Immune responses to amyloid beta and post-translationally modified variants

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Declaration:

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to these or any other university for a degree, and does not incorporate any material already submitted for a degree.

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Date:

Abstract

Alzheimer's disease (AD) is a neurodegenerative condition affecting approximately 30 million people worldwide. The abnormal build-up of amyloid beta (Aβ) peptide has been implicated in AD and is thought to cause damage to neurons and inflammation contributing to disease progression. In addition to the 'normal' Aβ there are also a number of post translationally modified (PTM) variants of the peptide. It is thought that the immune system is able to recognise Aβ, through different immune receptors including toll-like receptors (TLR)2 and 4 and T-cell receptors (TCRs), contributing to both inflammation and clearance of Aβ from the brain. There is a possibility that the PTM variants of Aβ will cause an altered immune response. Unmodified Aβ peptide and three PTM variants were tested, their ability to aggregate was investigated and their morphology was determined using transmission electron microscopy (TEM). ELISA was used to investigate the release of cytokines in response to the four Aβ peptides from human embryonic kidney293 (HEK293) cells expressing TLR2 or TLR4-MD2-CD14, primary human monocytes and peripheral blood mononuclear cells (PBMCs) from patients with AD and age-matched controls. A cohort of 40 patients with AD and 40 age-matched controls was recruited to investigate antigen-specific T-cell responses to the peptides using flow-cytometry to measure proliferation and phosphorylation of protein kinase C (PKC)-ζ and PKC-δ. TEM analysis of the Aβ peptides showed that they were all able to form mature fibrils although the modified Aβ peptides had a fibrillar morphology earlier than the unmodified form. IL-8 was detected in the supernatants of HEK293 cells expressing TLR2 but not TLR4 after stimulation with fibrillar Aβ (fAβ) peptides and there were no differences observed between unmodified and PTM variants. No IL-1β or TNFα was detected from isolated monocytes but IL-1β was released from PBMCs after stimulation with fibrillar forms of all the peptides tested, the use of flow cytometry showed that the IL-1β was released from monocytes. The addition of neutralising antibody for TLR2 was able to reduce the IL-1β signal in some participants but not in others. In contrast, the use of a second batch of the same peptides was unable to induce a response from PBMCs. It was not possible to see Aβ-specific activation of T-cells leading to proliferation, or phosphorylation of PKC-δ or PKC-ζ. These results suggest that modifications to the Aβ peptide are able to alter the aggregative properties of the peptide but they do not change the immune responses observed. Fibrillar Aβ peptide and its PTM variants can be recognised by TLR2, however other mechanisms are likely to be involved in cytokine release. For example, T-cells may be able to influence the release of cytokines from monocytes. Published data looking at Aβ-specific T-cell activation shows varied results, in this study it was observed that unaggregated Aβ peptide was not able to activate antigen-specific T-cells. There is, however, a possibility that an aggregated form of the peptide would have an effect on T-cells. It was also clear in this study that different batches of peptide are able to induce different biological responses.

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Abbreviations

AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotein
ADDLs	Aβ-derived diffusible ligands
AP-1	Activating protein 1
APC	Antigen presenting cells
APH-1	Anterior pharynx
APLP	Amyloid-like protein
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid-beta peptide
AβpE3	Pyroglutamate modified Aβ at amino acid 3
BACE1	β-secretase
BBB	Blood brain barrier
BFA	Brefeldin A
CD	Circular Dichromatism
CD14	Cluster of differentiation 14: co-receptor for TLR4
Cit5	Citrulline modification at amino acid 5
CLIP	Ii-derived class II invariant chain peptide
CMV	Cytomegalovirus
CNS	Central nervous system
CTFβ	C-terminal fragment
DAMPs	Danger associated molecular patterns
DC	Dendritic cell
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide

DPBS	Dulbecco's phosphate buffered saline
E3	Ubiquitin protein ligase
ELISA	Enzyme linked immunosorbant sandwich assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FSC-A	Forward scatter -area
FSC-H	Forward scatter - height
FSL-1 for TLR2/6	Pam2CGDPKHPKSF: synthetic diacylated lipopeptide: ligand
FTIR	Fourier transform intra-red spectroscopy
GI	Gastro-intestinal
GILT	gamma-IFN-inducible lysosomal thiol reductase
HBSS	Hank's buffered saline solution
HCl	Hydrochloric acid
HEK	Human embryonic kidney cells
HFIP	Hexafluoroisopropanol
HLA	Human leukocyte antigen
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IKK	IκB kinase
IL-1β	Interleukin 1 beta
IP-10	INFγ-inducible protein 10
IRAK	IL-1 receptor associated kinase
IRF3	IFN regulatory factor

ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LCMS	Liquid chromatography mass spectrometry
LFA	Leukocyte function antigen
LPB	LPS binding protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LTA	Lipoteichoic acid
MALDI	Matrix Assisted Laser Desorption/Ionization
MAP	Microtubule associated protein
MAPK	Mitogen activated protein kinase
MD2	Co-receptor for TLR4
MHC	Major histocompatability complex
MKK	MAPK kinases
MMSE	Mini-mental state exam
MTT	Methylthiazoletetrazolium: Cell viability assay
MyD88	Myeloid differentiation primary response protein 88
NEMO	NF-κB essential modifier
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHSBT	National health service blood and transplant
NK	Natural killer
NLR	Nod-like receptor
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PAD	Peptidyl arginine deaminase
PAM3	Pam_3CSK_4: ligand for TLR1/2

PAMPS	Pathogen associated molecular patterns
PB	Phosphate Buffer
PBMC	Peripheral blood mononuclear cells
PD	Parkinson's disease
PEN-2	Presenilin enhancer 2
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PKC	Protein kinase C
PMB	Polymyxin B
PPD	Purified protein derivative
PrP	Cellular Prion protein
PRR	Patter recognition receptor
PRUH	Princess royal university hospital
PTM	Post translational modification
QLS	Quasi-elastic light scattering
RAGE	The receptor for advanced glycation end products
RIP1	receptor interacting protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSCH	Royal Sussex county hospital
SEB	Staphylococcus enterotoxin B
SPPS	Solid phase peptide synthesis
SSC-A	Side scatter - area
TAB	TAK-1 binding protein
TAK	Transforming growth factor activated protein kinase
TANK	TRAF-associated NF-κB activator
TAP	Transporter associated with antigen presentation

TBK	TANK-binding protein
TCR	T-cell receptor
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TMB	3,3,3-Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
TRAF	TNF receptor associated factor
TRIF	TIR domain containing adaptor protein inducing IFN-β
α-TLR2 (neutralising antibody)	anti-toll like receptor 2 neutralising antibody
α-TLR2	anti-TLR2 antibody

1. Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease and is the most common form of dementia, affecting more than 35 million people worldwide [1, 2]. As AD is predominantly a disease of old age, the number of people with the disease is set to increase as the world's population continues to age. It has been estimated that by 2040 the number of people with AD will have tripled[2] [3]. There are two different forms of AD; familial or early onset and sporadic or late onset AD. Despite the contribution that age makes to the disease others factors are also thought to be involved. In the familial form of the disease it is believed that there is a much stronger genetic component, mutations in single genes have been identified and so far three genes are believed to carry mutations: these are found in the gene encoding the amyloid precursor protein (APP) gene, and in the gene encoding presenilin 1 and 2, all of which are involved in the production of amyloid beta (Aβ) [4-9]. Although the exact cause of sporadic AD remains unclear, there is evidence of some genetic polymorphisms causing a predisposition to the disease [10-12]. The strongest risk factor with sporadic AD is age, however there is also evidence for factors such as heart problems, obesity, high cholesterol and stroke [13, 14]. Currently there is no cure for either form of the disease but there are drug treatments used, with limited success, to treat the symptoms of the disease. These include: Donepezil, Rivastigmine and Galantimine, all of which are acetylcholine inhibitors, as well as Memantine, an N-methyl-D-aspartate (NMDA) receptor antagonist [15].

There are a number of symptoms and hallmarks associated with the disease that are present in both forms of AD, the main symptoms being progressive memory loss, emotional changes and anxiety [16], although the symptoms never present in exactly the same way in different people. Additionally, a variety of changes occur in the brains of people with AD. The main neuropathological change that occurs is progressive cortical atrophy focussed in the frontal, parietal and temporal brain regions. There is also considerable atrophy of the hippocampus and sometimes the amygdala as well as the enlargement of the ventricles [17-19]. The two main physiological hallmarks are extracellular deposits of Aβ and intracellular deposits of hyperphosphorylated tau protein. In addition to these changes there is also widespread inflammation, suggesting a role for the immune system in AD [20-23].

Despite the now general agreement for the genetic basis of familial AD, relatively little is known about the causes of sporadic AD. There are, however, a number of possible theories that have been proposed. Firstly, one of the most researched areas of AD is the 'amyloid cascade hypothesis', which suggests a pathogenic role of the Aβ peptide [22, 24, 25]. Secondly, there is a potential role for the other peptide found in AD, hyperphosphorylated Tau, and finally the genetic involvement of genes such as apolipoprotein E (APOE).

1.1.1 Theories in Alzheimer's disease

Although there is a strong genetic basis for familial forms of AD, the genes implicated in this form of the disease are not thought to be as important in the

sporadic form. However, there is still believed to be a genetic predisposition to sporadic AD, involving the apolipoprotein E gene (*APOE*). This is the only strongly confirmed genetic risk factor for both sporadic and familial AD [26-28].

It is specifically the type 4 allele that is thought to be a risk factor in AD, interestingly it has been suggested that the type 2 allele has protective potential [29, 30]. In the brain, APOE is expressed in greatest abundance in non-neuronal cells such as astrocytes and is involved in transporting cholesterol to neurons. The precise involvement of APOE in AD progression is not clear; however there is a strong association with $A\beta$. It has been suggested that APOE functions as an $A\beta$ binding protein inducing increased aggregation through increased β-sheet conformational changes.

There is also evidence to suggest that tau is involved in the cause of AD. Tau is a microtubule associated protein (MAP) found in the greatest abundance in neuronal axons and has an important role in the assembly and stability of the neuronal microtubule network.

The finding that has most implicated tau in AD is that the neurofibrillary tangles (NFTs), characteristic of the disease, are composed of hyperphosphorylated tau. It has been observed that the phosphorylation of tau reduces its ability to stimulate microtubule assembly therefore contributing to the degeneration of neurons in the brains of people with AD [31-33]. It has also been suggested that an increase in unbound tau under pathological conditions leads to an increase in the chance of conformational changes that lead to aggregation and fibrillisation, resulting in NFTs [34]. In addition to

AD, tau has also been implicated in several other neurodegenerative diseases suggesting a general role in neurodegeneration [34, 35].

However, probably the most popular and researched area in AD has been into the involvement of the peptide Aβ in disease progression. Interest in this area started after the discovery that senile plaques, one of the two major physiological hallmarks of AD, are composed mainly of different isoforms of Aβ [36, 37]. This discovery led to the development of the amyloid cascade hypothesis. The theory is based on the idea that a build-up of Aβ in the brain leads to a series of downstream events causing damage to neurons and synapses, inflammation and the phosphorylation of tau, all of which contribute to AD[25, 38]. In addition, all the genes involved in both sporadic and familial AD point towards Aβ playing a role in the disease. It is the role of Aβ that will be the focus of this study.

1.2 Amyloid beta

One of the major hallmarks of the disease is the presence of insoluble, extracellular plaques made up of the peptide Aβ. These plaques are found in specific areas of the brain, the cerebral cortex and hippocampus. Aβ is formed from the sequential, proteolytic cleavage of the APP, which is expressed in the greatest abundance in neuronal synapses, although it can also be found in several other tissues in the body. The actual function of APP is unknown; however, several physiological roles have been suggested for it, for example, cell adhesion, synaptic plasticity and neuronal survival [39-42].

 The human *APP* gene is one member of a family of genes including, amyloid-like protein 1 (*APLP1*) and *APLP2* (in humans) [41, 43-45]. They

are single transmembrane proteins with large extracellular NH_2-terminal domains and shorter cytoplasmic COOH-terminal domains; however, APP is the only member of the family that is able to produce the Aβ fragment [46, 47]. There are three major isoforms of APP, composed of 695, 751 or 770 residues, formed from alternative splicing. The isoforms are expressed in different cells types: APP_{695} is expressed on neuronal cells whereas APP_{751} and APP_{770} are more widely expressed on non-neuronal cells [47-49]. It is co-translationally translocated into the endoplasmic reticulum and matured through the secretory pathway, during which it can be proteolytically cleaved by enzymes known as secretases [50, 51].

There are three secretases known to be involved in APP processing: α-, β- and γ-secretase. The initial step is performed by α- or β-secretase resulting in the release of either sAPPα or sAPPβ. α-secretase is a metalloproteinase, specifically ADAM10 (a disintegrin and metalloproteinase 10), that cleaves APP at the α-secretase cleavage site, positioned between Lys^{16} and Lue^{17} [41, 52] within the Aβ domain therefore eliminating the possibility of Aβ being released. After action by α-secretase an 83-residue COOH-terminal fragment, known as CTFα, is retained in the membrane. In contrast, β-secretase is a transmembrane aspartyl protease known as β-site APP-cleavage enzyme 1 (BACE1). Cleavage by β-secretase generates the N-terminal of Aβ leaving a 99-residue COOH-terminal fragment, called CTFβ, retained in the membrane [41, 51, 53-55].

The subsequent cleavage, regardless of whether the initial step was performed by α or β secretase, is performed by γ-secretase. γ-secretase is a multi-subunit

enzyme complex made up of four subunits: presenilin 1 or 2, nicastrin, APH-1 and PEN-2, that is able to cleave APP at several sites within the transmembrane domain [56, 57]. This results in the generation of p3 (initial cleavage performed by α-secretase) a peptide with no known biological action [58] or Aβ (initial cleavage performed by β-secretase). As γ-secretase can act at a variety of cleavage sites a number of Aβ isotypes are formed between 38 and 42 amino acids in length. It has been estimated that more than 90% of Aβ produced is Aβ40 whereas less than 10% of Aβ secreted in Aβ42 [41]. However, a mutation in the APP gene and PSEN1 and 2 all result in an increase in the Aβ42 isotype. Comparisons between Aβ40 and Aβ42 have shown that Aβ42 is the more neurotoxic and amyloidogenic of the two isoforms and is therefore thought to play the more significant role in AD. Aβ processing is shown in figure 1.1.

