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Understanding cause and effect in Alzheimer's pathophysiology: implications for clinical trials

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Data Availability Statement

The manuscript is a review of the literature on our current knowledge of the pathophysiology of Alzheimer's disease and thus the authors do not have data to share.

6548 words

2 Figures

Abstract

Alzheimer's disease (AD) pathology is multi-faceted, including extracellular accumulation of amyloid-β (Aβ), accumulation of tau within neurons, glial activation and loss of neurons and synapses. From a neuropathological perspective, usually at a single time-point and often at the end-stage of the disease, it is challenging to understand the cause and effect relationships between these components. There are at least four ways of trying to unravel these relationships. Firstly, genetic studies demonstrate mutations that influence Aβ production, but not tau, can initiate AD; whereas genetic variants influencing AD risk are related to innate immunity and lipid metabolism. Secondly, studies at early time points show that pathology begins decades before the onset of dementia and indicate different anatomical locations for initiation of Aβ and tau accumulation. Thirdly, cause and effect can be studied in experimental models, but most animal models do not fully replicate AD pathology. However, induced pluripotent stem cells (iPSCs) to study live human neurons has introduced a new perspective. Fourthly, clinical trials may alter AD pathology giving insights into cause and effect relationships. Therefore, a sequence of (i) neocortical Aβ accumulation followed by (ii) a microglial inflammatory reaction to Aβ, causing neuritic dystrophy which promotes (iii) spread of tau from the limbic system to the neocortex with (iv) progressive tau accumulation and spread resulting in (v) neurodegeneration, explains the evidence. It is proposed that different therapeutic targets are required for different stages of the disease process: Aβ for primary prevention, microglia for secondary prevention, and tau for established disease.

Introduction

Dementia is a major problem for our ageing societies. The global figures are concerning with around 50 million people living with dementia, a number projected to increase to over 150 million in 2050 [1]. All regions of the world are affected. In some developed countries, dementia is now recognised as the commonest cause of death based on death registration certificates in men and women combined (Office for National Statistics, England), exceeding those due to ischaemic heart disease, cerebrovascular disease, chronic lower respiratory diseases and bowel cancer. However, the scale of the problem is anticipated to increase most rapidly in developing countries in which changes in life expectancy are occurring rapidly. Overall, 5% of people over the age of 60 are living with dementia globally [2].

Understanding Alzheimer's pathophysiology, how the disease process is initiated and how it progresses, is key to developing rational approaches to therapy which can then be tested in clinical trials. In turn, the findings from clinical trials in Alzheimer's disease (AD), which have been taking place for more than 20 years, can provide feedback to help understand how the pathological features of AD are related in terms of cause and effect and which AD features are most closely causally related to the development of dementia. The pathology of AD is multifactorial involving: accumulation of amyloid-β protein (Aβ) in the cerebral cortex in the form of plaques and in the blood vessel walls as cerebral amyloid angiopathy (CAA); phosphorylated tau which occurs intraneuronally in the form of tangles, neuropil threads and plaque-associated dystrophic neurites; glial activation including microglia and astrocytes; neuronal and synaptic dysfunction and loss, and associated cerebral atrophy.

1. The genetics of AD

In a complex disease process, genetic information can be helpful in attempting to unpick which of the features are important in causation of the disease because a person's genetic make-up is present before the onset of the disease.

Firstly, it has been known for some years that there are single gene mutations that can cause AD [3]. Point mutations in the Amyloid Precursor Protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes occur in families in which AD is inherited as an autosomal dominant trait. In addition, trisomy 21, the chromosome on which the *APP* gene is located, associated with Down's syndrome [4] and isolated APP gene duplication, both cause early onset AD [5, 6]. Such genetic abnormalities

all *cause* an increase in the production of Aβ, implying that the accumulation of this peptide can initiate AD pathogenesis.

Secondly, there are gene polymorphisms that can influence risk for AD. The association of *APOE* genotype with AD has been known since the 1990s [7]. People with a single copy of the *APOE* ε4 allele, approximately 30% of the population, are at a two to fourfold increased risk of developing AD; with ε4 homozygotes at fourteen times increased risk of developing AD, whereas people with the ε2 allele are relatively protected, having approximately half the risk [7, 8]. The effect is so profound that a person who is ε4 homozygous who lives to 85 years of age has a lifetime risk of AD of >50%, comparable to the risk associated with BRCA1 mutation in breast cancer [8]. The effect of *APOE* ε2 is so marked that ε2 homozygotes have an exceptionally low likelihood of developing AD; ε2 homozygotes had a 87% lower odds ratio than ε3 homozygotes, and a 99.6% lower odds ration than ε4 homozygotes [9], raising the prospect of replicating the effects of *APOE* ε2 by gene editing, protein-reducing, protein-modifying, or other treatments. ApoE protein co-localises with Aβ in plaques and blood vessel walls [10] and *APOE* ε4 genotype is strongly associated with accumulation of Aβ, providing a suitable mechanism for the genetic risk [9]. In addition, *APOE* genotype has been associated with microglial activity, synaptic plasticity and other effects, so multiple mechanisms may be in operation. More recently, genome wide association studies (GWAS) have identified numerous other genes in which variation can influence risk of AD, although generally with a much smaller effect size. These genes are mainly related to inflammation, more specifically innate immunity [11, 12]. This implies that inflammatory processes, and more specifically microglia as the mediators of innate immunity in the brain, are important in AD pathogenesis but are not the initiating factor.

Thirdly, striking by their absence in terms of causing AD, are mutations in the tau gene. Tau mutations can cause other forms of dementia (i.e. primary tauopathies [3]), but not AD itself. However, in AD, tau pathology is a good correlate with the severity of dementia [13, 14] and this suggests that tau pathology is important in later stages of AD pathogenesis.

