. Privagen appeared to prevent viral entry into cells and act synergistically with acyclovir, leading the authors to suggest that the combination of IVIG and acyclovir may be beneficial in the treatment of AD [32, 238].

HSV-1 vaccine

Lin and coworkers showed that a vaccine of mixed HSV-1 glycoproteins had a protective effect against HSV-1 infection of mouse brain. The vaccine significantly reduced HSV-1 latency in the CNS of mice that had been infected peripherally with HSV-1 [239]. Although not yet developed, a human vaccine for HSV-1 administered to prevent HSV-1 infiltration into the brain might prevent some cases of AD [239].

Treatment of CMV, EBV, and HHV-6

Anti-viral therapy for asymptomatic CMV infection in immunocompetent patients is not feasible because of toxicity, limited number of approved drugs, and the potential for drug resistance [82]. Thus, there is no available suppressive therapy for CMV comparable to the usage of valacyclovir or acyclovir for HSV suppression. Ganciclovir (GCV) and valganciclovir, the oral prodrug of GCV, are nucleoside analogues. GCV is phosphorylated to its active form in CMV-infected cells. These medications are currently the most commonly prescribed drugs for the prevention and treatment of CMV in immunocompromised patients [240]. Although limited, current evidence suggests that targeted antiviral therapy with GCV or valganciclovir is appropriate for severe CMV disease in immunocompetent adults [241]. There are no FDA approved medications for the treatment of EBV, and anti-viral therapy is generally ineffective for this virus [242]. No therapies are approved for the treatment of HHV-6 currently; however, small studies and individual case reports have reported intermittent success with drugs such as cidofovir, GCV, and foscarnet [243]. To date there are no FDA approved vaccines for CMV, EBV, or HHV-6 [240].

Treatment of Chlamydophila pneumoniae

Acute *C. pneumoniae* infections are susceptible to antibiotics that interfere with DNA and protein synthesis, including tetracyclines, macrolides, quinolones, and rifamycins [110]. All of these antibiotics cross the BBB except for the macrolides [244]. *C. pneumonia*e becomes spontaneously persistent following infection of monocytes [112]. *C. pneumonia*e within infected lymphocytes *in vitro* demonstrated resistance to single antibiotics including minocycline and tosufloxacin and did not show uniform susceptibility within infected monocytes [245]. Nine months of combination doxycycline and rifampin were used in a successful clinical trial as treatment for chronic *Chlamydia*-induced reactive arthritis. This study suggests that persistent chlamydia infection responds poorly to single antibiotic therapy but appears to be susceptible to combination antibiotic regimens [246].

Tetracyclines possess both immunomodulatory and antiapoptotic properties [247] and have been shown to be anti-inflammatory and neuroprotective in various models of neurodegenerative disease [248]. Tetracycline and doxycycline exhibit anti-amyloidogenic activity *in vitro* by inhibiting the self-aggregation capacity of $A\beta_{42}$ and disassembling pre-formed $A\beta_{42}$ fibrils [249]. The semi-synthetic, second generation tetracycline analog minocycline inhibited increases in p-eIF2α and reduced neuronal cell death in $A\beta_{42}$ peptide treated nerve growth factor-differentiated rat pheochromocytoma (PC12) cells *in vitro* [161]. Minocycline also reduced neuronal cell death and attenuated deficits in learning and memory after $A\beta_{42}$ infusion in a rat model of AD [161].

Rifampin crosses the BBB [244, 250] and attenuates all chlamydial gene transcription [246]. Rifampin-induced upregulation of LRP1 and p-glycoprotein at the BBB enhances Aβ clearance from the mouse brain [251].

In a clinical trial, Loeb *et al*. treated probable AD patients diagnosed with mild to moderate dementia with doxycycline and rifampin versus placebo for 3 months. There was significantly less decline in cognition as measured by the Standardized Alzheimer's Disease Assessment Scale cognitive subscale at 6 months in the antibiotic treatment group compared to the placebo group [252]. In a clinical trial by Molloy and colleagues, mild to moderate AD patients were treated for 12 months with doxycycline and rifampin. These drugs, given both alone or in combination, had no effect on cognition or function [253]. Preclinical AD patients and biomarker endpoints were not assessed in this study. In addition, these medications have not been tested in combination with valacyclovir.