In addition to the 'normal' Aβ there is extensive evidence of post-translationally modified (PTM) forms of the peptide [59-66]. Among these the most abundant PTM variants contain pyro-glutamate modifications at the N-terminal of the peptide [63-65]. This type of modification most commonly occurs at either the 3rd (AβpE3) or 11th (AβpE11) amino-acid and involves the truncation of the peptide. In the case of AβpE3 this requires the removal of the first two amino acids and results in the enzyme glutaminyl cyclase (QC) catalysing the dehydration of the exposed glutamate to form pyro-glutamate [63]. A second modification in the Aβ peptide, which has very recently been identified in the brains of patients with AD, is the citrullination, or deimination, of arginine residues. There is only one arginine residue in the Aβ1-42 sequence at position 5, which can be converted to a citrulline through

the action of the peptidyl arginine deiminase (PAD) family of enzymes [67, 68]. There are several PAD enzymes, however in AD it has been reported there is an increase in the expression of PAD2 and citrullinated proteins in hippocampal astrocytes in patients with AD as well as the presence of PAD2 and PAD4 in hippocampal neurons and neurons from the entorhinal cortex [60, 61, 69]. The different amino acid sequences for the Aβ peptides used in this study are shown in figure 1.2.
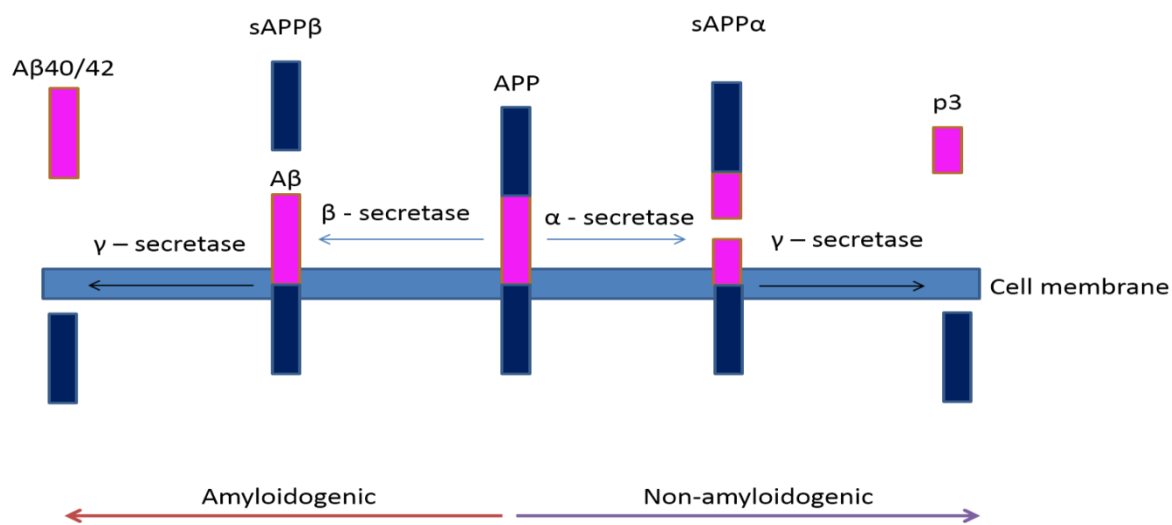
Figure 1 . 1: Processing of Aβ

The processing of amyloid beta by secretases to form the amyloidogenic and non-amyloidogenic products. For the release of Aβ APP is first cleaved by β-secretase followed by γ-secretase. [513]

Wildtype	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
AβpE3	3-pEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβcit5	DAEF-cit-HDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
AβpE3cit5	3-pEF-cit-HDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

Figure 1 . 2: Four amino acid sequences of Aβ

Four Aβ peptides were used in this study. The first is the unmodified Aβ 1-42 peptide, the second is truncated and has a common modification pyro-glutamate (AβpE3 3-42), the third has a recently discovered modification for Aβ (Aβcit5 1-42) and the final one had both modifications (AβpE3cit5 3-42)

All forms of the Aβ peptide are released from the membrane as monomers. Very little is known about the action and structure of Aβ monomers due to its propensity to aggregate, although it has been proposed to have physiological roles [70, 71]. The process through which Aβ aggregates is known as fibrillogenesis and is one of the properties of Aβ that has been suggested to be implicated in AD, both through the formation of Aβ plaques as well as the potential action of its intermediates. Furthermore it has been proposed that PTM variants of Aβ have altered aggregative properties compared with the unmodified form, for example AβpE3 has been reported to have an increased propensity to aggregate [63, 72-76].

1.2.1 Amyloid Beta Fibrillogenesis

Aβ has several properties that have all contributed to the interest in it over the last couple of decades. One of these properties is its ability to form a variety of aggregates culminating in the insoluble fibrils that form extracellular plaques. The exact process by which Aβ aggregates is not entirely clear; however possible processes have been hypothesised. A wide variety of techniques have been employed to try and find a more definitive explanation of how Aβ aggregates including: Solid state NMR, electron microscopy (EM), X-ray fibre diffraction, Fourier transform infra-red spectroscopy (FTIR), circular dichromatism (CD), sedimentation analysis and quasi-elastic light scattering (QLS) [32, 77-81].

It has been reported that the unaggregated monomers of Aβ have an α-helix conformation but in aggregates of the peptide the conformation is predominately β-sheet. It has been proposed that this conformation shift

towards β-sheet is responsible for the aggregation of Aβ peptides and the N-terminal is thought to be important in its initiation. One proposed mechanism for the conformational switch involves the destabilisation of the α-helix. This may be due to the deprotonation of the side chains: Asp^7, Glu^{11}, Glu^{22} and Asp^{23} at a pH higher than 4 as well as the protonation of $His^{6, 13 \text{ and } 14}$ [82-85].

It is now widely accepted that Aβ is produced as monomers released from membranes after the processing of APP [86]. It is also believed that a variety of different structures are also produced during fibrillogenesis before mature fibrils are formed. The initial phase of fibrillogenesis is known as the lag phase, it is a thermodynamically unfavourable process that involves the formation of nuclei from monomers. This is followed by the growth phase which occurs rapidly to form higher order aggregates, including oligomers and protofibrils. Oligomers is an umbrella term that encompasses Aβ-derived diffusible ligands (ADDLs) [87-89], globulomers [90, 91], Aβ*56 [92-94] and Aβ-oligomers all of which have a varying structure. It has been proposed that some of these oligomeric structures, mainly globulomers and ADDLs, are the aggregational end-point [89, 95-98] whereas others such as Aβ-oligomers are precursors to mature fibrils [85, 99].

Oligomers appear as small globular structures typically made up of 15-20 monomers with a molecular weight greater than 40kDa [94]. It has often been reported that these structures are the earliest detectable morphology of the peptide due to its ability to aggregate [3, 100] and the presence of them appears to be equilibrium with the higher order aggregates known as

protofibrils. Some studies have shown that oligomers align and fuse together to form these small protofibrils [3].

Experimental techniques, including electron microscopy and NMR, have shown that protofibrils are flexible fibres approximately 2.7-4.5nm in diameter and are around 40% of the size of full-length fibrils. They have a beaded structure that elongates throughout aggregation to form full-length fibrils and have been observed during the aggregation of both Aβ40 and Aβ42 [85, 101-103]. In addition it has been observed that protofibrils have an extensive β-sheet rich, un-branched structure and become more rigid as they convert to fibrils. The mechanism by which protofibrils have been proposed to elongate remains to be fully elucidated, however, lateral association and end-to-end association are two methods that are hypothesised to be involved [3, 103-105].

As the protofibrils continue to associate and elongate they eventually form full-length, mature fibrils. These are insoluble, unbranching fibres that are approximately 7-10nm in diameter, with a molecular weight of 150-250kDa. X-ray diffraction and solid-state NMR studies providing further information about the structure of Aβ-fibrils show that they have a β-turn-β conformation as well as a sharp reflection at 4.7Å in the same direction as the fibre and diffuse reflection perpendicular to the fibre direction at 10 and 11Å [101, 106, 107]. It is believed that it is these fibrils that make the extracellular plaques observed in AD.

Due to a relatively high presence of PTM variants of Aβ in the brains of people with AD, studies have also been performed to look at differences in

the aggregation of some of these peptides. One peptide that has been highly researched is the AβpE3 peptide. It has been reported that this peptide has increased aggregation propensity, potentially due to an increase in hydrophobicity caused by the loss of charge to the peptide during the truncation of the peptide [75, 108]. Despite the proposed increase in the propensity to aggregate, the final structure of the mature fibrils has been reported to be the same/similar to the unmodified form of the peptide [74, 109] (data in unpublished manuscript). In contrast, there are no current studies looking into the effect of the citrulline modification on the aggregation kinetics or the structure of the peptide. However, due to the changes in the peptide, for example the loss of a charged arginine for a neutral citrulline, could have potential effects. The differences observed in the peptides could also mean that they have different effects in the disease.

Monomers   Oligomers   Protofibrils   Mature fibrils
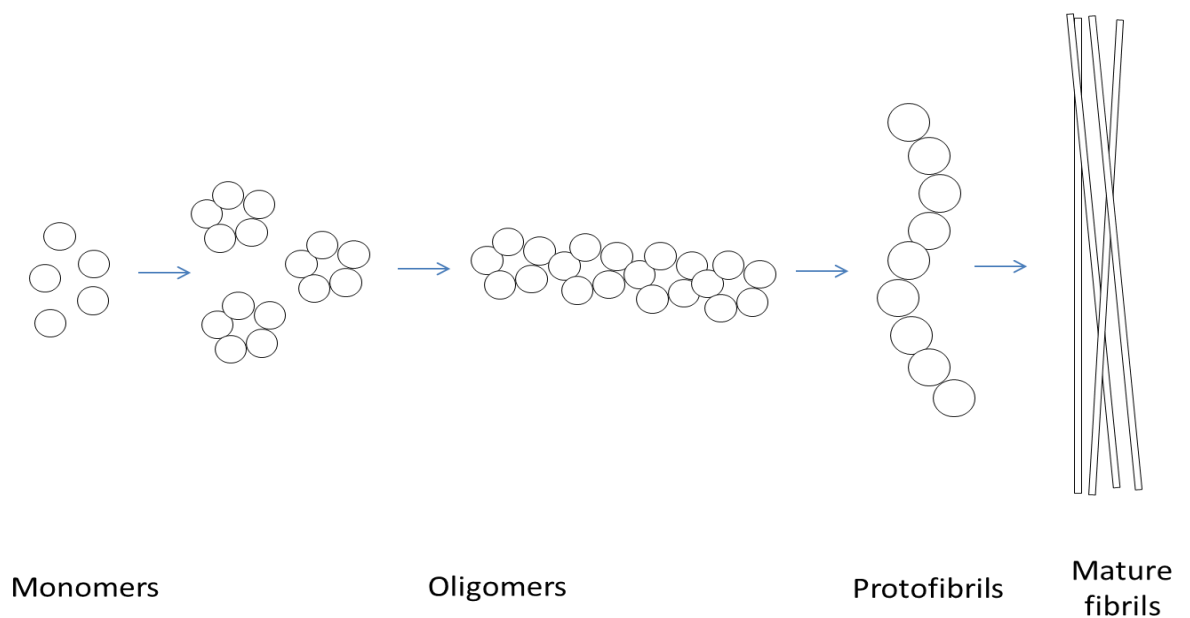
Figure 1 . 3: Aggregation of Aβ peptide

The process of aggregation from monomers to mature fibrils. The peptide is released from the membrane as monomers then aggregates to form oligomers, protofibrils and finally mature insoluble fibrils. [514]

1.2.2 The amyloid cascade hypothesis

The amyloid cascade hypothesis was originally proposed in 1992 by Hardy and Higgins and posited that Aβ plays a central role in AD progression. The importance of Aβ stems from the discovery that extracellular plaques in AD brains are made of deposits of the peptide. The hypothesis suggested that these deposits are the causative agent of AD pathology. Additionally, intracellular neurofibrillary tangles, cell loss, vascular damage and dementia occur as a direct result of Aβ deposition [25].

The amyloid cascade hypothesis is based on the idea that APP processing is the initiating factor in AD pathogenesis leading to the release and aggregation of Aβ, in particular Aβ42. Aβ42 has been shown to be the more amyloidogenic and neurotoxic isoform of Aβ when compared to Aβ40. The role of APP processing in the pathogenesis of AD has been backed up by a range of evidence. For example, many people with Down's syndrome (DS) develop advanced AD within the first 40 years of life. This is thought to be due to people with DS having trisomy 21, the chromosome on which the APP gene is located. Furthermore, in familial AD the three missense mutations that have been implicated in AD causation, *APP, PSEN1* and *PSEN2*, have all been shown to increase the ratio of Aβ42:Aβ40, as well as the APP mutation increasing the production of Aβ overall [5, 110-113].

Despite the evidence to suggest that fibrillar deposits of Aβ are responsible for the progression of AD, there are a lot of criticisms of the hypothesis. These have mainly highlighted discrepancies between the presence of fibrillar Aβ and symptoms of AD. For example, deposits of fibrillar Aβ cannot explain

the specificity of neuron loss in the brain [27]. There is also poor correlation between cognitive decline and the presence of fibrillar Aβ [114]. It has therefore been proposed that fibrillar Aβ may not be a causative agent in AD as had previously been suggested. Attention, however, has still remained on Aβ in AD research but the focus is now on pre-fibrillar, oligomeric species of Aβ.

There is a far stronger correlation for oligomeric species of Aβ with specific neuronal death, in the cerebral cortex and hippocampus, as well as with the cognitive decline associated with the disease [114]. Additionally, the oligomeric species of Aβ is more neurotoxic than fibrillar Aβ and there is also evidence that oligomeric Aβ can cause long term potentiation (LTP) [27, 95], a revised version of the amyloid cascade hypothesis is shown in Fig 1.3. However, although it is now widely accepted that oligomers are more likely be involved in the cause of AD it does not rule out the role that fibrillar forms of the peptide may play in other aspects of the disease, such as in the widespread inflammation observed in people with AD [23, 115, 116].

Aβ is produced both by people who are healthy and by people with AD, however it is believed that in healthy individuals there is a balance between the amount of Aβ that is produced and the amount that is removed, meaning that the abnormal build-up associated with the disease does not occur. It has been proposed that the amount of Aβ produced by healthy people and people with AD is very similar, but there is a reduction in the removal of the peptide from the brain causing an increase in patients [117]. There is evidence to suggest that the immune system is involved in removal of Aβ from the brain

and that changes in the ability of the immune system to perform this role may

be responsible for an increase in the peptide [118-123]. Furthermore, the

immune system has been shown to function less efficiently in older age which

may additionally contribute to the reduced removal of Aβ [124-126].

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┌─────────────────────────┐
│  Failure of Aβ to be     │
│  cleared causing         │
│  increase in the brain   │
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┌─────────────────────────┐        ┌──────────────────────┐
│  Increase in oligomeric  │───────▶│  Impaired LTP,        │
│  Aβ species in the brain │        │  increase in          │
└─────────────────────────┘        │  cognitive decline,   │
            │                       │  neuronal cell death  │
            ▼                       └──────────────────────┘
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│  Aggregation of the      │
│  peptide to higher       │
│  order aggregates        │
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│ Deposition   │◀─│ Formation and       │─▶│ Triggering of    │
│ of hyper-    │  │ deposition of Aβ    │  │ inflammation,    │
│ phosphory-   │  │ fibrils in          │  │ oxidative stress │
│ lated Tau    │  │ insoluble plaques   │  │                  │
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                  │ Neuronal            │
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                  │ death,              │
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Figure 1 . 4: An update on the amyloid cascade hypothesis

An updated version of the original amyloid cascade hypothesis, proposed by Selkoe and Hardy. Based on recent research into the role of oligomers and fibrils in AD, modified from

[127]

1.3 The immune system

The immune system is a combination of specialised cells and molecules with the role of protecting against infection and damage. There are two overlapping systems that make up the immune response, the innate immune system and the adaptive, or acquired, immune system [128]. Each of the two systems has specific roles to play within the immune response and although they are thought to generally work separately of each other there is lots of interaction between them.

1.3.1 Innate immunity

The innate immune response is the first and most rapid line of defence against infection and includes anatomical barriers and molecular and cellular components [128-131]. There are a number of anatomical barriers that exist to help prevent the entrance of pathogens from outside into the body. There are a variety of epithelial surfaces, such as the skin, surfaces of the gastrointestinal (G.I) and respiratory tract and the cornea, which play a major role as barriers. One feature of epithelial cells is the formation of tight junction between cells which prevents easy entry of microbes through the surface. In addition different surfaces have developed different ways to reduce the risk of microbial infection, for example desquamation of the skin removes infectious agents adhering to the skin surface. The skin also provides an unsuitable environment for bacteria to survive, by secreting sebum, a complex group of oils that are known to have innate antimicrobial activity [132]. Inside the body epithelial surfaces such as those in the G.I and respiratory tract are coated in mucus. This mucus contains proteins associated with the innate

immune system that are able to scavenge, kill and immobilise infectious agents preventing them from both adhering to the epithelium and from moving around inside the body. Specialised hair cells in the lung, known as cilia, are able to 'beat' microbes from the lung and along with coughing remove them from the body [133]. Mucus plays a similar role in the G.I tract, where the action of peristalsis is able to move bacteria and other infectious agents out of the body [134]. The epithelial cells of the cornea are able to secrete cytokines to activate innate immune defences, IL-1α is passively released from corneal epithelial cells upon injury or infection of the membrane [135, 136]. The body has further barriers, such as tears and saliva, which prevent the entrance of microbes through the eyes and mouth.

In addition to epithelial cells, endothelial cells are also thought to be important in immunity. It has been reported that endothelial cells act as sentinels for gram-negative bacteria and are able to recruit cells of the innate immune system such as neutrophils. In addition endothelial cells have been observed to express toll-like receptor 4 (TLR4) and are activated in responses to lipopolysaccharide (LPS) [137]. It is not only TLR4 that has been found to be expressed on endothelial cells, a number of studies have observed that TLR2, TLR9 and the co-receptors CD14 and MD2 as well as the adaptor protein myeloid differentiation primary response protein 88 (MyD88) [138-140].

In the event that microbes are able to get past the anatomical barriers there are several cellular and molecular processes in the innate immune system that are able to respond to the infection. The main cells involved in this type of

immune response are monocytes, macrophages, dendritic cells and neutrophils. Innate immunity is not considered to be antigen-specific, in that it responds equally well to a variety of organisms and responses to specific antigens do not improve upon repeated exposure, as in the case of the adaptive immune system [128].