These genetic data in themselves are therefore consistent with a hypothesis comprising the following sequence of events in the pathogenesis of AD: (i) Aβ deposition is the key initiating event; (ii) this is followed by a microglial inflammatory reaction to the presence of the Aβ and (iii) tau accumulation within neurons is a consequence of the microglial activation. This proposed sequence of events is not new and was suggested many years ago by Griffin and McGeer based on appreciation of cellular interactions [15, 16]. However, it is noteworthy that consideration of the genetic data alone provides strong support for this sequence. It then becomes pertinent to ask the

question: where in the brain do these three features, the Aβ, microglia and tau, appear together, i.e. where are they spatially co-localised, in order to interact?

2. Human neuropathology: A key role for the neuritic plaque - where Aβ, microglia and tau collide

The only component of AD pathology in which these three key features (i.e. Aβ, microglia and tau) are present together is in the neuritic plaque, and it follows that important interactions in relation to AD pathophysiology must occur here (Figure 1). It is therefore logical to elaborate the hypothesis that the initiating feature in AD pathophysiology is the appearance in the cerebral cortex of non-neuritic plaques (i.e. diffuse plaques lacking the microglia and dystrophic neurites which define the neuritic plaque) (Figure 1). Diffuse plaques are present in the brains of many middle-aged and elderly people who are cognitively normal (diffuse plaques were present in the brains of 69% of non-demented community-dwelling people aged 60-103 years [17]), implying that accumulation of Aβ by itself does not cause cognitive dysfunction. Whether such diffuse plaques represent pre-clinical AD, which given sufficient time would progress to AD, or whether such subjects are resistant to the development of AD is not currently known. Furthermore, although diffuse plaques are generally present before neuritic plaques, whether an individual diffuse plaque can actually transform into a neuritic plaque or whether diffuse and neuritic plaques are different *de novo* is not entirely clear at present. There are differences in the components of the aggregates, for example, the Aβ in neuritic plaques tends to be more fibrillar, more likely to form dense cores, and has a more varied composition with the presence of Aβ40, 42, 43, n-terminus truncated Aβ and other post-translationally modified forms [18, 19]. It is possible that these Aβ alterations accumulate over time in a deposit that is initially diffuse, provoke a microglial reaction and consequently initiate the transformation into a neuritic plaque. Alternatively, plaques may initially form with this varied composition and therefore be destined to become neuritic, whereas plaques lacking this variation begin as and remain diffuse deposits. However, we propose a key step occurs when the microglia, which for many years presumably have been unresponsive to the diffuse plaques, begin to recognise and react to their presence. Our immune systems have evolved largely to combat infective organisms. Consequently, microglia possess pattern recognition receptors, such as macrophage scavenger receptors, which recognise bacterial proteins, including those associated with bacterial biofilms which form amyloid fibrils [20-23]. An appealing mechanism is that, in effect, the microglia "see" the fibrillary amyloid plaques as invading bacteria and therefore mount an appropriate

response [24]. This results in the activation of microglial mechanisms evolved to kill invading microorganisms, with neuronal damage as a collateral or bystander effect.

According to the hypothesis stated above, this microglial reaction to Aβ causes the appearance of the tau-containing dystrophic neurites, to produce the fully-fledged neuritic plaque. The question then arises as to whether the neuritic dystrophy precedes and causes the tau accumulation or the tau accumulation causes the dystrophy. It seems more likely that the neuritic dystrophy occurs first and the tau accumulation is a consequence of this, because plaque-associated dystrophic neurites can occur in the absence of tau. Firstly, this can be clearly seen in APP transgenic mice which develop plaque–associated dystrophic neurites lacking tau [25], and secondly in the human brain, not all dystrophic neurites contain aggregated tau, with tau negative dystrophic neurites detected by APP immunohistochemistry [26, 27], particularly in the absence of dementia (Figure 1). The appearance of APP in dystrophic neurites seems likely to reflect disruption of intracellular transport in a manner analogous to APP accumulation in the white matter in diffuse axonal injury [28], whether due to trauma or other causes. Therefore in this context, the accumulation of APP is unrelated to its role as the precursor for Aβ, but simply reflects the fact that APP is transported within neuronal processes by fast axonal/dendritic transport as other proteins (e.g. clusterin) also accumulate when transport is disrupted [29]. Precisely how the microglial reaction to Aβ causes the neuritic dystrophy is, therefore, an important question. McGeer has proposed a mechanism by which activation of the complement cascade forms pores in the membranes of neurites via the membrane attack complex (MAC, C5b-9) [30-32]. MAC has evolved to form transmembrane channels in the cell membranes of micro-organisms and thereby kills the target cell by osmotic lysis. The hypothesis is that insertion of MAC into the cell membranes of neurites results in the irregular thickening and tortuosity of the neurites within the plaque [33]. Further support comes from study of “high pathology controls”, subjects with abundant neocortical Aβ plaques and entorhinal tangles but without dementia, compared with subjects with dementia and AD pathology showing that MAC within Aβ deposits and microglial activation correlated strongly with synapse loss and dementia [34]. A subsequent key step to understand following the development of neuritic dystrophy, is how and why tau aggregates in the damaged neurites.

A very long-standing debate in the Alzheimer field has been how Aβ and tau are inter-related and whether Aβ or tau “comes first”. A difficulty with this debate is that Aβ and tau begin to accumulate, in an apparently unrelated fashion, in different anatomical brain regions. Accumulation of Aβ starts in the form of diffuse plaques in the cerebral cortex (Thal stage I) before progressing to involve the entorhinal cortex and hippocampus, then subcortical regions, including the caudate nucleus, basal forebrain, thalamus and hypothalamus and finally the brainstem and cerebellum [35,

36]. In contrast, tau accumulation in the cerebrum first appears in the entorhinal cortex and limbic system (Braak stage I [36, 37]), only later spreading to the neocortex. Indeed, it has long been known that tau accumulation can be present in medial temporal lobe structures relatively early in life and certainly in some cases, before any Aβ aggregates have appeared in the cerebral cortex. This condition has now been labelled "Primary Age-Related Tauopathy" (PART) and is recognised as an extremely common condition which is almost universally detectable at autopsy among elderly individuals and which may be asymptomatic or associated with memory impairment [38].