Treatment of spirochetes

The CNS infection Lyme neuroborreliosis caused by the spirochete *B. burgdorferi* has been successfully

treated in clinical trials with 10–14 days of intravenous ceftriaxone or oral doxycycline [254, 255]. In one trial, 79% of the ceftriaxone-treated patients and 72% of the doxycycline-treated patients had completely recovered by 6 month follow-up. Ceftriaxone and oral doxycycline used as single agents were found to be effective, safe, and convenient for treatment of Lyme neuroborreliosis [254]. Abramson *et al*. studied the bactericidal activity of antimicrobial agents *in vitro* for 17 strains of treponemes. Most treponemes, including human oral spirochetes such as *T. dentolytica*, were sensitive to tetracycline, doxycycline, and cephalothin [256].

Treatment of periodontal pathogens

Improved oral hygiene and meticulous dental care may be beneficial for patients with dementia. Kikutani *et al*. studied the effects of oral care in a group of nursing home vascular dementia and AD patients. The oral care group had a high level of dental care, as nurses and caregivers cleaned the patients' teeth and mouth with a toothbrush for approximately 5 minutes after each meal for one year. The oral care group had significantly less decline in MMSE scores compared to the non-oral care group at six months and one year [257].

Systemic antibiotics are widely used in the treatment of periodontal infections; however, clear guidelines for the use of systemic antibiotics to treat periodontitis in general clinical practice are not yet available [258, 259]. Adjunctive antibiotic treatment in moderate periodontal disease for 14 days with either amoxicillin or metronidazole in addition to traditional scaling and root planing has been shown to markedly reduce bacterial counts for pathogenic species including *B. forsythus*, *P. gingivalis*, and *T. denticola*, which remained lower than baseline at one year post-treatment [259]. Several clinical trials treating patients with mild to moderate periodontal disease have shown microbiological and/or clinical benefits by using azithromycin or metronidazole combined with scaling and root planing or pocket reduction surgery when compared to scaling and root planing alone [260–262], or surgery alone [263]. Subantimicrobial dose doxycycline (20 mg doxycycline twice daily) has also been used successfully to treat periodontitis [247]. Feres *et al*. extensively reviewed randomized clinical trials performed over the last decade involving antibiotic treatment of periodontitis where advanced microbial diagnostic testing had been performed. Patients treated with adjunctive antibiotic therapy had improved microbiological and clinical outcomes [258]. The authors recommended that patients with advanced or very advanced periodontitis should be treated with the adjunctive use of metronidazole or combination metronidazole plus amoxicillin for 14 days in addition to traditional scaling and root planing [258]. Antibiotic treatment does not appear to create lasting changes in the percentage of resistant isolates or sites harboring resistant species [260, 264].

Treatment of Helicobacter pylori

Treatment of *H. pylori* involves triple or quadruple regimens using a proton pump inhibitor such as omeprazole plus various combinations of amoxicillin, Clarithromycin, metronidazole, and tetracycline taken orally for 7 to 14 days. Sequential, concomitant, or hybrid regimens are chosen depending on resistance rates and other clinical factors [265, 266]. Clarithromycin and metronidazole resistance has been increasing in certain populations. Levaquin triple based and bismuth quadruple based regimens have been used successfully to treat resistant *H. pylori*. Patient-specific therapy is based on factors such as cost, allergy history, and known or suspected patterns of resistance [266]. Treatment success rates with current regimens are variable, ranging from 50% to over 95% in clinical trials depending on factors such as length of treatment, rates of antibiotic resistance and patient specific cytochrome P450 2C19 genotype [265].

Kountouras *et al*. evaluated AD patients with gastric biopsy-proven *H. pylori* infection. Patients were treated with combination omeprazole, clarithromycin, and amoxicillin triple based therapy to eradicate *H. pylori* from the gastric mucosa. At the 2-year clinical endpoint, cognitive (MMSE and Cambridge Cognitive Test) and functional (Functional Rating Scale for Symptoms of Dementia) parameters significantly improved in the subgroup of AD patients with successful *H. pylori* eradication, but not in the subgroup of AD patients in which *H. pylori* eradication was not achieved [152]. In another clinical trial, successful treatment and eradication of *H. pylori* in a group of probable AD patients was associated with a significantly lower 5-year mortality risk than a control group of probable AD patients who did not achieve eradication of the bacterium [267].