The innate immune system has a number of roles in the immune response, one of which is causing inflammation. Immune cells such as macrophages are able to detect the conserved motifs found on organisms, known as pathogen associated molecular patterns (PAMPs), via pattern recognition receptors [141-143]. The activation of these receptors leads to downstream signalling cascades, involving adapter molecules, kinases and transcription factors, resulting in the production of various molecules such as cytokines, chemokines and cell adhesion molecules.

In addition to inflammation an important role of the innate immune system is the phagocytosis of pathogens. Macrophages are the main phagocytic immune cell, although they are not the only cell able to perform this function. The main role of phagocytosis is to endocytose and digest invading pathogens and debris, to remove them from the body. In addition to removing pathogens, phagocytosis also breaks them down so they can be displayed on antigen presenting cells (APCs) in order to activate cells of the adaptive immune system.

In addition to activating downstream signalling cascades within cells, activation of immune cells also activates the complement system extracellularly. This contributes to the immune response in a variety of ways:

the recruitment of immune cells such as macrophages for phagocytosis, aiding in clearance of immune complexes and apoptotic cells, marking bacteria for phagocytosis (opsonisation) and the regulation of antibodies.

1.3.6 Toll-like receptors

Toll-like receptors (TLRs) are important immune receptors that are involved in both the innate and adaptive immune systems [144-146]. Their primary role is as pattern recognition receptors (PRRs) involved in the recognition of PAMPs from invading pathogens or danger associated molecular patterns (DAMPs) from damaged tissue [147-149]. In general the activation of these receptors leads to the release of inflammatory mediators, however, additional to the innate immune response they are also involved in linking the innate immune system with the activation of the adaptive immune system [145, 146, 150].

There are at least 11 TLRs, however not all of them are functional in humans, for example TLR11. All TLRs are type1 transmembrane receptors and contain an extracellular domain with leucine-rich repeats and a cytosolic Toll/IL-1 receptor (TIR) domain. Each domain has a specific role in TLR activation: The extracellular domain is responsible for the recognition of PAMP/DAMPs and the cytosolic domain for the initiation of downstream signalling [143, 151]. Despite the general structure being the same not all receptors are expressed in the same part of the cells and there are differences in the PAMPs/DAMPs they recognize. TLR1-2 and TLR4-6 are expressed on the cell surface and mainly recognise microbial membrane components

whereas TLR3 and 7-9 are expressed on intracellular structures, such as the endoplasmic reticulum, and recognise microbial nucleic acids [143].

The majority of TLRs form homodimers; however this is not the case for TLR2 which forms heterodimers with either TLR1 or TLR6. Some TLRs also require the involvement of co-stimulatory molecules in order to stimulate downstream events. In the case of TLR4, MD-2 and CD14 are both needed for responses to be seen [152] and in some cases CD14 and CD36 have been shown to play a role in activation of both TLR1/2 and TLR2/6 [153-157]. Collectively TLRs are able to respond to all known infectious agents [158], although different agents are recognised by different receptors. For example, lipopolysaccharide (LPS) is recognised by TLR4 and lipopeptides by TLR1/2 and TLR2/6. A more detailed description of ligands recognised by TLRs is shown in table 1 [150, 158].

Table 1 . 1: Overview of toll-like receptors, their origin and ligands

[150, 158]

TLR	Origin	Ligand
TLR1/2, TLR2/6	Gram-positive bacteria, fungi	Lipopeptides: e.g. Pam_2CSK_4
TLR3	Viruses	Poly I:C, dsRNA
TLR4	Gram-negative bacteria	LPS
TLR5	Bacterial Flagellin	Flagellin
TLR7	Viruses	Small anti-viral compounds: ssRNA, resiquimod, imiquimod, loxoribine
TLR8	Viruses	Small anti-viral compounds
TLR9	Bacteria	Unmethylated DNA, CpG-DNA

There are two signalling pathways that can be activated by TLR stimulation, based on the adaptor molecules involved. The two pathways are known as MyD88-dependent and MyD88-independent pathways. All TLRs except TLR3 are able to activate the MyD88-dependent pathway, but only TLR3 and in some cases TLR4 activate the MyD88-indenpendent pathway [143, 159].

The binding of a ligand to its TLRs results in MyD88 associating with the cytosolic part of the TLR and sequentially recruiting and activating IL-1 receptor-associated kinase (IRAK) 4, 1 and 2. IRAK1 and 2 then associates with TNF receptor-associated factor 6 (TRAF6), which acts as ubiquitin protein ligase (E3) and catalyses the synthesis of K63. K63 binds to transforming growth factor-activated protein kinase 1 (TAK1), TAK1 – binding protein (TAB) 2 and 3 and the IκB kinase (IKK) subunit, NF-κB essential modifier (NEMO). The recruitment of TAB2 and TAB3 to TRAF6 activates TAK1, which activates either the IKK or mitogen-activated protein kinase (MAPK) pathway.

The IKK pathway is activated by TAK1 coupling to the IKK complex (made up of NEMO, IKKα and IKKβ) causing the phosphorylation and therefore activation of the IKK complex. This activates NF-κB allowing its nuclear translocation and subsequent production of cytokines, including TNF-α and IL-1β.

TAK1 is also able to phosphorylate MAPK kinases (MKKs) leading to phosphorylation of either p38 or c-Jun N-terminal kinase (JNK). These both cause the activation of activator protein-1 (AP-1) and therefore the production of cytokines.

The second pathway activated by the binding of ligands to TLR3 involves the adaptor protein TIR domain-containing adaptor inducing IFN-β (TRIF). TRIF recruits receptor interacting protein 1 (RIP1) which forms a complex with TRAF6, activating TAK1. This leads to IKK-mediated activation of NF-κB and the MAPK pathway. TRIF is also able to recruit TRAF3 leading to the activation of a complex containing TANK-binding protein 1 (TBK1) and IKKε. This causes the phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF3), leading to the production of type 1 IFN [143, 160-162].

It has been proposed that the reason for the diversity of PAMPs that are recognised by TLRs is due to differences in the LRR domain. There is believed to be a consensus LRR that is made up of a series of amino acids and repeats throughout the extracellular domain. Further to this, it has been suggested that non-consensus LRRs that contain different insertions after residue 10 and 15 are responsible for the ligand specificity [118, 163]. These insertions provide binding sites for different ligands, for example in TLR5 LRR insertions provide the binding site for flagellin.

It has been reported that TLRs are involved in the recognition of Aβ in patients with AD, the two receptor types most implicated in this are TLR2 and TLR4.
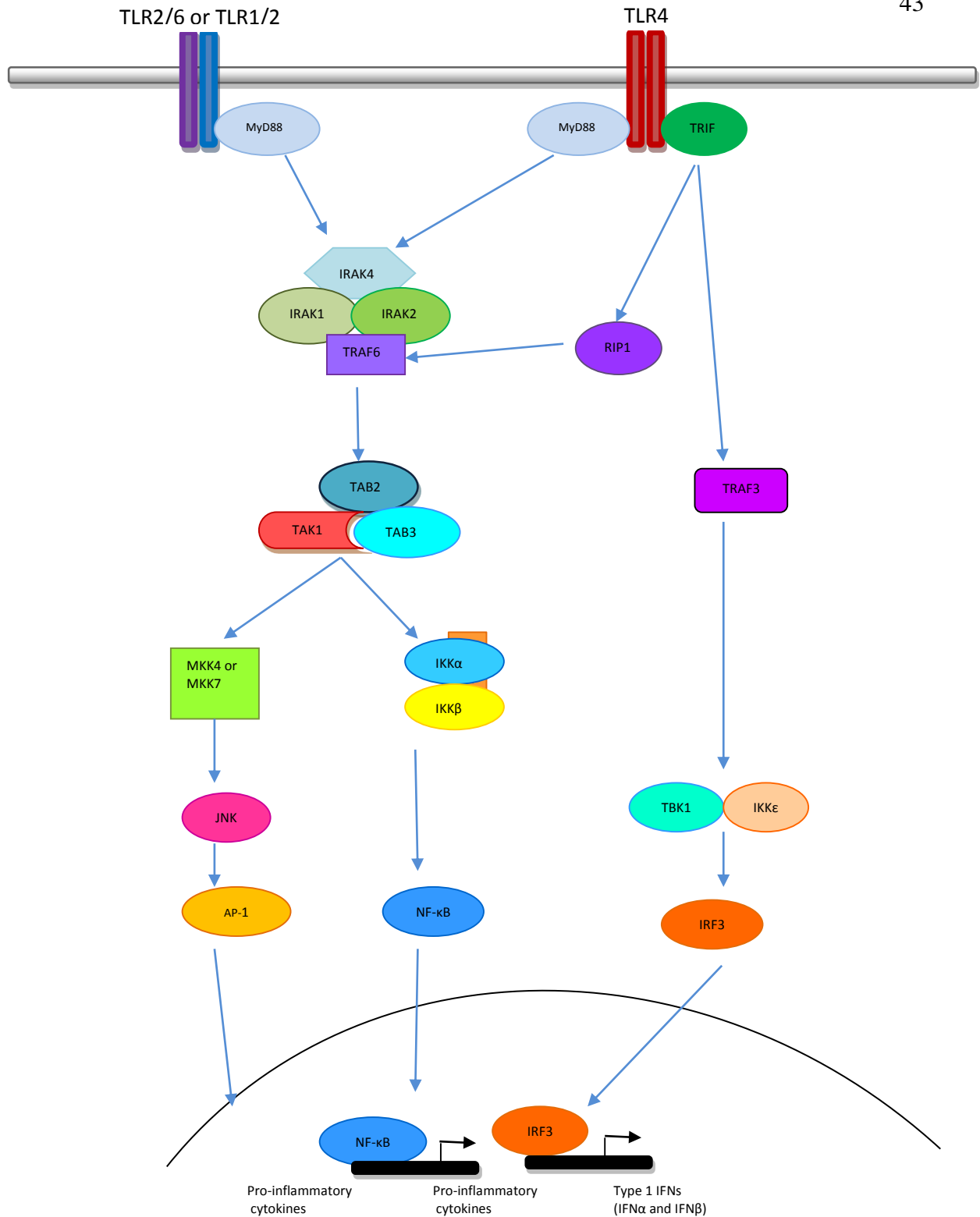
Figure 1 . 5: TLR signalling cascade

An overview of the main signalling molecules and complexes formed after TLR activation. Showing both the TLR2 and TLR4 signalling. For TLR4 both the MyD88 dependent and MyD88 independent pathways are shown. Adapted from a similar diagram from Takeda et al.

[164]

1.3.7 Toll-like receptor 2

TLR2 is often thought to be able to recognise the greatest variety of microorganisms, originally it was believed that LPS was the ligand for TLR2 however subsequent studies showed that it was actually due to contaminating bacterial lipoproteins in the LPS preparations [165]. These lipoproteins are from sources such as gram-negative bacteria, mycoplasma and spirochetes in addition there is a vast array of microbial products including: peptidoglycan and lipoteichoic acid (LTA) from gram-positive bacteria [166-168], Zymosan [169, 170], lipoarabinomannan [171, 172] and glycolipids from *Treponema maltophilum* [173, 174].

 TLR2 is also able to recognise some ligands as a homodimer. In particular, ligands such as LTA, a component of the Gram-positive cell wall, lipoarabinomannan, a mycobacterial cell wall component and atypical LPS produced by *Legionella, Porphyromonas gingivitis* and *Leptospirainterrogans*. The properties of the atypical LPSs differ to that of 'normal' LPSs in both function and structure, notably in the number of acyl chains in the lipid A component which may account for the recognition by either TLR2 or TLR4 [164].

The ability of TLR2 to recognise such a variety of microbial products may be due to it forming heterodimers with other TLRs, TLR1 and TLR6. There is evidence that the two heterodimers are involved in the recognition of different ligands, for example, TLR1/2 recognizes lipoproteins from mycobacteria and menigococci whereas TLR2/6 recognizes peptidoglycans or lipoproteins from mycoplasma. One proposed difference between the two heterodimers is that

TLR1/2 is involved in triacylated lipopeptide recognition but TLR2/6 is involved in diacylated lipopeptide recognition.

Further to the ability of TLR2 to recognise diverse bacterial components it is also able to recognize fungal and protozoan molecules such as Zymosan. The structural differences between these molecules and bacterial ones show how diverse the recognition of TLR2 is. It is likely that this diversity is due to the combination of LRR domains on each TLR [163]. For example, it has been shown that the ability of TLR1 and 6 to discriminate between acylated lipoproteins is mediated by LRRs 9-12 [164, 175]. In addition, specific regions of LRRs in TLR2 are have been shown to be needed for certain response [176, 177]. The studies into the specific structure of these LRR regions on both TLR2 and its two heterodimeric partners have suggested that heterodimers bind ligands cooperatively and may therefore be able to increase the diversity of their PAMP specificities.

It has also been demonstrated that TLR2 is able to utilise additional receptors that are involved in PAMP recognition. Traditionally, the CD14 co-receptor has been associated with signalling through TLR4, however there is also evidence to suggest that it is involved in recognition by TLR2. CD14 has been shown to enhance TLR2 mediated responses to several PAMPs including, peptidoglycans and lipopeptides, potentially by transferring them to TLR2 [165, 178, 179]. In addition to CD14 it has also been suggested that CD36, a type 2 scavenger protein, is needed for the effective activation of NF-κB and production of cytokines through TLR2/6 mediated recognition of diacylated lipopeptides and LTA [156, 163].

1.3.8 Toll-like receptor 4

One of the most well characterised TLRs is TLR4, it is mostly thought of as the receptor for LPS, however it has been shown to respond to a variety of other ligands as well. For example, fibronectin, hyaluronic acid and heparin sulphate as well as several heat shock proteins such as HSP 60. However, it has also been reported that these ligands require a much higher concentration in order to elicit responses observed with much smaller concentrations of LPS, it has even been suggested that responses observed to other ligands may be due to LPS contamination [164].

LPS is a major component of the outer surface membrane in Gram-negative bacteria and are thought to be the most potent activator of all known TLR ligands [180]. Even trace amounts of LPS are able to cause the production of inflammatory mediators such as TNFα and IL-1β. The structure of all forms of LPS are believed to be comparable and are composed of three regions: lipid A, an oligosaccharide core and an O-antigen [181, 182]. The O-specific chain is made up of repeating oligosaccharide units, usually up to 50, that maintain the hydrophilic domain of the molecule. A high level of variation occurs between species, therefore the main determinants for recognition resides in this domain [181, 182]. The oligosaccharide core is made up of an outer core found closest to the O-antigen domain and the inner core found proximal to the lipid A domain. The two can be distinguished by their monosaccharide composition, with the inner core showing much less variability [182]. The last region, and arguably the most important in immune responses, is the lipid A domain. It was in 1975 that Galanos et al showed that

lipid A exhibited strong endotoxin properties in the absence of the rest of the LPS molecule. Lipid A is the hydrophobic region of LPS and acts as the anchor to the bacterial membrane, the rest of the molecule then projects outwards from the membrane. It is composed of a diglucosamine backbone with ester-linked and amide-linked fatty acid chains.

The first step in LPS signalling through TLR4 is the binding of LPS to the LPS-binding protein (LPB), an acute phase protein that circulates in the blood and forms high affinity complexes with the lipid A domain of LPS [183]. The primary role of LPB appears to be to transfer LPS to the TLR4 receptor complex, however in doing so it first forms a complex with the co-receptor CD14 which allows LPS to be transferred to TLR4 complex. The TLR4 complex consists of TLR4 and the secreted glycoprotein MD-2, which is believed to be essential for the activation of TLR4 by LPS. It has been proposed that LPB-bound LPS is transferred to MD-2 bound to the membrane surface, MD-2 then associates with the TLR4 via the extracellular LRR domain causing the oligomerisation of TLR4 and the subsequent activation of the signalling cascade [183].

1.3.2 Adaptive Immunity

In contrast to the innate immune system, the adaptive immune system is regarded as more evolved and complex. One difference is the cell type involved in the response; the two main cell types are T-cells and B-cells, so called because of their maturation in the thymus or the bone marrow respectively. The two cells types play different roles in the immune response. B-cells are responsible for the release of immunoglobulins (Ig), or antibodies,

that specifically target the invading pathogen. There are a number of different Igs: IgG, IgM, IgA, IgE and IgD, named because of amino acid differences. The production of an antibody is considered to be the humoral response of the immune system. On the other hand, T-cells are thought to be responsible for the cell-mediated branch of the adaptive immune response [184-186].