It therefore seems clear that Aβ and tau each begin to aggregate in separate neuroanatomical locations and meet in the cerebral neocortex in the neuritic plaque (Figure 2). The appearance of dystrophic neurites in a plaque, as a consequence of the microglial reaction to the Aβ, in some way facilitates or permits the spread of tau aggregation from the limbic system into the cerebral neocortex [39-41]. Several lines of evidence support this idea: imaging studies show that tau spread is accelerated by the presence of Aβ [41]; injection of human tau from AD brains into APP-knock in mice with tau-negative neuritic plaques forms tau aggregates initially in plaque-associated dystrophic neurites, before tangles appear [42]; and in humans, tau can aggregate initially in dendrites before tangles appear in the neuronal soma [43] (Figure 1). This "collision" of Aβ and tau, mediated by microglia, in the cerebral neocortex has devastating consequences in terms of promoting neurodegeneration and the consequent development of dementia.

Microglial phenotype in human AD

Microglia are the innate immune cells of the brain, with some functions common to macrophages, but different in morphology and different in the pattern of genes expressed [44-46]. We explored the role of microglia in relation to AD pathology using 299 *post-mortem* cases, with subjects ranging in age from 77 to 93 years [47]. These cases were derived from the MRC Cognitive Function in Ageing Study (CFAS), a large UK community-based longitudinal multicentre study looking at health and cognitive function in the elderly. We were interested in immunophenotyping microglia, the concept of applying multiple antibodies to proteins each associated with different specific microglial functions. Antibodies employed in this study included: Iba1, commonly considered a pan-microglial marker and associated with motility [48]; Macrophage Scavenging Receptor (MSR)-A, a scavenging receptor [21]; HLA-DR involved in antigen presentation [49]; CD68 involved in phagocytosis [50]; and CD64, an Fcγ receptor 1 binding antibody [51]. The evidence relating each of these microglial proteins to particular functions is mainly based on studies of

macrophages, which by extrapolation are likely also to apply to microglia, a conclusion that remains somewhat tentative at present.

Important findings from this study include the relationship of expression of these different microglial proteins to dementia status and cognitive function. The presence of dementia was associated with high expression of CD68, MSR-A and CD64 and conversely with low expression of Iba1. In relation to cognitive function as assessed by the Mini Mental State Examination (MMSE), in subjects without dementia, better cognition was associated with higher levels of Iba1 and low levels of CD68 expression. However, in those subjects with dementia and concomitant AD pathology, higher levels of CD68, HLA-DR and MSR-A and low expression of CD64 related to worse cognitive function. Associations were assessed between the microglial markers and specific features of AD pathology, including meningeal and parenchymal cerebral amyloid angiopathy (CAA), diffuse plaques, neuritic plaques and tangles. There were striking differences in these relationships in subjects without dementia, who nevertheless being elderly often had some AD pathology, and subjects with dementia. In subjects without dementia, the significant relationships observed between microglia and AD pathology were mainly negative, except for diffuse plaques which were positively related with four of the five microglial markers (Iba1, CD68, HLA-DR, CD64). In contrast, in the participants with dementia and Alzheimer's pathology, the relationships were mainly positive and stronger than those in the participants without dementia. The highest odds ratios in subjects with dementia were for the associations between CD68 and tangles and neuritic plaques, MSR-A and neuritic plaques, and CD64 with diffuse plaques [47]. This striking change in the associations between different microglial markers and features of pathology in subjects with and without dementia are compatible with the concept that a switch in the microglial response to Aβ occurs as AD pathology develops.

Additional study of cases lacking dementia in the face of significant Alzheimer pathology suggests a protective role for down-regulated inflammation, in the form of a pattern of expression of cytokines associated with pathogen clearance and/or resolution of inflammation, reduced expression of chemokines associated with microglial recruitment and reduced microglial CD68 [52]. In the context of the hypothesis under discussion, and with the support of the genetic data implicating microglia in the evolution of AD pathophysiology, it seems likely that some of these associations reflect causal processes, in particular the microglial activity mediating the link between Aβ and tau [Fig 2]. In addition, it is inevitable that part of the microglial response in AD is simply a reaction of the microglia to neuronal degeneration, as in any neurodegenerative condition. The strong relationship between MSR-A [20, 21] and neuritic plaques is potentially in the causal pathway, with MSR-A acting as a mediator of the microglial response to fibrillary Aβ. Conversely, the

strong associations of CD68, a lysosomal protein reflecting microglial phagocytic activity, with the neuritic plaques and tangles are likely, at least in part, due to microglia phagocytosing neuronal and synaptic debris as a consequence of the neurodegeneration which occurs once the tau accumulation has started.

As noted above, *APOE* genotype is the major genetic risk factor for sporadic AD and is known to influence Aβ deposition, both in the form of plaques and CAA [53]. In this study, as microglia are also implicated in apoE metabolism, we investigated *APOE* genotype in relation to the microglial activity. The finding was that, overall, possession of the protective *APOE ε*2 allele was significantly related to a high expression of Iba1 and MSR-A and a reduced amount of CD68 and HLA-DR. The association of *APOE* ε2 with MSR-A might appear to contradict our suggestion that it plays an important role in the response of microglia to Aβ plaques; alternatively, microglia may detect the presence of plaques but fail to mount a toxic response in ε2 carriers. This observation potentially points to the complex role of microglia in AD pathogenesis and requires further investigation. Conversely, possession of the risk factor allele *APOE ε4* was significantly related to greater expression of CD68, HLA-DR and CD64, but a reduced amount of Iba1 [47]. This is consistent with (i) the concept that Iba1 expression, being related to microglial motility, may have a beneficial homeostatic role which is lost in AD [48], and (ii) the experimental findings that *APOE* ε4 modulates microglial responses to neurodegeneration [54]. Our previous studies indicated an inverse association between Iba1 and Aβ42 loads in AD; whereas microglial activity, as assessed with multiple microglial markers, was closely related to the level of tau in the same cohort [55].