CONCLUSION

Evidence supports the hypothesis that pathogens interact with susceptibility genes and are causative in

AD. Pathogen induced neurodegeneration occurs by both direct effects on brain cells and indirect inflammatory and oxidative effects. HSV-1, *C. pneumoniae*, and spirochetes are able to infect the brain and induce formation of Aβ and hyperphosphylated tau proteins. Chronic CNS infections lead to glial cell activation, resulting in neuroinflammation and neuronal apoptosis. Peripheral infections including CMV, periodontal pathogens, and *H. pylori* induce systemic inflammation with elevated levels of pro-inflammatory molecules. Pathogen induced pro-inflammatory cytokines are transported across the BBB into the brain where they induce CNS inflammation and contribute to AD pathology in genetically susceptible individuals. In addition, pathogen induced pro-inflammatory cytokines and Aβ interact with genetic susceptibility factors to damage the BBB, resulting in increased BBB permeability. Chronic CMV infection with reactivation in peripheral blood or organ systems induces systemic activation of IFN-γ-producing T cells, which may enter the brain and contribute to the pathogenesis of AD. CMV also plays a role in immunosenescence, which damages the immune system and is associated with the reactivation of other *Herpesviridae* such as HSV-1. Thus, in addition to direct pathogen effects, AD pathogenesis results from interactions involving both the innate and adaptive immune responses to both CNS and peripheral systemic pathogens.

As proposed, the AD pathogen hypothesis would explain the multiple epidemiological studies showing increased risk for development of cognitive impairment and AD in patients with various CNS infiltrative and systemic chronic infections. Peripheral viral reactivation with resultant systemic inflammation is a potential mechanism involved in the association between HSV-1 seropositivity and cognitive impairment in younger patients ages 6 to 16 as found by Tarter *et al*. [87]. Systemic HSV-1 induced inflammation may also explain the association between HSV-1 positivity, cognitive impairment, and neurodegeneration on MRI in relatively younger schizophrenic patients as demonstrated by Schretlen *et al*. [34]. Younger subjects have been shown not to have HSV-1 in the brain [107] so presumably, the patients in these studies did not have direct HSV-1 CNS infiltration by the virus. Limited studies to date have failed to detect HSV in postmortem brain tissue from patients with schizophrenia as well [268], including one study using nested PCR [269].

The *APOE*-ε4 allele with resultant ApoE4 phenotype impacts the pathophysiology of AD by increasing the pathogen load in the brain, specifically for HSV-1 and *C. pneumoniae*. ApoE4 also interacts with pathogens to enhance the human innate pro-inflammatory response and contributes to the breakdown of the BBB.

HSV-1 may be a primary CNS infiltrative pathogen in the pathophysiology of AD. There is substantial evidence for HSV-1 causation in AD involving studies in epidemiology, neuropathology, and molecular biology as cited in this review. There is the high prevalence of HSV-1 in both elderly normal brains and AD brains [4, 41] (Table 1) and the presence of both HSV-1 in brain and carriage of the *APOE*-ε4 allele significantly increases the risk for sporadic AD [49]. Data for both *C. pneumoniae* and spirochetes (Table 3 and Fig. 3) shows a high prevalence of these pathogens in AD brains only [5, 126], suggesting that secondary *C. pneumoniae* and/or spirochete infection of brain may occur after HSV-1 and other co-factors have already initiated AD pathogenesis. Additional studies need to be done to confirm this hypothesis.

A comprehensive antimicrobial treatment strategy for AD must be developed and tested in clinical trials focusing on pre-clinical or early onset AD. Itzhaki [270] and Strandberg [28] have proposed a randomized controlled antiviral clinical trial using oral valacyclovir. The initial study would test the concept that HSV-1 in *APOE*-ε4 carriers is causative in AD [80]. Additional AD clinical trials could then evaluate the AD multi-pathogen hypothesis by testing the effectiveness of valacyclovir combined with the appropriate antibiotics used to treat spirochetes, chronic persistent *C. pneumoniae,* and systemic AD associated pathogens such as *H. pylori* and periodontal infections.

Safe, effective, and less toxic treatments for CMV and other *Herpesviridae* must be developed. Antimicrobial medication in combination with anti-inflammatory treatments may also be beneficial in the treatment of AD. Vaccines against CMV, HSV-1, and other *Herpesviridae* must be developed, as reducing primary infection or reactivation may be useful in AD prevention.

Treatment of HSV-1 and other pathogens present in the AD brain and peripherally may be the most efficacious way to reduce CNS inflammation. The goal of such treatment would be to lower production of pro-inflammatory molecules, reduce accumulation of Aβ, and lower the levels of hyperphosphorylated tau proteins. Antimicrobial therapy could decrease neuronal damage and apoptosis and ultimately aid in the prevention and treatment of AD.

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