It is possible to differentiate T-cells in different ways, for example there are populations of T-cells that have specific roles within the adaptive immune system, including: helper T-cells, cytotoxic T-cells and regulatory T-cells. T-cells can also be identified by their level of maturity, for example naive T-cells, which have not been exposed to any antigens, and antigen-experience T-cells, such as effector and memory T-cells [186].

T-cells are considered to be 'naive' until they come into contact with an antigen for which they have a high affinity. Before they become stimulated naive T-cells remain in resting state and are considered to be small lymphocytes with a diameter of 8-10μM.

Helper T-cells, also referred to as CD4$^+$ T-cells because of their expression of CD4$^+$ on the cell surface, have a number of different roles in the immune response, for example to help B-cells secrete antibodies, macrophages to phagocytose microbes and activate cytotoxic T-cells. There are several types of helper T-cells: Th1, Th2 and Th17. When activated Th1 cells secret interferon gamma (IFNγ), interleukin (IL)-2 and tumour necrosis factor alpha (TNFα) for macrophage activation and cell-mediated immunity. Th2 cells release IL-4, IL-5, IL-10 and IL-13 and are responsible for inducing the release of antibodies from B-cells. Th17 cells are thought to secrete IL-17 and

are able to recruit cells such as neutrophils to sites of infection [187-191].

Cytotoxic T-cells, also known as CD8^{+} cells because of their surface

expression of CD8, are thought to be responsible for the elimination of

intracellular microbes by killing infected cells [192]. Regulatory T-cells play

a very important role in maintaining immune homeostasis, these cells are

thought to be involved in peripheral tolerance to self-antigens, preventing

autoimmune disease and reducing the effect of chronic inflammatory disease

such as asthma. It has been hypothesised that there are a number of different

mechanisms through which Tregs are able to suppress immune responses: the

production of cytokines such as IL-10 and TGF-β, by cytolysis, via the

actions of granzyme A, by metabolic disruption or by suppressing dendritic

cell (DC) targeting, potentially by disrupting the maturation or function of the

DCs [193-195].

The effective activation of naive T-cells to effector T-cells requires the

antigen to be presented via APCs. In order for this to occur, antigen in

processed by the APC and presented on a complex known as major

histocompatability complexes (MHCs), the presented antigen is then able to

bind to the T-cell receptor. In addition to the antigen/TCR binding co-

stimulatory molecules such as CD80 and CD86 are also required [72, 196]

There are two classes of MHC, MHC I and MHC II, both of which are

polymorphic and therefore bind to a variety of peptides. Each class of MHC is

expressed on different cells types and is able to present antigen to different T-

cells. Firstly, MHC II molecules are expressed on APCs, including DCs,

macrophages and B-cells. The processed antigen presented by class II

complexes is then recognised via T-cell receptors (TCRs) on CD4^{+} (helper) T-cells. They are therefore responsible for the priming of naive T-cells as well as establishing specific immunity. In contrast, MHC I molecules are expressed on all nucleated cells and are recognised by CD8^{+} (cytotoxic) T-cells and natural killer (NK) cells. The binding of MHC I/antigen to cytotoxic T-cells leads to the activation of these cells and killing of the target cell via a number of mechanisms including the induction of cytotoxins that cause the apoptosis of the target cell [196-198].

In addition to the type of cell that the MHC molecules can activate, there are also differences in how pathogens or peptides are processed before they are presented to TCRs. MHC II molecules typically express antigens from outside the cell and process pathogens through an exogenous pathway. Cells producing MHC II, APCs such as DCs and macrophages, engulf the extracellular antigen and form phagosomes containing the antigen [199-202]. Phagosomes fuse with lysosomes inside the cells to form phagolysosomes, within the phagolysosome proteins undergo unfolding and reduction of disulphide bonds by gamma-IFN-inducible lysosomal thiol reductase (GILT) [201, 203]. After which proteinases, which work best in the acidic conditions within the phagolysosome, are able to break down the peptides [197, 199, 200, 204]. The MHC II molecules themselves are synthesised by ribosomes in the rough endoplasmic reticulum (ER), they are associated with the invariant chain (Ii) which prevents premature binding by peptides within the ER. Ii also guides the MHC II molecule through the golgi apparatus to be sorted into endosomes or phagosomes [205-208]. The Ii-derived class II invariant chain peptide (CLIP) remain associated with the MHC II molecule to ensure there is

no premature binding, HLA-DM is able to remove CLIP when MHC II comes into contact with its antigen [208-211]. The endosome containing the MHC II molecules fuses with the phagolysosome where the antigen comes into contact with MHC II binding grove. The peptide loaded MHC II molecules are then exocytosed to the surface of the APC where they are able to present the antigen to TCRs expressed on CD4^{+} T-cells [210, 212-214].

In contrast, MHC I molecules are associated with the presentation of cytosolic peptides [204]. Peptides to be degraded are first covalently modified by ubiquitination, which targets the protein for degradation and the proteins are then degraded by a proteosome complex in the cytosol. Once peptides have been processed they are translocated from the cytosol to the lumen of the ER, where MHC I molecules are synthesised, by the transporter associated with antigen presentation (TAP). In addition to transporting antigen, TAP is also involved in the binding of the antigen to MHC I molecules. Once peptide has bound to the MHC I molecule is transported through the Golgi apparatus to the plasma membrane [202, 204, 215, 216].

There are also structural differences between MHC I and MHC II molecules that allow the binding of different length antigens in the binding groove. The MHC I molecule is composed of an α-subunit and a β2-microglobulin subunit non-covalently linked, and the antigen. The N-terminal of the molecules has the α1 and α2 segments of the α-subunit which interact to form the binding groove of MHC I, these are also the polymorphic segments of molecules and are therefore responsible for the difference seen in different MHC I alleles. The binding groove recognises antigen of 8-11 amino acids and has closed

ends, so antigens with a longer length are not able to bind. The α3 segment of the α-subunit and β2m are invariant among MHC I alleles and are responsible for recognition by CD8$^+$ cells.

On the other hand MHC II molecules are composed of two non-covalently linked polypeptide chains. The α1 and β1 segments of these chains interact to form the binding groove of the molecule and the ends of the groove are open meaning that peptides containing 30 or more residues are able to bind, however MHC II molecules typical recognise antigens between 13 and 25 amino acids in length. Like in MHC I molecules the α1 and β1 segments are the polymorphic regions of the MHC II molecule and therefore provide variation among the different alleles, the α2 and β2 segments of the polypeptide chains are the invariant segments and are responsible for recognition by CD4$^+$ cells [59, 63, 217].

1.3.3 T-cell activation

T-cells are derived from bone marrow hematopoietic stem cells that migrate from the bone marrow to the thymus where they mature [218]. The thymus is a lymphoid organ located in the anterior superior mediastinum, in front of the heart and behind the sternum [219]. It is composed of two histologically distinct areas: the central medulla and the peripheral cortex. The thymus plays an important role in generating circulating T-cells and its two different areas are involved in different aspects of this process. The initial steps in T-cell maturation occur in the cortex of the thymus, it is here that T-cell receptor gene rearrangement and positive selective takes place. The later events then

occur in the medulla, the main one of which is negative selection against self-reactive T-cells [220, 221].

The mature T-cells are released from the thymus and until they come into contact with an antigen they are considered to be immunologically naive [222]. Naive T-cells circulate in the blood where their homeostasis is maintained by a number of extrinsic cues such as TCR engagement by self-peptide MHC complexes and IL-7 stimulation of IL-7 receptor (IL-7R) [223].

Naive T-cells express TCRs, which are responsible for antigen recognition. They consist of two different polypeptide chains, in two combinations. The vast majority of TCRs, 95%, are made up of TCR-α and TCR-β chains whereas the remaining 5% of TCRs are comprised of TCR-δ and TCR-γ. The two chains are linked by a disulphide bond and share a similar structure with the Fab fragment of immunoglobulin molecules. The chains have a variable (V) region found at the amino-terminal and a constant (C) region, in addition there is a cysteine residue found in a short stalk segment that is responsible for forming an interchain disulphide bond. Within the variable region of the chains there are areas of hypervariability that are responsible for the antigen specificity of the TCR and each individual T-cell expresses TCRs that are all able to recognize the same antigen meaning each T-cell is specific for a single antigen [60, 224-226]. In spite of this theory it has been shown that some T-cells are able to express more than one type of TCR allowing T-cells to recognise more than one antigen [227, 228]

Naive T-cells can be activated when they encounter an MHC-bound antigen for which their TCR has a high affinity and the recognition of this antigen

results in the initiation of an immune response. Activation of the TCR occurs at an interface, known as the immunological synapse, between the TCR and the APC. In addition to the recognition of antigen by TCR, a CD3 molecule is also required to associate with the TCR and initiate the intracellular signalling cascade in the T-cell [229]. In order for this initiation to occur it is critical that the TCR associates with immunoreceptor tyrosine-based activation motif (ITAM), found in the cytoplasmic tails of CD3 [230].

After the activation of T-cells the immune system is able to produce two distinct subsets of T-cells, those responsible for the short lived effector phase and those that are responsible for long-lived memory in case of re-challenge with the same antigen. These two subsets are commonly referred to as effector and memory T-cells [231].

Upon antigen stimulation, T-cells undergo clonal expansion, a process involving several rounds of cell-division [232]. Activated T-cells produce IL-2, which is known to promote the expansion of antigen specific T-cells [233]. There is an initiation phase within the first 24h of activation after which T-cells rapidly proliferate and reach a maximum population size after approximately a week. During this proliferation stage T-cells differentiate into effector T-cells, to combat the immediate infection [232]. There is further division of effector T-cells to become CD8$^+$ cytotoxic T-cells or CD4$^+$ helper T-cells leading to the production of cytokines and the killing of infected cells or pathogens (94). After the threat from the invading pathogen has been removed the effector cells go through a contraction phase whereby the majority of effector T-cells are killed by apoptosis, leaving a small proportion, 5-10%, of cells to become memory T-cells. The remaining

memory T-cells are maintained for long periods of time after the initial infection, this is in large part due to the actions of IL-7 and IL-15. These signals are thought to be involved in supporting cell survival and proliferation [234].

1.3.4 Interactions between the adaptive and immune system

Although traditionally the two systems of the immune response were thought to be independent of the other it is now believed that that there is considerable interaction between them. Furthermore, the adaptive immune system will not function effectively without the involvement of the innate system.

It has been suggested that dendritic cells, normally associated with the innate immune system, are perhaps the most important cells in linking the two systems together. Although it has been shown that DCs and macrophages are able to act as APCs, only DCs can prime naive T-cells [235-238]. It has been observed that DCs are able to act as APCs expressing both MHC class I and class II molecules on their surface, therefore allowing them to activate both $CD4^+$ and $CD8^+$ T-cells [235, 239-241]. However, in order to fully activate T-cells it is not enough for the DCs to be able to express these molecules they also have to express co-stimulatory molecules such as CD80 and CD86 [237]. For DCs to express these co-stimulatory molecules they must have matured, which occurs after exposure to a variety of signals including: PAMPs, cytokines and tissue factors. The maturation of DCs is therefore caused by direct stimulation through PRRs, like the TLRs, or indirectly through cytokines etc. that are produced by the stimulation of the innate immune system [237]. In addition to DCs, macrophages are also able to act as APCs,

however, unlike DCs they are not good at presenting antigen to naive T-cells, however they are very effective at activation of memory T-cells.

As mention above, TCRs associate with the surface receptor CD3 in order to initiate intracellular signalling cascades. CD3 is made up of 4 chains: δ, γ and two ε. In addition to CD3, TCRs also associate with a ζ chain and the combination of all three composes the TCR complex. It is the α:β TCR that interacts with MHC-molecules, however this interaction is a weak one. In order for stronger interactions to be formed accessory molecules are needed. The two most notable molecules are CD4 and CD8, these are expressed on either helper T-cells or cytotoxic T-cells and are therefore responsible for the recognition of either MHC II or MHC I molecules respectively. It has also been reported that co-stimulatory molecules are required for efficient activation of T-cells. The interaction of co-stimulatory molecules with their ligands occurs alongside the engagement of TCRs with antigen via MHC molecules and is considered the second signal in T-cell activation. One of the most dominant co-stimulatory molecules is CD28 a molecule constitutively expressed on 80% of human T-cells (on all CD4 and 50% of CD8 T-cells). It interacts with its ligands B7.1 (CD80) and B7.2 (CD86) on APCs [242, 243] and upon activation of T-cells the expression levels of CD28 increase.

Another aspect of the innate immune system that is important in the induction of the adaptive system is the TLR. TLRs are expressed predominantly on DCs and macrophages, two vital cells in the innate immune response. Aside from being involved in the indirect activation of T-cells via DCs, there is evidence to suggest that TLRs have a more direct effect on T-cells. Studies looking at mRNA levels of TLRs have observed that many different T-cell subset

express various TLR mRNA, however there are many inconsistencies between the levels from study to study [244]. Furthermore, it appears that TLRs expressed on T-cells can influence T-cells through modulation of responses triggered by the TCR [244]. For example, TLR2 is constitutively expressed on memory T-cells and expression is induced on naive T-cells when TCRs are activated. When the TLR2 recognises a pathogen it delivers a co-stimulatory signalling resulting in the production of cytokines and proliferation of T-cells. Similar co-stimulatory effects have also been seen from other TLRs [244-246].

The inflammatory mediators produced by both the innate and adaptive immune system play an important role in the interaction between the two systems. For example, an array of chemokines produced by DCs are important in attracting different cells, such as neutrophils, effector T-cells and naive T- and B- cells [247]. In addition, the production of specific cytokines has been shown to determine whether T-cells differentiate in Th1, Th2 or Th17/Treg cells [248]. For the development of Th1 cells, IL-12 is required, for Th2 cells Il-2 and IL-4 are required and for Th17 cells TGF-β and IL-6 are required, although there is also evidence for IL-21 and IL-23 also causing differentiation to Th17 [189, 249-251]. It has been shown that PAMPs can influence which form of Th cell develops: LPS binding to TLR4 results in IL-12 production from DCs and therefore causes differentiation to Th1 cells [237], whereas lipoprotein from Gram-positive bacteria that signal through TLR1/2 induce IL-10 as well as low levels of IL-12 that favour differentiation to Th2 or Treg cells [252]. All these cytokine can be produced by cells of the

innate immune system, although the main producer of IL-2 and IL-4 are T-cells [253-255].

Furthermore, it has been shown that in order for CD8^{+} T-cells to be effectively activated they may require a third signal, in addition to the antigen and co-stimulatory molecules; IL-12 and IFNα/β have been proposed to have signal 3 activity for CD8^{+} T-cells [256]. In addition to a third signal being needed for CD8^{+} T-cell activation it has also been suggested that the cytokine IL-1 has signal 3 activity for CD4^{+} T-cells. IL-1 has been shown to significantly increase proliferation and differentiation in response to antigen by acting directly on CD4^{+} T-cells [257].

In addition to the innate immune system influencing the activation of the adaptive system there is also evidence of the reverse being true. For example, the production of cytokines by T-cells are known to interact with innate immunity. IFNγ, produced by Th1 T-cells is involved in the priming of macrophages and in the cellular maturation of DCs [258, 259]. TNFα produced by both Th1 and Th2 has been shown to activate macrophages [259] furthermore IL-4 is involved in DC and macrophage activation [260]. These are just a few examples of the interactions of cytokine produced by the adaptive immune system with the innate immune system. In addition to the action of cytokines from the adaptive immune system, there is also evidence that T-cells can have a direct effect on the activation of immune cells such as monocytes. Studies have shown that contact activation occurs between T-cells and monocytes [261-263].

1.4 Role of the immune system in AD

One of the major hallmarks of AD is widespread inflammation in the brains of people with AD. This has been seen by a dramatic increase in a host of inflammatory mediators such as: cytokines, chemokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS). In addition to the brain it has also been reported that there is evidence of increased inflammation in the periphery of people with AD [115, 264]. It is still unknown whether inflammation is a cause or a consequence of the disease. Although it is accepted that it contributes to disease progression, there is also some evidence of a beneficial role for inflammation. There is now a fairly large body of evidence that supports the hypothesis that Aβ is able to cause inflammation in the brain through the activation of the immune system

One discovery that has implicated Aβ in the activation of the innate immune system was the observation that microglia, resident immune cells of the CNS, associate with plaques of Aβ in AD brains [265-270].The associated microglia have been shown to be reactive and have been seen in both humans with AD and transgenic mouse models of the disease [265-270]. It has been proposed that microglia play a role in the clearance of Aβ from the brain through phagocytosis of the peptide in healthy individuals. Microglia are able to interact with Aβ through a surface receptor complex that promotes the clearance of the peptide. However, despite Aβ-associated microglia in patients with AD displaying an activated phenotype they are unable to effectively remove Aβ [125, 126]. There are a number of hypotheses to explain why this occurs: microglia become dysfunctional with age, there is a

higher level of microglia dystrophy in brains with an increased Aβ load, and Aβ increases microglial senescence [121, 126].