Recent work using single-cell RNA sequencing to characterise microglia in the human brain has confirmed the existence of numerous microglial subtypes [56]. There are marked ageing effects, with young adults having predominantly “homeostatic” microglia whereas older adults express more inflammatory genes [57]. In AD, transcriptomic analysis of nuclear RNA found a cell population with increased expression of AD risk genes (APOE, TREM2, MEF2C, PICALM, and MHC Class II), many of which are expressed in microglia [58], to be associated with AD pathology [59]. *In vivo* positron imaging tomography (PET) imaging of microglia has been performed in AD using several different ligands, and will doubtless provide important information; however, the precise cell type and activation state the ligands signal is as yet unclear [60].

TREM2 in the human Alzheimer’s brain

The role of the Triggering Receptor Expressed on Myeloid cells (TREM)-2 in AD has been of great interest since the finding that a *TREM2* gene variant, although rare, if present can cause a risk effect size comparable to that of *APOE* genotype [61, 62]. Evidence suggests that in the context of AD, TREM2 acts as a key regulator which switches myeloid cells from a homeostatic to a neurodegenerative phenotype [63], and the TREM2 R47H variant may result in a loss of function [64]. To date, investigation of the localisation of TREM2 protein has mainly been performed on experimental models with little evidence for its presence in histological studies of the human brain. Using an antibody to TREM2 demonstrated as specific by Western blot and by immunoreactivity of osteoclasts and splenic macrophages [65], we screened the CFAS cohort described above for TREM2 expression [66]. In 284/299 cases, TREM2 labelled monocytes within vascular lumens only, but not microglia or perivascular macrophages. Of note, TREM2-positive cells with the morphology of monocytes were identified within acute infarcts in five cases (5/6 cases of acute infarcts), likely able to cross the blood-brain barrier which was disrupted by the infarction. These infiltrating monocytes appeared to lose TREM2 immunoreactivity as they matured into phagocytosing CD68-positive macrophages, the role of which is to phagocytose the necrotic tissue of the infarct. These observations and interpretations may be controversial and remain uncertain until more definitive evidence is available from the human brain, but seem to suggest that the role of TREM2 may be different in the human brain compared with laboratory rodents and may be a marker of recruitment of circulating monocytes into the brain. This finding, if confirmed, would seem consistent with evidence that TREM2-related systemic immune responses may play a role in the development of AD.

Several roles for innate immunity in AD

Innate immunity appears to have several roles in AD which likely change with the evolution of the disease (Figure 2):

(i) An *increase in "harmful" functions of microglia*, exemplified by microglia responding to the presence of Aβ in the cortex and inducing the neuritic dystrophy. Of note, MSR-A which is involved in the recognition of fibrillary Aβ by microglia, is expressed particularly by microglia clustered around plaques, presumably immobilising the cells there and initiating a "toxic" response which damages neurons as collateral damage ("bystander effect") [20, 55].

(ii) *A decrease in beneficial homeostatic functions of microglia* which include a role in homeostatic maintenance and synaptic remodelling for which Iba1 and P2ry12 appear to be relevant in this respect, and consistent with the findings from experimental models [63, 67].

(iii) *A non-disease-specific response of microglia to neuronal damage* with upregulation of phagocytic activity to remove damaged neurons and synapses, indicated by CD68 immunoreactivity of lysosomes

(iv) Interaction between *systemic inflammatory influences* and microglia in the brain [68, 69].

Presumably, these different functional changes in microglia each have different time-courses during the pathogenesis of AD, but little is known about this from pathological studies. In vivo PET imaging of microglia has suggested that there may be different phases of microglial activation. However, better understanding of the functional aspect of those phases has to await microglia-function specific ligands [60].

3. Modelling the role of microglia in AD pathophysiology

A limitation of human *post-mortem* studies is that although it is possible to show associations and relate findings to a specific hypothesis, it is not usually possible to demonstrate cause and effect. Consequently, various model systems are employed in order to perform manipulations and attempt to clarify cause and effect relationships. Animal models are extensively used for this purpose and to pilot ideas for therapy, although a limitation is that these are biased by the model chosen [70]. For example, mutant APP transgenic mice are very good models of Aβ accumulation; however most models lack tau and the severe neurodegeneration associated with the human disease. Despite the availability of more than 180 experimental models of AD, most do not fully replicate the complexity of human AD pathology. A further concern is that as yet poorly understood differences exist in innate immunity between mice and humans [70]. An example of this is the evidence that, in rodents, the immune response to the presence of plaques is partly due to circulating monocytes which enter the brain and develop into mature microglia [71-73], evidence for which in humans is lacking. An interesting recent animal study of direct relevance to the hypothesis under discussion relates to the use of a CSF1R inhibitor to ablate microglia in 5xFAD mice. This model has plaques with neuritic dystrophy, though lacking the tau accumulation characteristic of human neuritic plaques. CSF1R is necessary to maintain the presence of microglia in the brain [74] and its inhibition in the 5xFAD mice led to a reduction in neuritic plaque formation, indicating that the presence of microglia is necessary for the development of neuritic dystrophy [75].

Induced Pluripotent Stem Cell (iPSC) technology has evolved rapidly in recent years and has the advantage of allowing manipulation of human cells cultured *in vitro*. A recent study described the development of a 3D tri-culture system in which iPSCs derived from familial AD patient fibroblasts

were differentiated into neurons, astrocytes and microglia [76]. This co-culture system allowed identification of key signalling molecules including Tumour Necrosis Factor (TNF)-α, Interferon (IFN)-γ and C-C Motif Chemokine Ligand (CCL)-2 (also known as Monocyte Chemoattractant Protein (MCP)-1) which could potentially be manipulated for therapy. The system demonstrated that microglia migrate towards Aβ and subsequently play a role in neuronal/neuritic damage, again consistent with the hypothesis that microglia provoke neuritic dystrophy in the human brain. This technology has also been used to explore the mechanism by which the *APOE* gene polymorphism influences AD risk, which remains unclear despite 25 years elapsing since the discovery of its importance [77]. iPSC-derived microglia differing only in their *APOE* genotypes were generated and showed that the APOE4 *vs* APOE3 microglia displayed fewer and shorter processes, had an upregulation of immune response genes and a downregulation of movement-related genes and were considered more "reactive" and less "homeostatic" [78].

4. Inferences on cause and effect in AD from neuropathological alterations induced by Aβ immunotherapy

Further information on AD pathogenesis can be derived from analysing the effects of potential therapeutic agents employed in human clinical trials. In particular, we have had a unique opportunity to decipher cause and effect relationships in the human AD brain in relation to the amyloid cascade hypothesis. Based on the hypothesis that Aβ accumulation is a key initiating factor in AD pathogenesis [79], therapies emerged to target Aβ, with trials that began nearly 20 years ago following the seminal experimental studies of Dale Schenk and colleagues. Using PDAPP transgenic mice, they observed that peripheral immunisation with full-length Aβ42 peptide (AN1792) before the appearance of plaques prevented age-related Aβ accumulation; while immunisation of aged mice which already had extensive Aβ accumulation resulted in plaque reduction with evidence of plaque removal from the brain [80]. Additionally, there was evidence of functional benefit with Aβ immunisation ameliorating the memory loss associated with Aβ deposition in these animals [81, 82]. Subsequently, in the year 2000, the first clinical trial of Aβ immunisation in human AD was initiated using AN1792 (full-length Aβ42 peptide with QS21 as adjuvant). Eighty patients with mild to moderate AD (MMSE score 15 to 25/30) were enrolled; sixty-four subjects had the active agent and 16 subjects had the adjuvant alone as controls. Up to eight intramuscular doses were given over an 18-month period. This initial study was essentially a safety and immunogenicity study, to determine if elderly subjects immunised in this way would develop antibodies to Aβ [83], and was successful in

this regard. However, a subsequent larger trial with this agent was halted when 6% of patients developed an inflammatory complication [84].

Changes in Aβ induced by Aβ immunotherapy

After initial observations in a single case suggesting that Aβ immunisation in human AD provokes removal of plaques [85], as predicted from the experimental studies, we initiated a systematic long-term follow-up study of the neuropathology of the cohort from the original AN1792 trial. Our detailed neuropathological analysis on the brains of 22 of the 80 patients have revealed a number of important findings [86] (Table 1). A notable observation was that five of the 22 subjects had pathological causes other than AD for their dementia, highlighting the need for better diagnosis of the pathological substrate of dementia during life which is a current focus of endeavour. The most striking feature was a marked reduction in Aβ load (1.3% area fraction) in the 16 immunised AD cases compared with a group of untreated AD subjects matched for age , *APOE* genotype and dementia duration (6.8% area fraction), and similar to that of age-matched non-demented controls (1.9% area fraction) [87], when quantified in a consistent neuroanatomical region (inferior parietal lobule). However, there was a marked variation in the extent of plaque removal from case to case. Quantification in whole hemisphere sections confirmed evidence of plaque removal in 14/16 immunised AD cases with almost complete removal of plaques in five cases, extensive patches of plaque removal in four, and plaque removal limited to small patches in five cases. Only two immunised AD subjects had no evidence of plaque removal, similar to the single AD subject receiving the placebo who came to *post-mortem* examination [86]. There was an inverse correlation between the anti-Aβ antibody titres measured in serum and the plaque scores; those with high titres having relatively low plaque scores, consistent with the hypothesis that the anti-Aβ antibodies generated by the patients' immune systems were responsible for the removal of plaques [86]. This correlation was all the more remarkable bearing in mind that the antibody titres were measured during the trial period, which in most cases was many years before the *post-mortem* assessment of plaques. However, disappointingly, all the immunised AD patients declined to a terminal end-stage dementia [88], even those with the most extensive plaque removal, including the five subjects with complete removal of plaques in whom a more complete response to the immunotherapy could not be envisaged. We concluded that removing plaques from the brain in AD is not sufficient to halt the cognitive decline [86, 88].

In the context of the hypothesis being considered, Aβ accumulation activates microglia leading to tau accumulation - it is therefore logical to ask whether despite Aβ removal, the continued

progression of the cognitive decline might be related to persistent microglial activation and/or progressive tau accumulation.

Changes in microglial behaviour induced by Aβ immunotherapy

To investigate the behaviour of microglia after Aβ immunotherapy, we firstly performed multilabel confocal microscopy and demonstrated extensive phagocytosis of Aβ plaques by microglia in the immunised AD cases [21, 89], with Aβ visible within lysosomes labelled with CD68. The appearances were similar to that shown in microglia induced to phagocytose Aβ when incubated on slices of human AD brain *in vitro* and exposed to anti-Aβ antibody [90].

Immunohistochemistry for HLA-DR in AD showed enhanced microglial clustering around plaques after immunisation during the process of active plaque removal, and then substantially reduced labelling once plaques had been removed, with clusters of HLA-DR–labelled microglia no longer visible. CD68 immunoreactivity, reflecting phagocytosis, was widely scattered throughout the cortex in untreated AD, not clearly related to plaques; whereas after immunisation, CD68 was observed clustering around plaques in the process of being phagocytosed, and similarly once plaques had been removed, its expression was dispersed [89]. The interpretation is that although microglia cluster around plaques in untreated AD, there is little or no phagocytosis of Aβ. However, after immunisation, antibodies generated by the patient and circulating in the blood enter the brain and bind to the Aβ plaques resulting in opsonisation, with the Fc portion of the antibody binding microglial Fc receptors, prompting phagocytosis by nearby microglia. It is notable that although most of the antibodies generated by patients in response to AN1792 (i.e. full length AB42) were directed to the N terminus of Aβ, all plaque components are removed including: $Aβ_{40}$, $Aβ_{42}$, $Aβ_{43}$, N-terminus truncated Aβ, the amyloid as detected by thioflavin S and Congo red, and plaque detection by silver stains, as well as removal of plaque-associated dystrophic neurites and clusters of astrocytes and microglia [18, 91, 92].