Despite evidence that there is a reduced ability of microglia to phagocytose Aβ, it is thought that Aβ is able to induce the production of proinflammatory mediators from microglia. There is evidence to suggest that different receptors are involved in Aβ mediated inflammation, such as Nod-like receptors (NLRs) and RAGE, however, probably the most implicated receptors are TLRs [119, 268, 271-273]. A large number of studies have been performed exploring the role that TLRs play in Aβ recognition, these have pointed to the involvement of two specific TLRs: TLR2 and TLR4.

Several studies have investigated the activation of TLR2 and TLR4 in response to the Aβ peptide. These studies have been done using a variety of different cells, including THP-1, a human monocytic cell line, and HEK293 as well as primary microglia and a very small number on primary human monocytes [274-279].

One compelling discovery for the involvement of TLRs, in particular TLR4, in Aβ recognition was the observation that CD14 associates with Aβ. Fassbender et al, were able to show using a variety of techniques, that CD14 associated with fibrillar Aβ but not pre-fibrillar species of the peptide, it was proposed that the β-sheet structure of the peptide may be responsible for the difference in association. They demonstrated that antibodies against CD14 and animal models deficient in CD14 caused a reduction in the activation of microglia by CD14. In addition they also found an increase in expression of CD14 on microglia in the brains of animal models of AD [280].

It is not possible that CD14 alone is able to induce an inflammatory response, as it has no cytoplasmic domain; however its involvement with TLR4 and TLR2 activation suggests that the production of inflammatory mediators may be due to the activation of these two receptors. A number of studies have been performed to measure direct activation of cells expressing TLR2 and/or TLR4 [275, 277, 281]. THP-1 cells have been shown to produce TNFα in response to Aβ peptide, furthermore, upon the addition of neutralising antibody for TLR2 and TLR4 there was a reduction in the amount of cytokine produced [274]. Investigation of TLR2 expressed on HEK293 cells showed an increase in IL-8 production. They further showed that TLR1/2 heterodimers had a more enhanced response whereas TLR2/6 heterodimers showed a reduced response [275]. There is also a variety of work looking at mouse models deficient in TLR2 or TLR4 that show a reduced or absent ability to respond to Aβ peptide [275, 279, 281-283].

Combined these studies provide convincing evidence that TLR2 and TLR4 are involved in the recognition of Aβ. The majority of these studies have pointed to the need of the peptide to have a fibrillar morphology, however, there are some studies that have shown the ability of oligomeric Aβ to cause inflammation through PRRs, including TLRs [275, 284]. This suggests that Aβ is able to induce inflammation before it has formed the fibrils that are deposited in Aβ plaques.

Although it is thought that TLRs are expressed mainly on innate immune cells such as monocytes, there is also evidence of cells such as T-cells expressing these receptors [244]. It is therefore possible that Aβ can activate a variety of

cells through these receptors. In addition to the potential role of TLR activation on T-cells there is also a number of studies have pointed towards antigen-specific T-cells that respond to Aβ peptides.

Despite the belief that the brain is an immune-privileged site, meaning that traditional immune cells such as T-cells and B-cells are not present, there is now evidence that T-cells frequently enter the brain, although in small numbers [285, 286]. It has been proposed that in AD there is an increase in the numbers of T-cells that enter and remain in the brain. This could be due to damage to the blood brain barrier (BBB), therefore allowing increased infiltration of the CNS by T-cells. Immunohistochemistry studies have shown that there is increased numbers of helper T-cells and cytotoxic T-cells in AD brains compared with both non-AD demented patients and healthy controls. Although, they did also suggest that these were most likely not effector T-cells as no proliferation markers could be detected [287]. It has further been reported that there are difficulties in accurately measuring the number of T-cells in the brain as they may not actually enter brain parenchyma but a space between cerebral vasculature and the brain parenchyma, known as the Virchow-Robbins space [288].

In animal models of AD, active and passive immunisation with Aβ has been shown to have a number of effects including a reduction in the deposition of Aβ and an improvement in cognition [289]. Studies using a variety of mouse models containing mutations seen in the familial form of AD have used active immunisation to show several positive effects for example in a PDAPP model Aβ immunisation significantly increased serum anti-Aβ antibody titres when compared with untreated controls. These antibodies caused an almost

complete prevention of Aβ deposition as well as gliosis and neuritic dystrophy [290, 291]. Furthermore immunisation of PDAPP mice with an existing amyloid burden reduced the extent and progression of AD [291]. Studies using and APP +PS1 mouse model observed that subcutaneous injection of human Aβ42 protects the mice from learning and memory deficits that are normally seen in the mouse model. To test for deleterious effects of Aβ immunisation the working memory of immunised mice was tested using a radial-arm water-maze test in which the immunised mice performed to the same level as the non-transgenic control and far superior to the untreated APP +PS1 mice [292-294].

Due to the apparent success of immunisation in mouse models a phase 1 clinical trial was set up in human using a vaccine known as AN1792 which contained a pre-aggregated Aβ42 peptide and QS21 as an adjuvant (later in the study polysorbate 80 was added [295]. The phase 1 study found that there were positive anti-AN1729 titres in at least half the patients, disability assessment for dementia (DAD) showed less decline in the treated group and no safety concerns arose during the study. One patient died of menigoencephalitis but this was deemed to be unrelated to the vaccine study [295]. The vaccine then went into phase 2a trials, AN1729 was given with QS21 in the polysorbate 80 formulation. However, due to several of the patients developing menigoencephalitis the study was stopped [296]. Despite the interruption of the study there were a number of follow –up tests carried out and several observations made: autopsy results showed some clearance of parenchymal plaques which in most cases was associated with phagocytosis by microglia, amyloid remained in cerebral vessels, tau-immunoreactive

neurofibrillary tangles were found in regions where plaque clearance had occurred [297-299], there was evidence in some patients of deleterious T-cell activity pointing towards an excessive T-cell response [296]. When an antibody response occurred, which wasn't in all patients, there was a modest improvement in cognition and slowed rate of disease progression [295, 300] and furthermore when the antibody titres were high there was a significant improvement in memory function tests than both non-responders and the placebo group [301].

A long term follow up of the phase 1 trial was performed six years after the initial vaccination to investigate the degree of plaque removal and long-term clinical outcomes [302]. The study showed that although there was a significant reduction in the amyloid plaque burden in the immunised patients compared with the controls there was no differences in time to severe dementia or death between the groups. This has been proposed to be because Aβ immunisation was performed after neurofibrillary tangles of tau had begun to form. It may therefore be possible that if immunisation occurred earlier it may have a more significant effect on cognitive functions.

In addition to the evidence of Aβ-specific T-cells in the brain there is also evidence that they exist in the periphery. However, only a few studies have looked directly at the activation of Aβ-specific T-cells from the periphery. In addition to the small number of studies the results observed vary quite significantly. There is some evidence that Aβ specific T-cells from patients with AD have an increased proliferative response to Aβ when compared with middle aged control [303, 304]. Although very similar studies have shown that the opposite is true, in that patients with AD respond less than healthy

controls [305]. Furthermore some studies have reported that no proliferative response was observed or that when proliferation could be detected it was in a very small proportion of cells [306]. These differences in response have several potential causes, one was the peptide used, i.e. $A\beta40$ or $A\beta42$, the methods used to measure proliferation, or that only people with a specific HLA-type are able to respond to the peptides [303, 307]. It has also been reported that patients with AD display small subsets of peripheral $A\beta$-specific T-cells that are characterised by increased phosphorylation of PKC-δ and ζ [308, 309]. This has been proposed as a potential method for detecting AD. It is clear that there is still a lot of research needed into the responses and roles of $A\beta$-specific T-cells in AD.

Further to clearance of $A\beta$ by the immune system there is also evidence that a substantial proportion of $A\beta$ is removed from the brain through other mechanisms. The glymphatic system is a macroscopic waste clearance system that facilitates efficient removal of soluble proteins and metabolites from the brain. It involves the continuous interchanging of cerebral spinal fluid (CSF) and interstitial fluid (ISF). CSF is transported through the ventricles and foramina of the brain into subarachnoid spaces from which it is forced into the Virchow-Robins spaces. From here CSF is transported into the deep brain parenchyma through AQP4 channels located in the astrocytic end-feet that enclose brain vasculature [310, 311]. The movement of CSF into the brain parenchyma drives the ISF within the tissue towards perivenous spaces where it drains into the cervical lymphatic system [312, 313]. Recent studies have highlighted the presence of $A\beta$ monomers, oligomers and aggregates in ISF and CSF and the glymphatic system has been proposed as a possible pathway

for Aβ removal from the brain. It has been observed in rat models that Aβ is cleared through the glymphatic system and that a larger proportion of Aβ is removed this way than via the BBB [311]. It is possible that a combination of glymphatic system removal and transport across the BBB is sufficient in healthy people to clear Aβ from the brain, however when the CSF bulk flow is no longer adequate, which often happens with age, it leads to a build-up of Aβ in the brain [314].

Altogether it is clear there is still a large amount of research that needs to be done into the role of the immune system in the recognition of Aβ in AD.

1. 5 Aims and Hypotheses

The hypothesis of this thesis was:

Amyloid beta (Aβ) peptides and their post-translationally modified (PTM) variants are able to induce immune responses through recognition by T-cells and monocytes resulting in inflammatory responses that contribute to the progression of Alzheimer's disease.

The hypothesis was tested by addressing the following aims:

1. To identify TLR stimulating Aβ 1-42 peptides and PTM variants;

Do any of the peptides tested induce a response through TLR2 or TLR4? Human embryonic kidney cells (HEK293) cells expressing TLR2 or TLR4 were stimulated to determine whether modified Aβ42 peptides are able to induce an immune response through either of these receptors and to investigate whether there are any differences when compared with the unmodified form of the peptide (Chapter 4).

2. To characterise T-cell activation by Aβ peptides and PTM variants in patients with AD and age-matched controls.

Are any of the peptides able to induce a response from T-cells from patients with AD or age-matched controls? The activation of T-cells, isolated from the peripheral blood mononuclear cells (PBMCs) from patients with AD and age-matched controls, after stimulation with Aβ peptide was investigated. Proliferation and phosphorylation of PKC-δ and PKC-ζ in response to the peptides was measured (Chapter 5).

3. To characterise TLR dependent monocyte activation by Aβ1-42 and PTM variants.

Do primary human monocytes show activation after stimulation with Aβ peptide and its PTM variants? Monocytic responses to Aβ peptides were investigated to determine whether the peptides were able to induce cytokine secretion and whether there were any differences when comparing the four peptides. In addition responses observed from patients with AD and age-matched controls were measured to investigate whether people with AD respond the same way as people without AD (Chapter 4). To determine whether any responses observed were due to TLR activation, the receptors were blocked and any reduction in cytokine production indicated the peptides were recognised by that TLR.

2. Materials and Methods

Table 2 . 1: Table of Reagents

Reagent	Manufacturer	Product number
Aβ 1-42, Aβcit5 1-42, AβpE3 3-42, AβpE3cit5 3-42 peptides	Charité, Institut für Medizinishe Immunologie, Berlin, Germany	Custom peptide synthesis
Aβ 1-42 rPEP, recombinant	Stratech Scientific Ltd, Suffolk, UK	A-1163-1-RPE
Aβ 1-42 synthetic	JPT Peptide Technologies, Berlin Germany	Custom peptide synthesis
BD CompBeads	BD, Oxford, UK	552843
BD FACs Lysing solution	BD, Oxford, UK	349202
BD FACSDiva CS and T Research beads (Diva 8)	BD, Oxford, UK	655051
BD^{TM} Cytometer setup and tracking beads kit (Diva 6)	BD, Oxford, UK	642412
Blasticidin	Invivogen, Toulouse, France	ant-bl-1
Bovine serum albumin (BSA) (Flow cytometry)	Acros Organics, Geel, Belgium	
Bovine Serum Albumin (BSA)(ELISA)	Sigma Aldrich, Gillingham, Dorset, UK	A7030-50G
CD Leukocyte cones	National Health service blood and transplant,	NC24
DMSO	Fisher Scientific, Loughborough, UK	BP231-100
Dry DMSO	Acros Organics, Geel, Belgium	326881000
Dulbecco's Modified eagle media (DMEM)	PAA, Yeovil, Somerset, UK	E15-843
Dulbecco's Phosphate Buffered Saline(DPBS)	Gibco, Life technologies, Paisley, UK	14190-094
FACs permeabilising solution 2 (10x)	BD, Oxford, UK	340973
Ficoll-Paque^{TM} PLUS	GE Healthcare	17-1440-03
Foetal bovine serum (FBS)	Gibco, Life technologies, Paisley, UK	10500
Formaldehyde solution	Merck, Darmstadt, Germany	1.04002.2500
HEK293 wild-type	Invivogen, Toulouse, France	
HEK293-TLR2	Invivogen, Toulouse, France	293-htlr2
HEK293-TLR4-MD2-CD14	Invivogen, Toulouse, France	293-htlr4md2cd14
Hygromycin (Hygrogold)	Invivogen, Toulouse, France	Ant-hg-1
IgA2 isotype control	Invivogen, Toulouse, France	
IL-1β capture antibody	R and D systems, Abingdon, UK	MAB-601
IL-1β detect antibody	R and D systems, Abingdon, UK	BAF-210
IL-1β standard	Peprotech, London, UK	200-01B
IL-8 Capture antibody	R and D systems, Abingdon, UK	MAB-208
IL-8 detect antibody	R and D systems, Abingdon, UK	BAF-208
IL-8 standard	R and D systems, Abingdon, UK	208-IL-010
Interferon α	Cambridge Bioscience, Cambridge, UK	OPPAO1628
Ionomycin	Sigma Aldrich, Gillingham, Dorset, UK	I0634-1MG

Isopropanol	Fisher Scientific, Loughborough, UK	P/7507/17
Lipopolysaccharide (LPS)	Axxora, ENZO life sciences LTD, Exeter, UK	AXL-581-010-L00Z
Lympholyte	Cedarlane, BH Bio LTD, Gateshead, UK	CL5020
MTT	Sigma Aldrich, Gillingham, Dorset, UK	M5655-500MG
NaCl	Sigma Aldrich, Gillingham, Dorset, UK	
Pam2CGDPKHPKSF (FSL-1)	Invivogen, Toulouse, France	Tlrl-fsl
Pam3CSK4 (PAM3)	Invivogen, Toulouse, France	Tlrl-pms
PBS citrate	Abliance, Compiègne, France	DY9405
Penicillin- Streptomycin	Gibco, Life technologies, Paisley, UK	15070-063
Penicillin-Streptomycin-Glutamine	Gibco, Life technologies, Paisley, UK	10378-016
Percoll	GE Healthcare life sciences, Buckinghamshire, UK	17-5445-01
Phytohemagglutinin (PHA)	Sigma Aldrich, Gillingham, Dorset, UK	L1668
PKH67 Green fluorescent cell linker midi kit for general cell membrane labelling	Sigma Aldrich, Gillingham, Dorset, UK	MIDI67-1KT
PMA	Sigma Aldrich, Gillingham, Dorset, UK	P8139-1MG
Purified protein derivative (PPD)	BD, Oxford, UK	Custom lyophilised stimulation plate
Rainbow calibration particles (8 peaks)	BD, Oxford, UK	559123
RPMI 1640	Gibco, Life technologies, Paisley, UK	31870-074
Sodium Azide	Fisher Scientific Ltd, Loughborough, UK	S/2380/48
Staphylococcus enterotoxin B (SEB)	Sigma Aldrich, Gillingham, Dorset, UK	S4881
Sterile Tissue culture grade phosphate buffer	PAA, Yeovil, Somerset, UK	H15-011
Sterile Tissue Culture grade water	PAA, Yeovil, Somerset, UK	S15-012
Streptavidin HRP	R and D systems, Abingdon, UK	DY998
TMB microwell peroxidise substrate system	Insight Biotechnology, Wembley, Middlesex, UK	50-76-00
Tryphan blue	Sigma Aldrich, Gillingham, Dorset, UK	T8154
Uranyl Acetate	Agar Scientific, Stansted, Essex, UK	
Zenon ®Fluoroscein rabbit IgG labelling kit	Invitrogen, Life technologies, Paisley, UK	Z-25342
αTLR2 neutralising antibody	Invivogen, Toulouse, France	