The initial response to immunotherapy and the process of plaque removal seems to involve migration of microglia to the plaques and increased phagocytic activity. Interestingly, in a larger group of immunised AD cases, including long-term survivors, and using multiple microglial markers such as Iba1, CD68, CD64 and MSR-A, microglial activation appears to be down-regulated after immunisation compared with an untreated AD cohort [55]. This implies that persistent microglial activation after Aβ immunisation is not the cause of the continued cognitive decline.

Changes in Tau pathology induced by Aβ immunotherapy

We then investigated the question of whether persistent tau accumulation could explain the continued cognitive decline following Aβ removal. Initial observations on the first case clearly showed that clearance of Aβ was associated with removal of the plaque-associated tau-containing dystrophic neurites [85]. Acquisition of further cases permitted quantitative studies using several complementary approaches.

Firstly, quantitative analysis of phosphorylated tau in anatomically similar regions of cerebral cortex and hippocampus, regardless of whether or not plaques had been removed from that location, showed a significant reduction in tau load comparing the immunised *vs* non-immunised AD cases [87, 88]. Quantification of the individual features of tau pathology in this study demonstrated a reduction in dystrophic neurites and neuropil threads, but not tangles [91].

A second approach took advantage of the patchy nature of plaque removal in many of the immunised AD cases, comparing tau pathology in areas with and without plaque removal [86]. Although in this context the anatomical regions compared were not identical, the advantage was that each case was acting as its own control. The findings confirmed the significant reduction in the dystrophic neurite clusters but also showed a reduction in tangles in areas of cortex in which Aβ had been removed [86]. The demonstration that a reduction in tau occurs in areas freed of Aβ supports a link between Aβ and tau accumulation as stated by the amyloid cascade, further supported by the finding that Aβ removal is associated with a reduction in the tau-phosphorylating enzyme GSK3β [93]. Stereological study of hippocampal changes in a separate cohort of AN1792-immunised subjects demonstrated similar findings and additionally showed evidence of decreased tau phosphorylation [94].

A third approach was to evaluate tau pathology using Braak staging which assesses the degree of spread of tangles through the brain [95], and it was notable that 15 of the 16 AD subjects immunised against Aβ were assessed as Braak stage V-VI, indicating extensive spread of tau pathology within the brain. The single immunised AD case not at this advanced stage of tau distribution was a patient who had died just four months after the first immunisation dose, from a cause unrelated to the treatment, and had a Braak stage of IV. It is noteworthy that when the subjects were recruited to the trial, they had MMSE scores in the range 15-25/30. An unrelated study of a large cohort of elderly people indicated that MMSE scores of 15-25/30 corresponds to Braak stages between III-V [96]. Therefore, despite the localised relative reduction in tau after Aβ immunotherapy, the advanced Braak stages of the immunised cases at death implies a continuation in tau spreading through the brain, potentially explaining the cause of the progressive cognitive decline.

These findings are consistent with a scenario in which Aβ initiates and then continues to drive and support local tau aggregation within the neocortex during the course of AD, consistent with the amyloid cascade hypothesis. However, in addition, tau aggregation has its own self-propagating nature and, once it has started, tau spreads through the cortex according to the Braak sequence, putatively in a "prion-like" manner, independently of Aβ [97, 98]. The likely complex relationship between tau pathology and cognition following Aβ immunisation is poorly understood. Further understanding of the effect of Aβ immunotherapy-induced removal of amyloid on the spread of tau through the brain, and the relationship with cognition, will likely be gained from PET imaging studies of both amyloid and tau currently being performed in immunotherapy clinical trials.

Relevance to subsequent immunotherapy trials

In summary, Aβ immunotherapy caused clearance of Aβ plaques in established AD in humans, as predicted from the preclinical studies; however, the patients still died with severe dementia. Microglial phagocytosis was increased transiently after immunotherapy as one of the mechanisms to remove Aβ, but microglial activity then quietened down in the long-term. A relative reduction in tau locally where Aβ has been removed was observed. Nevertheless, there is a suspicion that tau was still spreading through the brain in a "prion-like" manner, potentially underlying the continuing cognitive decline. Aβ immunotherapy trials have represented huge investments in time and resources. The early immunotherapy trials following AN1792 were mostly passive immunisation in which antibodies directed to Aβ were provided, rather than relying on the ageing immune system to generate them. This has advantages in that the exact levels of antibody available are known and the treatment can be stopped if side-effects occur. However, in retrospect it seems the initial passive immunotherapy trials did not result in very extensive plaque removal, possibly impacting on the trial outcomes. Amyloid PET scanning has been developed and employed to assess plaque extent before and after treatment scans confirming than immunotherapy can remove plaques. Overall, the degree of amyloid removal in most of the trials of passive immunotherapy appeared low and no significant effect on cognition was obtained. A recent shift in the field of immunotherapy has been to trials at earlier stages of the disease (i.e. mild cognitive impairment and "prodromal" AD). Higher antibody doses have been employed and, in some cases, have provoked very extensive plaque removal, comparable to our *post-mortem* observation of the AN1792 trial. Early data have suggested some protection against cognitive decline, but this has not yet been confirmed [99].

The question that remains unanswered 20 years after the first clinical trial is whether AD can be prevented by Aβ immunotherapy. The initial experimental study using active Aβ immunotherapy in

mice at a young age showed inhibition of Aβ plaque formation [80]. Therefore, the outstanding question in humans is whether Aβ accumulation can be prevented by Aβ immunotherapy if provided at an early age, and then whether this will prevent the microglial activation, tau accumulation and neuronal and synaptic degeneration. Relevant clinical trials including preventative immunotherapy in families with AD-causing point mutations are in progress and should provide an answer to this crucial question.

Therapy targeting microglia

Since data indicating that people with rheumatoid disease taking long-term non-steroidal anti-inflammatory drugs (NSAIDs) appeared protected against AD, considerable interest has been generated in "anti-inflammatory" therapy for AD [100]. Although evidence implies that NSAIDS reduce microglial activity in AD [101], a recent prospective study of NSAIDs was unsuccessful [102]. Other more tailored approaches have been suggested involving complement, TREM2 and TNF-α [103], for example. However, neuropathological studies of microglia, such as those described above, highlight the complexity of the microglial responses that require to be better understood and a therapeutic approach to manipulate specific microglial activities may be more fruitful [31, 32].

Therapy targeting tau

Therapy targeting tau has been explored only relatively recently [14]. Normal functions of tau include stabilising microtubules and regulating intracellular trafficking. There are many disorders in addition to AD in which abnormally phosphorylated tau accumulates, mainly within neurons. Therapeutic approaches have included reducing tau expression using antisense oligonucleotides or siRNA, inhibiting tau phosphorylation, preventing tau aggregation and tau immunotherapy. With regard to tau immunotherapy, perhaps surprisingly, antibodies have been demonstrated to enter neurons [104] and interest has also been focused on targeting extracellular tau [105], putatively involved in the cell-to-cell spread of tau through the brain. As clinical trials targeting tau are performed in coming years, not only potential clinical benefits, but also observations of any neuropathological changes, will be of interest.

Conclusion

In conclusion, different therapeutic approaches are likely to be necessary to tackle the different stages of Alzheimer's disease, and may ultimately be administered either singly or in combination. Early Aβ immunotherapy and manipulation of apoE may have a role in disease prevention; agents modulating specific aspects of the microglial response/phenotype to Aβ, proposed as the intermediary between Aβ and tau, may be important in subjects in midlife in whom Aβ accumulation has begun in the form of diffuse plaques in the cerebral cortex [102]; whereas treatment of established AD requires inclusion of agents directly targeting tau accumulation and the neurodegeneration itself.

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Conflict of interest

The authors declare that they have no competing interests.

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Legends

Figure 1

The neuritic plaque - where Aβ, microglia and tau collide. **Step i** (first column): Aβ deposits appear in the cerebral neocortex, initially lacking a microglial reaction (HLA-DR), APP immunoreactive dystrophic neurites and ptau (i.e. diffuse, non-neuritic plaques). **Step ii** (second column): microglia recognise and respond to the Aβ deposits, forming clusters of activated microglia (HLA-DR) and inducing neuritic dystrophy demonstrated here by accumulation of APP within the abnormal neurites, which lack ptau at this stage. **Step iii** (third column): Dystrophy of the neurites renders them vulnerable to aggregation of ptau, facilitating spread of ptau to the cerebral neocortex from the limbic system where it has already been accumulating (Primary Age-Related Tauopathy; PART). **Step iv** (fourth column): ptau spreads from the plaque-associated dystrophic neurites throughout the neuron, forming neuropil threads and tangles in the cell bodies (to left of plaque in illustration). Ptau then spreads in a "prion-like" fashion from neuron to neuron extensively throughout the cerebral neocortex causing widespread neuronal dysfunction, manifest as dementia.

Illustrations from cortex of inferior parietal lobule, cases as follows: 82 year old man, non-demented; 78 year old woman, non-demented; 73 year old man, non-demented; 80 year old man, dementia, Alzheimer's disease. Antibodies employed: Aβ (4G8, Covance), HLA-DR (CR3/43, Dako), APP (22C11, ThermoFisher Scientific), ptau (AT8, ThermoFisher Scientific). Slides digitised on a Panoramic Scan slide scanner (3DHistech Ltd.) and images captured at x40 using Case Viewer software. Scale bar = 20μm. Images captured from slides prepared for a previous study [19, 69]. Tissue sourced from South West Dementia Brain Bank (NRES Committee South West Central Bristol, REC reference: 08/H0106/28+5).

Figure 2

Hypothesis for interactions of Aβ, microglia and tau in causing Alzheimer's disease. Genetic causes of AD all result in increased production of Aβ, indicating that Aβ accumulation is the initiating event, including in sporadic AD **(step i)**. However, many elderly and middle-aged people have diffuse cortical Aβ plaques (i.e. plaques lacking microglia, dystrophic neurites and ptau) and are cognitively normal, indicating that the diffuse Aβ plaques by themselves do not cause dementia. **Step ii:** Microglia recognise the presence of Aβ plaques and respond by migrating towards the plaques, abandoning their normal homeostatic functions, acquiring activated morphology, secreting neurotoxic substances and initiating the complement cascade. Most genetic factors increasing risk for, but not causing, AD are associated with microglia and operate at this step. As an end product of the complement cascade, membrane attack complex forms pores, evolved to killed invading micro-organisms, in nearby neuronal processes; the resultant metabolic dysregulation disrupts the neuritic cytoskeleton and neuronal transport, causing the characteristic irregularly thickened and tortuous dystrophic neurites of the neuritic plaque which accumulate APP as a reflection of the disturbed transport (APP+/ptau- dystrophic neurites). **Step iii:** The associated neuritic dysfunction facilitates spread of aggregated ptau, which has already begun to accumulate in limbic structures as part of the ageing process (Primary Age-Related Tauopathy), to the neocortex, first appearing in the plaque-associated dystrophic neurites (APP+/ptau+ dystrophic neurites). **Step iv:** Ptau spreads within neurons from the

dystrophic neurites to the neuronal somata resulting in neurofibrillary tangles. Subsequently, ptau spreads by cell-cell transfer throughout the neocortex resulting in progressive neurodegeneration and dementia. [Artwork by Dr Jennifer M Dewing]

Microglial functions are altered in three ways: (a) loss of normal homeostatic functions which support neuronal and synaptic health; (b) a toxic reaction to Aβ plaques resulting in bystander damage to neuronal processes, facilitating ptau accumulation and (c) clearance of neuronal debris. *APOE* ε4, the main genetic risk factor for AD, has multiple actions including: increased Aβ accumulation, enhanced microglial reactivity and impaired clearance of neuronal debris.