Table 2 . 2: Table of antibodies

Antibody	Company	Product code
CD14-PerCP/Cy5.5	Biolegend, London, UK	325622 (100 tests)
CD282(TLR2)-AlexaFluor 647	Biolegend, London, UK	121810 (100 tests)
CD282(TLR4)-PE	Biolegend	312806 (100 tests)
CD3 Pacific Blue	Biolegend, London, UK	300431 (100 tests)
CD40L-PE	BD, Oxford, UK	555700 (100 tests)
CD4-PerCP	Biolegend, London, UK	317432 (100 tests)
CD8-APC H7	Biolegend, London, UK	344714 (100 tests)
FITC 2°	Biolegend, London, UK	
HLA-DR-PE/Cy7	Biolegend, London, UK	307616 (100 tests)
IL-1β-FITC	Biolegend, London, UK	508206 (100 tests)
IL-2-APC	BD, Oxford, UK	341116 (100 tests)
PKC-δ (Thr507)	Santa Cruz Biotechnology inc,	sc-11770
PKC-ζ (Thr 410)-R	Santa Cruz Biotechnology inc,	sc-12894-R
TNFα-Alexa Fluor 700	BD Pharmingen, Oxford, UK	557996 (0.1mg)

Table 2 . 3: Table of Equipment

Equipment	Manufacturer	Product number/model
96-well plate		
Acquisition software	BD, Oxford, UK	BD FACSDiva 6 BD FACSDiva 8
Centrifuge	Heraeus,	Multifuge 3S-R: Rotor 75006445
ELISA plates	Fisher Scientific, Loughborough, UK	DPS-110-090D
Flow cytometer	BD, Oxford, UK	LSR II
Flow cytometry analysis software	FlowJo LLC, Ashland, OR, USA	FlowJo v10.0.8
Mr Frosty	Nalgene, Thermo Scientific, UK	
Plate reader	Biotek, Swindon, UK	
Plate reader software	Biotek, Swindon, UK	Gen5
Software		Gatan Ultrascan 1000CCD
Spectrophotometer	JENWAY, Stone, Staffordshire, UK	JENWAY 6305
T-75 culture flasks		
TEM camera		Hitachi-7100
TEM Grids	Agar Scientific, Stansted, Essex, UK	AGS162-4
Ultrawave Sonicating water bath	Wolf Laboratories, Pocklinton, York	U100H
Vacuum desiccator	Eppendorf, Stevenage, UK	
Zeba Desalt spin columns	Thermo Scientific, UK	89890

2.1 Amyloid Beta Peptides

Table 2 . 4: Full length Aβ peptides

Aβ 1-42	Unmodified peptide	1-DAEFRHDSGYEVHHQKLVFFAEDVGSNK GAIIGLMVGGVVIA-42
Aβcit5 1-42	Citrullination – Amino acid 5	1-DAEF-cit-HDSGYEVHHQKLVFFAEDVGSNK GAIIGLMVGGVVIA-42
AβpE3 3-42	Pyro-glutamate – Amino acid 3	1-DA-pE-FRHDSGYEVHHQKLVFFAEDVGSN KGAIIGLMVGGVVIA-42
AβpE3cit5 3-42	Citrullination – amino acid 5 and Pyro-glutamate – amino acid 3	1-DA-pE-F-citr-HDSGYEVHHQKLVFFAEDVGSNK GAIIGLMVGGVVIA-42

Table 2 . 5: Short Aβ peptide fragments

Peptide designation	Amino acid chain
Aβ 1-15	1-DAEFRHDSGYEVHHQ-15
Aβ 5-19	5-RHDSGYEVHHQKLVF-19
Aβ 9-23	9-GYEVHHQKLVFFAED-24
Aβ 13-27	13-HHQKLVFFAEDVGSN-27
Aβ 17-31	17-LVFFAEDVGSNKGAI-31
Aβ 21-35	21-AEDVGSNKGAIIGLM-35
Aβ 25-39	25-GSNKGAIIGLMVGGV-39
Aβ 28-42	28-KGAIIGLMVGGVVIA-42

Aβ peptides were purchased from a number of sources: the first batch was prepared by Dr Zerrin Fidan at Charité (Institute für Medizinische Immunologie at Charité Centrum für Innere Medizin und Dermatologie) using the method of solid phase peptide synthesis (SPPS). In addition to the Aβ 1-42 peptide, three more were also synthesised to contain the modifications being investigated (citrullination of arginine at amino acid position 5, pyro-glutamate modification at amino acid position 3 and a final peptide containing both modifications). The second peptide was a recombinant Aβ 1-42 peptide from rPeptide expressed in E.coli and containing no PTMs. The final peptide was a synthetic Aβ 1-42 peptide from JPT. This peptide was derived from the N-terminal end of the Aβ peptide and contained no modifications.

2.2 Amyloid beta peptide preparation

Several peptide preparation protocols were used in order to find the method that produced fibrils as well as a variety of different intermediate species.

2.2.1 Preparation 1

This protocol was adapted from the method by Broerson et al. [315] Lyophilised peptide was dissolved in hexafluoroisopropanol (HFIP) to a concentration of 1mg/ml. The dissolved peptide was vortexed for 60 sec and sonicated in a water bath for 60 sec. The HFIP was evaporated off using dry nitrogen gas leaving a peptide film and any remaining moisture was removed by vacuum desiccation for 30min. Zeba Desalt spin columns were equilibrated by washing three times with 50mM phosphate buffer (PB), pH7.4, by centrifugation at 4°C, 3000rpm for 2 min. After desiccation the

peptide film was dissolved in dry dymethyl sulfoxide (DMSO) to a

concentration of 1mg/ml and then vortexed and sonicated, each for 60sec.

Chilled 1.5ml eppendorf tubes were attached to the bottom of the Zeba spin

columns. 250µl of the peptide in DMSO was added to each column with 50µl

50mM PB and centrifuged for 2min, 4°C, 3000rpm (using the Heraeus

centrifuge). Once the peptide had been collected in eppendorf tubes it was

centrifuged for 30min, 16000rcf, 4°C and the supernatant was then transferred

to fresh, chilled eppendorf tubes. The absorbance of the peptide at 280nm was

measured using a spectrophotometer and the concentration was determined

using the equation:

$$\frac{A_{280}}{\varepsilon 1490} \times 10^6 = X\mu M$$

The peptide was then incubated at room temperature for 24h, 48h or 72h.

When this failed to produce fibrils a longer incubation different peptide

preparation protocols were tested.

2.2.2 Preparation 2

This protocol was adapted from Dahlgren et al. [316]. The peptide was

dissolved in HFIP to a concentration of 1mg/ml and then vacuum desiccated

to remove the alcohol. The peptide film was resuspended in dry DMSO, to a

concentration of 200µM. In order to form oligomers and fibrils the peptide

was diluted to 100µM in either growth media (DMEM+FBS etc.) or 10mM

hydrochloric acid (HCl) and incubated at 4°C or 37°C respectively for 24h.

2.2.3 Preparation 3

This final protocol was adapted from the protocol used by Udan et al. [274].
Lyophilised Aβ peptides were dissolved in HFIP to a concentration of 1mg/ml
and then incubated at room temperature for 1h in order to depolymerise
aggregates. 100µl of the peptide was aliquoted into sterile 1.5ml eppendorf
tubes and vacuum desiccated at room temperature for 45min. To each aliquot
250µl sterile water or sterile 50mM PB was added to dissolve the peptide film
and the peptides were vortexed and then pooled together. The absorbance of
the peptide was measured at 280nm and the concentration of the peptide was
determined using the equation:

$$\frac{A_{280}}{\varepsilon 1490} \times 10^6 = X \mu M$$

The peptides were allowed to aggregate at 4°C for 24h – 240h.

2.2.4 Transmission electron microscopy

In order to determine the morphology of the peptide transmission electron
microscopy (TEM) was used. TEM grids were prepared using samples of the
peptide after they had been incubated for the desired length of time. 4µl of the
peptide was added to a 400 mesh carbon/formvar-coated copper grid for
30sec and was removed using filter paper being careful to touch only the very
edge of the grid. The grid was washed using 0.2µm filtered MiliQ water and
the water was removed using filter paper. The grids were negative stained
with 2% Uranyl acetate solution. This was added for 30sec and then removed
and the step repeated. The grid was allowed to air dry and then stored until

imaging on the TEM. Micrographs of the TEM grids were taken using a Gatan Ultrascan 1000 CCD camera.

2.3 Cell culture

2.3.1 Human Embryonic Kidney 293 cells

Human embryonic kidney 293 (HEK293) wild type cells, HEK293 cells stably expressing toll-like receptor 2 (TLR2), TLR4 or the TLR4md2CD14 receptor complex were all obtained from invivogen. All HEK293 cells were maintained in Dulbecco's modified eagle's medium (DMEM) growth media containing 4.5g L-glucose, 10% foetal bovine serum, 50U/ml penicillin, 50µg/ml streptomycin. However, HEK293-TLR2, HEK293-TLR4 cells and HEK293-TLR4md2CD14 also required the addition of the selection antibiotics: blasticidin (10µg/ml) for TLR2 and TLR4 cells or blasticidin (10µg/ml) and hygromycin (50µg/ml) for TLR4md2cd14 cells. Cells were cultured in T-75 flasks, incubated at 37°C, 5% CO_2 and passaged when approximately 80% confluent. Cell passaging was performed the same way each time; Growth media was removed from the flasks and 5ml DMEM media without FBS and Pen/Strep was added. A cell scraper was used to remove the adherent cells from the bottom of the flask into the media. A further 5ml serum-free media was added to each flask and the cells were gently pipetted to distribute the cells evenly throughout the media. 1ml of media containing cells was transferred to a new T-75 flask, 9ml normal growth media (DMEM with FBS, P/S and relevant antibiotics) was added and the cells were returned to the incubator.

In order to use the cells in experiments, once the adherent cells were scraped from the T-75 flasks into 5ml of serum free media; 5ml normal growth media (without antibiotics) was added to the cells and they were transferred to a 50ml falcon tube. The tubes were vortexed to distribute the cells throughout the media and counted using a neubauer chamber. The cells were centrifuged (1200rpm, 10 min, RT, using the heraeus centrifuge), supernatant removed and cells resuspended at a density of 2×10^6 cells/ml. 100µl of cells, containing 2×10^5 cells was added to wells of a 96-well plate and were incubated at 37°C, 5% CO_2 until 80% confluent.

Before the cells were used in experiments they were tested with the ligands for TLR1/2, TLR2/6 and TLR4. The cells were treated as above and plated at a density of 2×10^5 cells/well and incubated until approximately 80% confluent. The supernatants were removed and replaced with 200µl media containing either PAM3 (TLR1/2) FSL-1 (TLR2/6) of LPS (TLR4). Each of these ligands were used at a concentration of 1ng/ml, 10ng/ml and 100ng/ml and the cells were incubated overnight at 37°C, 5% CO_2 and an IL-8 ELISA was performed on the cell supernatants. The ligand concentration that produced the best result was then used in all remaining experiments. Media alone was used as the negative control.

2.3.2 Primary human monocytes

Human blood was obtained from the National Health Service blood and transplant service (NHSBT) and monocytes were isolated and stored in liquid nitrogen. First blood was diluted in Hanks balanced salt solution (HBSS) to a final volume of 50ml. After thorough mixing an equal volume of blood was

carefully layered over 16ml lymphoprep in 50ml falcon tubes and the tubes were centrifuged 2200rpm, 25 min, no brakes. The lymphoprep allows the separation of sections of the blood meaning that the peripheral blood mononuclear (PBMCs) cells can be isolated. This was done by aspirating the entire layer of PBMCs to a fresh sterile 50ml Falcon tube. It is important to remove as little lymphoprep as possible with the PBMCs as it is toxic for the cells. The aspirated PBMCs were resuspended in HBSS up to 50ml and then centrifuged: 1200rpm, 10 min, full brakes. The supernatant was very carefully removed leaving the cell pellet as whole as possible. The pellet was again resuspended in HBSS and centrifuged, with the same settings as the first wash, and the supernatant removed. This step was repeated a second time but the pellet was resuspended up to 25ml HBSS (all centrifuge steps were carried out using the heraeus centrifuge).

Isosmotic percoll was used in order to separate the monocytes from the PBMCs. Isosmotic percoll consists of 1vol 1.5M NaCl and 9vol Percoll dissolved at a ratio of 1:1 with PBS citrate. The isolated PBMCs in HBSS were layered onto 16ml isosmotic Percoll, the layer is very fragile so the cells needed to be pipetted very gently and slowly. The tubes were centrifuged (2200rpm for 15 min, no brakes) and the monocyte layer was aspirated using Pasteur pipettes. The monocytes were resuspended in HBSS and centrifuged to wash (1200rpm, 10 min, full brakes). This was repeated a second time before the cells were counted. Once the number of cells was known the tubes were centrifuged a final time and resuspended in freezing media at a density of 20×10^6 cells/ml. Freezing media is made up of FBS with 10% DMSO. 1ml of the cell-freezing media suspension was added to each labelled

cryovial. The cyrovials were placed in a Mr Frosty freezing container filled with isopropanol and transferred to -80°C to slowly freeze down. They were then transferred to liquid nitrogen for long term storage. All centrifuge steps were performed using the heraeus centrifuge.

In order to defrost monocytes, the cyrovials were removed from liquid nitrogen and quickly placed in a 37°C waterbath until completely defrosted. The cells were washed with growth media and resuspended at a density of 2 x 10^6 cells/ml. Monocyte growth media is made up of RPMI-1640 containing 5% FBS and 50U/ml penicillin, 50µg/ml streptomycin and 2mM L-glutamine.

2.3.3 Peripheral blood mononuclear cells

Human PBMCs were isolated from fresh heparinised blood, obtained from control subjects or patients with AD, using a Ficoll gradient. Initially the blood was diluted 1:1 with sterile Dulbecco's PBS (DPBS) and mixed before very carefully being layered over 15ml Ficoll-Paque and centrifuged 1000g, 20min, no brakes. The Ficoll allows the separation of the blood and therefore the PBMC layer can be aspirated from the rest of the blood, care was taken to remove as little Ficoll as possible. The cells were resuspended in 50ml DPBS and centrifuged 300g, 10 min to wash. The supernatants were carefully removed and washed again in 50ml DPBS (centrifuge: 200g, 10 min). Once the cells had been washed they were counted and for all experiments (excluding PKH/proliferation assays) resuspended in RPMI (supplemented with 10% foetal bovine serum, 50U/ml penicillin, 50µg/ml streptomycin and L-glutamine). The density of the cells suspended in media depended on the

experiments performed. All centrifugation steps were performed using the heraeus centrifuge.

For phosphorylation of PKC-δ and ζ and the measurement of intracellular IL-1β and TNF-α the cells were at a density of $1x10^6$cells/ml and 500µl was added to each FACS tube (approximately 5×10^5 cells). For cells that were plated in 96-well plates for detection of IL-1β and TNF-α by ELISA the cells were suspended at a density of $2x10^6$cells/ml and 100µl was added to each well (approximately $2x10^5$cells/well). Treatment of cells for proliferation assay is described later.

2.3.4 Methylthiazoletetrazolium (MTT) cell viability assay

Solutions: MTT reaction solution – 5mg/ml MTT powder in 1xPBS

   MTT stop solution – 10% sodium dodecyl sulphate (SDS) in 0.01M hydrochloric acid (HCl)

Methylthiazoletetrazolium (MTT) reaction solution was diluted by a factor of 1:10 in normal growth media (depending on cell type using) and 100µl was added to each well of a 96-well plate. The cells were incubated at 37°C, 5% CO_2 for approximately 4h, or until crystals appear. 100µl of MTT stop solution was added to each of the wells and incubated at 37°C, 5% CO_2 for 4h or until the crystals had all dissolved. The absorbance of each well was read at 580nm to determine the viability of the cells after stimulation.

2.4 ELISA

Solutions: Blocking solution – 1g BSA in 50ml PBS (2% BSA solution)

ELISA buffer – 0.5% BSA solution

ELISA wash buffer – 1L 1x PBS and 100µl Tween20 (1:10000

Tween20:1xPBS)

The general protocol for ELISA experiments was the same regardless of what

output was being measured, however the concentration of antibodies used for

each was different. Firstly, capture/primary antibody was diluted to the

correct concentration in PBS and the ELISA plates were coated and incubated

at 37°C for 1h. The antibody was then removed by flicking the plate upside

down over a container. Each plate was then blocked with 2% bovine serum

albumin (BSA) for 1h at room temperature on a shaker and washed three

times with ELISA wash buffer.