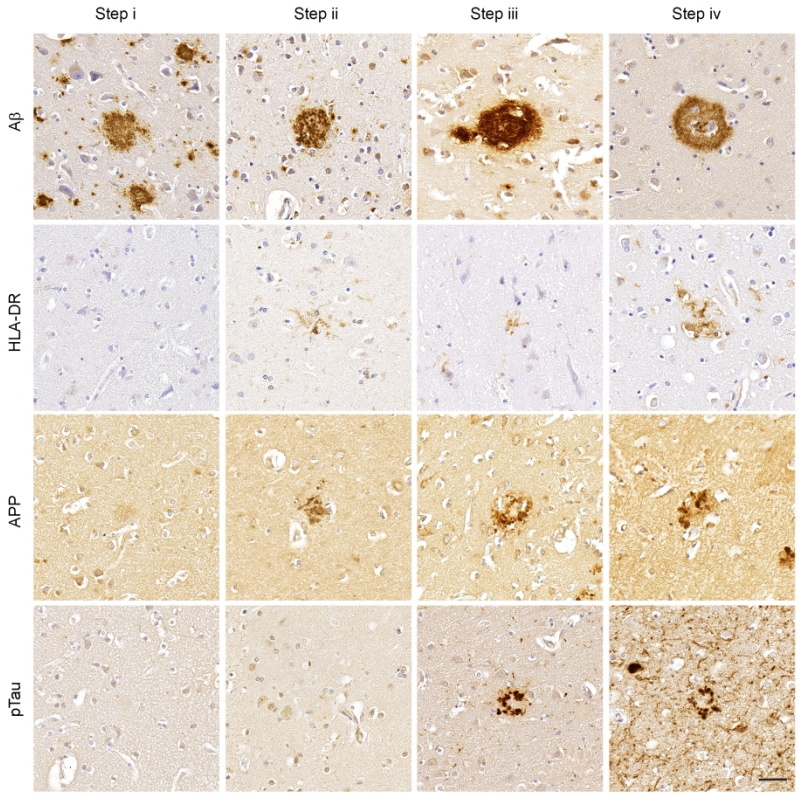
This hypothesis implies different therapeutic targets are appropriate for different steps of AD pathogenesis: *step i* - primary prevention, disrupt Aβ aggregation (e.g. active Aβ immunotherapy); *step ii* – secondary prevention, modulate microglial activity; *steps iii/iv* – established AD, target ptau.

Table 1

Main findings from *post-mortem* neuropathological study of AD patients who received active Aβ immunotherapy (AN1792)

Aβ
• Reduction of Aβ/removal of plaques ([85], *[106], *[107], [18], [88], *[94], *[108], [91], [86]) • Phagocytosis of Aβ by microglia ([85], [18], [89]) • Alterations in soluble Aβ ([109])
Tau
• Resolution of plaque-associated dystrophic neurites ([85], [91], [86]) • Reduction of phosphorylated tau in neuronal processes ([91], [94], [87]) • Reduction in the tau phosphorylating enzyme GSK3β ([93]) • Focal reduction in tangles where Aβ removed, but probable continued "prion-like" spread of tangles ([91], [86])
Vascular
• Increased cerebral amyloid angiopathy ([110]) • Increased microhaemorrhages ([110]) • Meningoencephalitis (c.f. Amyloid-related imaging abnormalities, ARIA) ([85], [84]) • Increased cerebral amyloid angiopathy-associated vasculopathy ([10]) • Redistribution of apoE from plaques to cerebral blood vessel walls ([10])
Inflammation
• Meningoencephalitis/ARIA ([85], *[106]) • Alterations in microglial activation ([89], [55]) • No effect on total IgG, C1q or T lymphocytes ([55])
Neurons
• Reduction in neuritic curvature (*[94], [111]) • Increased interneuronal distance/reduction in neuronal density ([111]) • Increased cerebral cortical neuropil degeneration (status spongiosis) ([111]) • Lack of beneficial effect on synapses ([112]) • Reduction in proteins associated with apoptosis and autophagy.

*These studies included subjects immunised with AN1792 in a subsequent clinical trial.



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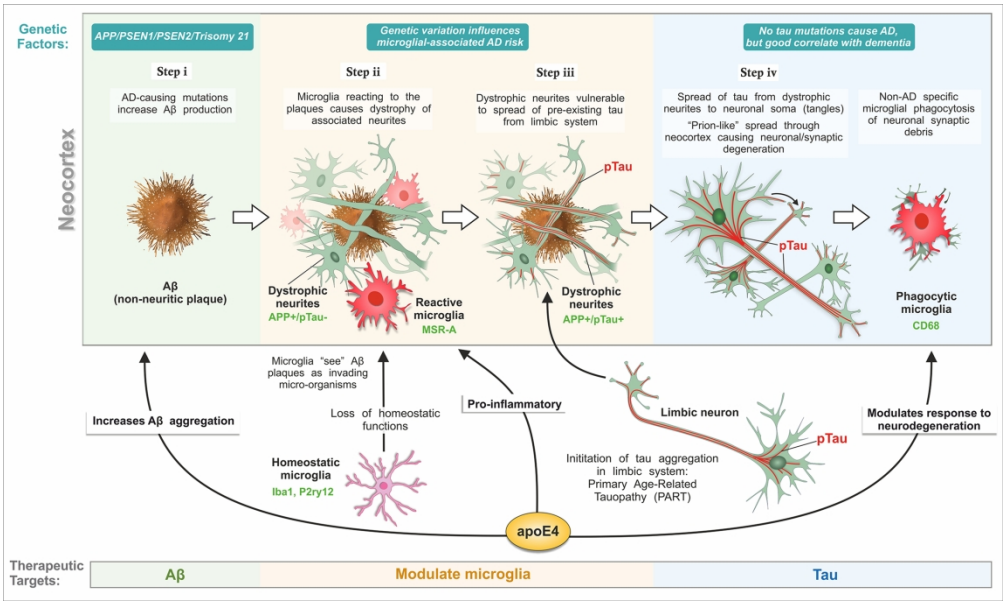

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