Standards of each cytokine being measured were prepared in ELISA buffer

and added to the plates in triplicate at this point the sample supernatants were

also added. Depending on the cytokine being measured and the strength of

activation, supernatants were added undiluted or diluted to an appropriate

factor. The plates were then left over night at 4°C. The plates were washed

and then secondary/detect antibody diluted in ELISA buffer was added and

incubated at room temperature for 1h. After the plates were washed again

streptavidin HRP was diluted at a factor of 1:400 in ELISA buffer and then

added to the plates and incubated for 1h at room temperature. 3,3,3-

Tetramethylbenzidine (TMB) peroxidise substrate (Insight Biotechnology)

was used to produce a colour change in the standards and the samples

producing the cytokine. TMB microwell peroxidise substrate kit was used, solution A and B were mixed together in equal measures and 50μl was added to each well until the standards have changed colour. The general rule used when watching for colour change was to stop the reaction when the second most dilute standard has begun to change colour. The reaction was stopped using ELISA acid stop solution (H_2SO_4), the addition of 50μl of this turns the solution in the well yellow and can be read on a plate reader at 450nm and compared to the standards using BioTek Gen5 software.

Table 2 . 6: Antibody concentrations for ELISA

Cytokine	Capture antibody	Detect antibody	Max standard concentration
IL-8	2μg/ml	0.2μg/ml	5ng/ml
IL-1β	2μg/ml	0.5μg/ml	10ng/ml
TNF-α	4μg/ml		5ng/ml

2.5 Flow cytometry
Solutions: ELISA wash buffer – 1L distilled water, 5g BSA and 1g sodium azide

2.5.1 Antibody Titrations

To ensure that the optimum concentration of antibody was used in each experiment all antibodies were titrated before use in experiments. PBMCs were isolated as usual and were resuspended in normal RPMI growth media at

a density of 1 x 10^{6}cells/ml. 1ml was aliquoted into each FACS tube ready to be used.

Most of the surface markers being tested were present on cells without the need for cell activation, therefore for titration of these antibodies cells could be used immediately. For cell surface markers that are only present if the cells are activated, cells were stimulated with 10ng/ml LPS for 4h before titration of the antibodies. For intracellular markers, cells were also stimulated for 4h with 10ng/ml LPS and 10μg/ml BFA.

To titrate the surface antibodies PBMCs were centrifuged for 8 min at 400g and the supernatants were discarded. One antibody was then added to each FACS tube, the antibodies were used at the concentration recommended by the manufacturer and also at 2x, 4x and 10x less than the recommended amount. For example for CD3-FITC 20μl/1x10^6 PBMCs was recommended so 20μl as well as 10μl, 5μl and 2.5μl were also tested. PBMCs were incubated for 30min, 4°C in the dark and the cells were lysed. This was done by adding 1ml BD FACS^{TM} lysing solution diluted 1:10 with deionised water into each FACS tube and incubating at room temperature in the dark for 10min. The cells were washed (3ml FACS wash buffer and centrifuged at 400g for 8min, 4°C),the supernatants were discarded and the cells were either acquired immediately or stored at 4°C in the dark for no longer than 48h before acquisition. All concentrations were tested in duplicate and were acquired using an LSRII. For the surface markers only one antibody was present in each tube therefore compensation as not required.

For intracellular antibody titrations it was necessary to use surface markers to isolate the cell types that produce each specific marker. For example, if the intracellular marker was found in T-cells CD3$^+$ and CD4$^+$ or CD3$^+$ and CD8$^+$ cells were isolated. If the intracellular marker was found in monocytes, HLA-DR$^+$, CD14$^+$ and CD3$^-$ cells were gated.

To titrate the intracellular antibodies cells were treated as described above, however all the required surface antibodies were added to every tube before lysis and washing. In addition the cells were permeabilised using 500µl BD FACS^{TM} Permeabilising solution 2 (diluted 1:10 in deionised water) and incubated at room temperature in the dark for 10min and washed. After permeabilisation the intracellular antibodies could be titrated. They were added as described for the surface antibodies and incubated for 30min in the dark at 4°C. They were then washed, fixed with 1ml 0.5%PFA for 5min and washed again before acquisition or storage in the dark at 4°C for up to 48h. The volumes of antibody used in experiments are the amounts found to produce the best result.

2.5.2 Antibody staining

The protocol used to stain cells with antibody depended upon the type of marker being investigated. For surface markers PBMCs were isolated as normal, resuspended in media at the density needed for the experiment, usually 1-2 x 10^6 cells/ml, and aliquoted into FACS tubes for the experiment. The cells were centrifuged, to remove the growth media, at 400g for 8 min, without brakes at 4°C and the supernatants were discarded. All centrifugation was performed using the heraeus centrifuge

Master mixes containing all the surface antibodies at the required volume were made and FACS wash buffer was added. $100\mu l/10^6$ cells of the master mix/wash buffer was added to each of the FACS tubes, and cells were incubated at 4°C in the dark for 30min. $1ml/10^6$ cells BD lysis solution (1:10 dilution with deionised water) was added to each tube to lyse any remaining red blood cells and to fix PBMCs and they were incubated for 10 min in the dark at room temperature. A washing step was then performed, 3ml FACS wash buffer was added and cells were centrifuged for 8min , 400g, 4°C no brakes (using the heraeus centrifuge) and the supernatants were discarded. The cells were then ready for acquisition. If acquisition was not performed immediately, cells were stored in the dark at 4°C for up to 48h.

For the intracellular staining the initial protocol was the same, however after lysing and washing the PBMCS BD Perm 2 was diluted 1:10 with deionised water and $500\mu l/10^6$ cells was added to each tube, the cells were incubated at room temperature in the dark for 10min and then washed. A second master mix was made using the intracellular antibodies in the panel and FACS wash buffer and $100\mu l/10^6$ cells was added to the tubes. The cells were incubated for 30min at 4°C in the dark and then washed. $1ml/10^6$ 0.5% PFA (diluted in wash buffer from 4% stock solution) was added to each tube for 5min at room temperature in the dark and a final wash step was performed. The cells were either acquired immediately or stored at 4°C in the dark for no longer that 48h.

2.5.3 Proliferation assay panel (PKH-green)

Table 2 . 7: Surface antibodies for proliferation assay

	Marker	Amount/ 1 x 10^6 cells
B4 - PerCP	CD4	3μl
R3 – APCH7	CD8	1.5μl
V1 – Pacific Blue	CD3	3μl

2.5.4 P-PKC panel

Table 2 . 8: Surface antibody panel for P-PKC assay

Channel	Marker	Amount/ 1 x 10^6 cells
B4 - PerCP	CD4	3μl
R3 – APCH7	CD8	1.5μl
V1 – Pacific Blue	CD3	3μl

Table 2 . 9: Intracellular antibody panel for P-PKC assay

Channel	Marker	Amount/1x10^6cells
B1 - FITC	P-PKCδ/P-PKCζ	1μl
	P-PKCδ/P-PKCζ (unconjugated) + secondary FITC antibody	1μl + 1μl secondary FITC antibody
B2 - PE	CD40L	10μl
R1 - APC	IL-2	5μl

2.5.5 Intracellular cytokine panel

Table 2 . 10: Surface antibody panel for monocytes

Channel	Marker	Amount/ 1 x 10^6 cells
B4 – PerCP/Cy5.5	CD14	3μl
B5 – PE/Cy7	HLA-DR	10μl
V1 – Pacific Blue	CD3	3μl

Table 2 . 11: Intracellular antibody panel for monocytes

Channel	Marker	Amount/ 1 x 10^6 cells
B1 - FITC	IL-1β	3μl
R2 – Alexa Fluor 700	TNF-α	2.5μl

2.5.7 Acquisition using LSRII

The LSRII was used for the acquisition of all antibody stained cells. The configuration of the lasers was constant for all experiments; the configuration included 5 blue lasers, 3 red lasers and 5 violet lasers, however not all of these lasers were involved in experiments. The layout of the lasers is shown below (Fig 2.1) and the lasers that were used in experiments have been highlighted. The configuration of the lasers was set and checked before the acquisition of any data.

After the configuration was set the performance of the cytometer was checked. This was done before every acquisition. Performance checking is done using cytometer setup and tracking (CS and T) beads, the beads were vortexed and one drop is added to a FACS tube. Approximately 300µl of water was added to the drop and the tube was vortexed, it was then used to run CS and T on the cytometer. CS and T is an important step for acquisition, it initially sets up the baseline for the configuration used, it then optimises and standardises the setup and tracks how the cytometer is performing.
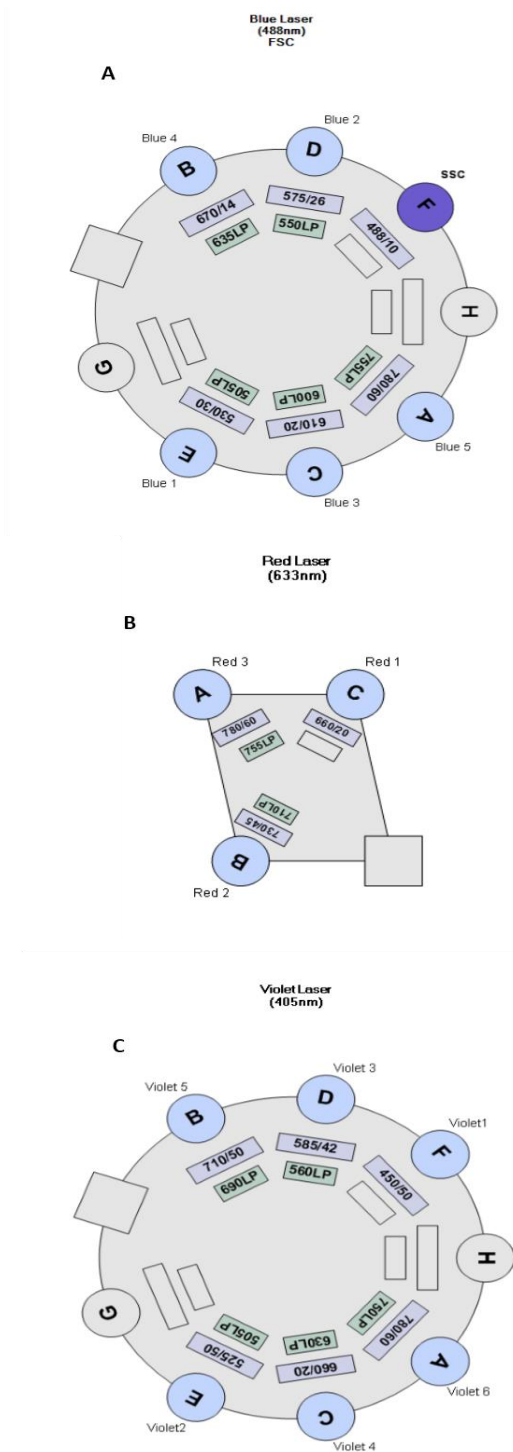
Figure 2 . 1: Laser configuration for flow cytometry acquisition

Configuration of lasers used in all acquistions, A) blue laser, B) red laser and C) violet lasers. The excitation and emission wavelengths are shown by the numbers in purple boxes and filters are shown in green boxes.

To further standardise the cytometer and therefore ensure that experiments can be compared, a rainbow profile was also carried out before each acquisition. The beads used in the experiment were 8-peak beads that contain a mixture of fluorochromes that are spectrally similar to many of the fluorochromes used in flow cytometry, they allow the excitation at any wavelength from 365nm to 650nm. The vial containing the beads was vortexed and one drop was added to a FACS tube with 500µl water and the tub was vortexed again. The beads were then acquired using the rainbow template. For each laser in the configuration there is a histogram on the rainbow profile that shows up to 8-peaks that have been gated. The gates remain in the same position between experiments but before each experiment rainbow beads were acquired and the voltages were changed so that each peak lies within the gate already set. Once all the peaks were within these gates the results were recorded and the settings were copied to the new experiment.

Some of the emission/excitation spectra from the fluorochromes used overlap, in order to overcome this problem fluorescence compensation was used. To do this, one drop of negative compensation beads and one drop of positive compensation beads was added to a FACS tube along with the half the recommended amount of antibody. One tube was prepared for each of the antibodies in the panel. All the channels that were not being used in the experiments were removed and then compensation was run. When each tube was run a histogram was displayed showing a negative and a positive peak, a gate was position around each of these and then compensation was calculated. At this point the cytometer was setup to acquire data from the experiments.

2.6 Cell stimulation with Amyloid beta peptides

The initial stimulations were performed on HEK293 cells expressing TLR2 and peptide was prepared using protocol 1. Cells were plated at a density of 1 x 10^4 cells/well in 100µl normal growth media in a 96-well plate. Plates were incubated at 37°C, 5% CO_2 for 48h or until approximately 80% confluent and the supernatants were removed before stimulation. The peptide was prepared as described previously and the concentration was measured. Peptides were either used immediately after preparation or after 24h of incubation at room temperature. The peptides were used at a final concentration of either 2.5µM or 10µM, stock solutions were diluted in normal growth media and 200µl was added to each well. The cells were then incubated for 18h at 37°C, 5% CO_2 before supernatants were collected and an IL-8 ELISA was performed. 100ng/ml PAM3 (TLR1/2) and 10ng/ml FSL-1 (TLR2/6) were used as positive controls and normal growth media alone or 10ng/ml LPS (TLR4 ligand) were used as negative controls.

A titration of the unmodified Aβ peptide was performed to determine the most appropriate concentration of peptide to use. The range of concentrations tested were 2.5µM, 5µM, 10µM and 20µM. The peptides were all prepared using preparation protocol 1 and diluted in normal growth media to the desired final concentration. Cells were plated 1 x 10^4 cells/well in 100µl normal growth media and incubated for 48h or until approximately 80% confluent. The supernatants were removed and 200µl of media containing the stimulant were added to each well. 100ng PAM3 and 10ng/ml FSL-1 were used as controls for the HEK293 TLR2 cells and media alone or 10ng/ml LPS

were used as negative controls. An MTT assay was performed on the cells once supernatants were removed. This was to check that the peptide was not damaging the cells.

As TLR2 and TLR4 have been implicated in the recognition of Aβ HEK293 cells expressing TLR2 or TLR4-MD2-CD14 were used. A longer aggregation time for the peptide was investigated. Aβ peptide was prepared using protocol 1 and left to aggregate at room temperature for 72h. In addition to investigate whether the negative results observed from these experiments was due to the preparation of the peptide, a new peptide preparation protocol was investigated (referred to as preparation protocol 2) alongside preparation protocol 1. Protocol 2 was designed to generate either oligomers or fibrils of the Aβ peptides and these were used to stimulate the HEK293 cells expressing TLR2 or TLR4-MD2-CD14. In order to determine whether there is a response to Aβ42 peptides only the unmodified peptide (Aβ 1-42) was used in these experiments. The cell types were all treated identically: First the cells were plated at a density of 1×10^4 in 100µl of normal growth media for 48h or until approximately 80% confluent, the supernatants were removed and the cells were stimulated. Peptides were diluted to 10µM in normal growth media and 200µl was added to stimulate. Peptide prepared by protocol 1 had been incubated for 72h and both oligomers and fibrils of the peptide prepared by protocol 2 were used. DMSO treated identically to the protocol 2 peptides, PB treated identically as protocol 1 peptides and normal growth media were used as negative controls and 10ng/ml LPS, 100ng/mlPAM3 and 10ng/ml FSL-1 were used as positive controls and an MTT was performed after these experiments.

2.7 Time-course with Aβ peptides: HEK293 cells

As no response was observed from stimulation with peptide prepared using protocol 1 and 2 a final protocol was tested (protocol 3). This protocol had been used to produce positive results in similar published experiments [274]. A time-course experiment was set up using the unmodified Aβ 1-42peptide to determine if a response could be detected. The peptide was prepared as described above, in either sterile water or sterile 50mM PB, at a concentration of 200μM and incubated at 4°C for 24 to 240h.

Peptide was used to stimulate HEK293 cells expressing TLR2 or TLR4-MD2-CD14, cells were plated at a concentration of 1×10^4 and allowed to incubate until 80% confluent (approximately 48h). After peptide preparation, every 24h a TEM grid was prepared to look at the aggregation of the peptide and the HEK293 cells were stimulated with 15μM peptide for 24h at 37°C, 5% CO_2. The supernatants were collected and stored at -20°C before an IL-8 ELISA was performed. Sterile H_2O, PB (treated the same as the peptide) and normal growth media were used as negative controls and 1ng/ml LPS, 100ng/ml PAM3 and 10ng/ml FSL-1 were used as positive controls.

This experiment was repeated using only fibrillar peptide (peptide incubated for 216h) to investigate the optimum concentration of Aβ peptide to use. The peptide was used at 15μM, 30μM and 50μM and the experiment was performed exactly as before.

These experiment provided information about the optimum conditions for the activation of TLRs by Aβ peptide and the three modified peptides (Aβcit5 1-42, AβpE3 3-42, AβpE3cit5 3-42) were used as well as the unmodified

peptides. For all further experiments the peptides were used at 30μM and were incubated for at least 216h to ensure a fibrillar morphology.

Peptides were prepared as described before, however only water was used for incubation of the peptide. Peptides were all incubated for 216h and were used at a concentration of 30μM. The stimulation of the HEK293 cells was performed exactly as for the timecourse experiment and the controls used were the same. Supernatants were collected and stored at -20°C until an IL-8 ELISA was performed.

2.8 Monocyte Stimulation

To further investigate the activation of TLRs by Aβ, peptides were used to stimulate primary human monocytes isolated from blood obtained from the NHSBT. In addition to the synthetic peptides a recombinant peptide (rPeptide) was also used. All peptides were treated with HFIP, which was then removed by vacuum desiccation, and the remaining films were diluted in sterile DMSO to produce 4mM stock solutions and were incubated for 72h at room temperature. For the monocyte stimulation peptides were used at either 10μM or 25μM. They were diluted to the desired stimulation concentration in normal growth media before stimulation.

Monocytes were thawed from frozen stocks, as described previously, and 2×10^5 cells in 100μl were plated in each well. Stimulation mixes were made up to twice the desired final concentration and 100μl was added to each relevant well. The plates were incubated at 37°C, 5% CO_2 for 18h and the supernatants were collected and stored at -20°C until an IL-1β and a TNFα ELISA was performed. An MTT assay was also performed on these cells. DMSO at the

same concentration as with the peptides and normal growth media were used as negative controls and 10ng/ml LPS, 100ng/ml PAM3 and 1ng/ml FSL-1 were used as positive controls.

In order to determine whether the response seen after stimulation with the commercial peptide was due to the peptide, 50ng/ml PMB was added to the stimulation mixes prior to stimulation of the monocytes. Peptides were prepared as described above but were used at a lower concentration, 0.1, 1 and 10µM. Monocytes were stimulation for 18h at 37°C, 5% CO_2, supernatants were collected and IL-1β and TNFα ELISAs were performed.

After the time-course experiment on HEK293 cells the peptides, prepared using protocol 3 and aggregated for 216h,were also used on monoyctes, all peptides were prepared using protocol 3 and were incubated until fibrillar (216h). The cells were plated at 2 x 10^5 cells in 100µl per well. The peptides were dissolved in normal growth media at twice the desired final concentration and 100µl was added to each relevant well. The peptides were all used at 30µM. The controls were the same as those used for the HEK293 cell experiments.

To ensure that the cryopreservation of the monocytes was not affecting responses to Aβ peptide, freshly isolated monocytes were also stimulated. They were treated exactly the same as the cryopreserved peptides: stimulated with 30µM fibrillar Aβ (prepared using protocol 3) for 18h at 37°C, 5% CO_2. An IL-1β and TNFα ELISA was performed on the supernatants.

2.9 Stimulation of PBMCs: ELISA

PBMCs were isolated from heparinised blood samples, from patients with AD and age-matched controls, as described before. PBMCs were counted and re-suspended at a density of 2×10^6 cells/ml and plated 100µl per well (2×10^5 cells per well). Aβ peptide was prepared using preparation protocol 3 and was aggregated at 200µM for 216h until fibrils had formed. Stimulation mixes of the peptide diluted in media were prepared at twice the final concentration and 100µl was added to the wells. Each stimulation condition was repeated in triplicate in the 96-well plate. 10ng/ml LPS was used as a positive control and media alone and water (treated as though used for peptide preparation) were used as negative controls.

The PBMCs were incubated for 18-24h at 37°C, 5% CO_2, the supernatants were collected and stored at -20°C. IL-1β and TNFα ELISAs were then performed on the supernatants. All four of the Aβ peptides, prepared using protocol 3, were used at a concentration of 30µM, the stimulation mixes were made at twice the final concentration in normal growth media and 100µl was added to the wells. LPS was used as a positive control and media alone and water (treated as though it contained peptide) were used as negative controls.

2.10 PKH/Proliferation assay

For the proliferation assay PBMCs were first isolated as described previously and were counted in 1ml DPBS, as FBS in media interferes with the reaction of the PKH-lipid dye. They were then centrifuged at 400g for 5 min at room temperature to remove the DPBS before staining. The supernatant was

discarded and the remaining pellet was resuspended in 1ml diluent C and carefully pipetted to mix. The reaction mixture was made by adding 4μl PKH dye to 1ml diluent C and mixing thoroughly. This was then added as quickly as possible to the cell suspension for 2min and was continuously gently pipetted throughout that time. 2ml FBS was added to the reaction for a further 2min to stop the reaction, again the cells were gently pipetted for the whole time. The cells were centrifuged at 500g, 10min at room temperature and the supernatant was discarded. The remaining cell pellet was resuspended in 25ml PKH-media (RPMI, L-glutamate, P/S and autologous serum (heat treated at 56°C for exactly 30min) and centrifuged again, 500g for 10min. This washing step was repeated twice, after the final wash supernatant was discarded and cells were resuspended in 1ml PKH-media. All of the centrifugation steps were carried out with the heraeus centrifuge). The cells were counted before being diluted to a density of 1×10^6 cells/ml and 1ml was aliquoted into FACS tubes for stimulation. At this point, 1 tube containing cells was put aside to use as compensation during acquisition.

For the first proliferation experiments, PBMCs were stimulated with Aβ 1-42, diluted from a 100μM stock solution in PB to a final concentration of 5μM and were incubated at 37°C, 5% CO_2 for 5 days. All four of the peptides were used to stimulate PBMCs. The peptides used in these experiments was prepared using protocol 1 and was used immediately after preparation to ensure the peptide had an un-aggregated morphology.

After incubation, PBMCs were stained with antibody in order to look at the proliferation of T-cells. The panel used for the initial proliferation experiments used CD3-PE . The general antibody staining protocol described

above was used and as only surface markers were being investigated the experiment was stopped after the lysis and wash steps.

As no proliferation could be seen in response to Aβ 1-42 in these first experiments the protocol was altered to include a restimulation step. After the initial stimulation, cells were incubated for 72h and then restimulated and incubated for a further 72h before being stained with antibody. Cells were restimulated by very carefully removing 100μl of media from the FACS tubes and adding 100μl of normal growth media containing the peptide. The peptides were prepared by dissolving them in DMSO to a concentration of 2mM, as had been described in published data [308, 309], and was used immediately at a concentration of 10μg/ml. In addition to the Aβ 1-42 peptide, a synthetic Aβ 1-42 peptide purchased from JPT was also used and treated exactly the same as the Aβ 1-42 peptide.

The antibody panel was changed to include CD4-BrillaintViolet 421. This allowed the gating of a more specific subset of T-cells. The staining of the PBMCs was performed using the general protocol for antibody staining and as only surface markers were being investigated, the experiment was stopped after the lysis and wash steps. The cells were then acquired using an LSR II flow cytometer.

These experimental changes made no difference to proliferative response of T-cells to Aβ so experiments were optimised further to determine if it was possible to observe a response. The Aβ peptide was treated as before, dissolved in DMSO to a concentration of 2mM and used to stimulate PBMCs at a concentration of 10μg/ml. PBMCs were still stained with the PKH dye,

stimulated and incubated for 72h before media was refreshed and cells were restimulated. However, it has been reported that the addition of interferon alpha (IFNα) reduces the amount of background proliferation so smaller responses could be more easily detected. For this reason IFNα was also included in the experiments. Before stimulating PBMCs, IFNα was added to the cells at a concentration of 125IU/ml. Each experimental condition included a duplicate with IFNα and a duplicate without IFNα. The different conditions are shown in table 2.3 below.

Table 2 . 12: Stimulation conditions for proliferation with IFNα

First Stimulation	Restimulation	IFNα
Aβ 1-42	Aβ 1-42	+
Aβ 1-42	Aβ 1-42	-
DMSO	Aβ 1-42	+
DMSO	Aβ 1-42	-
DMSO	DMSO	+
DMSO	DMSO	-
PHA	DMSO	+

After restimulation, PBMCs were stained with antibodies. To investigate in more depth whether different subsets of T-cell responded to the peptide a CD8 antibody was also included in the panel. Cells were stained with CD3-Pacific Blue, CD4-PerCP and CD8-APCH7. The staining process was performed using the general protocol and the experiment was stopped after the lysis and wash step.

In addition to the full length peptides, 8 shorter length Aβ peptides containing 15 amino acids were also used in the proliferation experiments. The MHCII binding predictions were made using the IEDB analysis resource Consensus tool [317, 318]. They were prepared using protocol 3, were used at immediately to ensure they did not have fibrillar morphology and at a concentration of 1μM (4μg/ml). This lower concentration was used as the literature suggests that less peptide epitope is needed than full length peptide. PBMCs were treated in exactly the same way as with the other PKH experiments; cells were incubated for 72h and then restimulated with the same concentration of peptide and incubated for a further 72h, the cells were then stained using the antibody panel that included CD3, CD4 and CD8 to determine if proliferation could be seen.

2.11 Phosphorylation of PKC-δ and PKC-ζ

Lanuti et al, have reported an Aβ-specific population of T-cells that exhibit a bright PKC-δ and PKC-ζ phosphorylation in patients with AD. To investigate whether this response could be replicated and whether the same could be seen with the PTM variants of Aβ, PBMCs were stimulated with Aβ 1-42 peptide and stained with antibodies for P-PKC-ζ and P-PKC-δ.

Initially only the Aβ 1-42 peptide was used, it was dissolved in DMSO to a concentration of 2mg/ml and used for stimulations. PBMCs were isolated as previously described, re-suspended at a density of 1×10^6 cells/ml in normal RPMI growth media and 1ml was aliquoted into FACS tubes. Aβ 1-42 peptide at a final concentration of 10μg/ml was used to stimulate PBMCs and the cells were incubated at 37°C, 5% CO_2 for either 72h. After this incubation

period 100µl of media was removed, taking care not to disrupt the cells, and 100µl fresh media containing Aβ was added to the tubes, the final concentration of the Aβ 1-42 peptide was the same as used originally. The restimulated cells were incubated for 2h at 37°C, 5% CO_2 before 10µg/ml BFA was added for the final 4h of incubation. This was in order to prevent the transport and release of intracellular markers. After the final incubation of the PBMCs cells were used for staining with antibody. A combination of 25ng/ml Phorbol-12-Myristate-13-Acetate (PMA) and 500ng/ml ionomycin was used as a positive control and DMSO and media alone were used as the negative controls.

The initial protocol for staining with antibodies was the same as described above, however after the permeabilisation step the protocol changes due to the antibodies used. Firstly, surface markers for T-cells were used: CD3-Pacific Blue, CD4-PerCP and CD8-APC/H7. This was followed by the normal lysis, permeabilisation and wash steps. The P-PKC-δ and P-PKC-ζ antibodies were not being conjugated to any fluorophore so an anti-goat FITC secondary antibody was used. After the cells were stained with surface antibodies, lysed and permeabilised the two P-PKC antibodies in their unconjugated form and the CD40L-PE and IL-2-APC antibodies were added to the cells for 30min, 4°C in the dark. A secondary FITC antibody (goatαrabbit from Sigma) was added to the tubes for a further 30min, 4°C in the dark. The stained cells were washed as normal and fixed with 0.5% PFA before a final wash was performed. They were then stored at 4°C until acquisition. Acquisition was always performed within 48h of antibody staining.

2.12 PBMC stimulation with fibrillar Aβ

To try to characterise the cell types that were able to produce cytokines in response to the Aβ peptides, intracellular staining of PBMCs was performed. The cells were isolated as normal counted, and resuspended at 1×10^6 cells/ml, 500μl of the cells were then aliquoted to each well. The PBMCs were stimulated with 30μM Aβ for 2h before 10μg/ml BFA was added for 14h at 37°C, 5% CO_2. The peptides were all prepared using protocol 3 and were incubated until they had a fully fibrillary morphology, 216h. 10ng/ml LPS and 100ng/ml PAM3 were used as positive controls (PBMCs were stimulated with BFA for 4h) and media alone and HFIP in water were used as negative controls.

After incubation cells were stained with antibodies using the same general protocol as all other experiments. These cells were stained with both surface and intracellular antibodies so the full protocol was used. The antibodies used in these experiments were: CD3-Pacific Blue, CD14-PerCP, HLA-DR-PeCy7, TLR4-PE and TLR2-AlexaFluor647 for the surface and IL-1β-FITC and TNFα-AlexaFluor700 were used intracellularly. Cells were acquired using LSR2 and were always acquired with 48h of staining.

2.13 Neutralising Antibodies

To investigate whether TLR2 was activated by Aβ peptides, antibody that neutralises this receptor was used. Firstly, the antibody were titrated to determine the best concentration to use: PBMCs were isolated as normal and resuspended at a density of 2×10^6 cells/ml and 175μl were aliquoted into

FACS tubes. Neutralising antibodies were first resuspended in sterile water to a stock concentration of 100µg/ml and were added to the cells at a concentration of either 1µg/ml, 5µg/ml or 10µg/ml and the tubes were gently vortexed. The cells were incubated with the antibodies for 1h at 37°C, 5% CO_2. To test whether the antibodies were working, the positive control for TLR2 (PAM3) was used at concentrations of 100ng/ml. 5µg/ml of BFA was then added to each tube and the cells and vortexed and the cells were incubated for 4h, 37°C, 5% CO_2.

The cells were then used for antibody staining following the normal protocol – the antibody panel used in these experiments contained CD3-Pacific Blue, CD14-PerCP and HLA-DR-PeCY7 and IL-1β-FITC and TNFα-ALexaFluor700. The stained cells were acquired using LSRII less than 48h after staining. After the titrations the concentration of α-TLR2 antibody used for all remaining experiments was 10µg/ml.

These antibodies were used to investigate whether cytokine signals observed after stimulation with Aβ peptides remains when TLR2 was blocked. For these experiments Aβ peptides made in two different batches were tested. As before PBMCs were isolated and 3.5×10^5 cells in 175µl were aliquoted to FACS tubes and neutralising antibodies were added, vortexed and cells were incubated for 1h. All four peptides from both batches were tested and were used at a concentration of 30µM, cells were stimulated for 2h at 37C, 5% CO_2 before 5µg/ml BFA was added for 14h. The cells were then stained as described for neutralising antibody titrations. Peptides from each of the batches were used alongside each other to ensure conditions were consistent and to confirm that any differences were not due to experimental conditions.

2.14 Statistical analysis

The statistical test performed depended on the experiment that had been carried out. For ELISA and ICS experiments where it was investigated whether there was a significant increase in the response observed after stimulation compared with the unstimulated control, a one-tailed t-test was used if the data had a normal distribution or a one-tailed Wilcoxon's matched paired test was used if the data did not have a normal distribution.

For experiments where the responses seen from different groups was compared, for example patient responses compared to control responses, a two-tailed unpaired t-test was used for normally distributed data and a two-tailed Mann-Whitney test was used if the data was not normally distributed.

To determine whether the data had a normal distribution or not a D'Agostino and Pearson omnibus test was performed. Statistics was only carried out when data had been replicated three times or more.

2. 15 Patient and control recruitment

Ethical approval was given for patient and control recruitment by the NHS research ethics committee (REC), Cambridgeshire 3 REC: application reference 10/H0807/98.

In order to investigate immune responses to Aβ peptides immune cells were isolated from blood samples obtained from patients with AD and age-matched healthy controls. All the participants were recruited from the Cognitive Disorders clinic at Hurstwood Park Neurological Centre at the Princess Royal University Hospital (PRUH) in Haywards Heath. Patients with AD were only

approached if they were attending the clinic and an invitation letter and information sheets about the study were sent a week before their appointment to allow them adequate time to read through the information.

There were a number of exclusion criteria for patient participation: Anyone with severe AD (based on a mini mental state exam (MMSE) score of <10), anyone with mixed dementias (i.e. AD with vascular dementia), people with dementia due to non-AD causes and any participant with inadequate venous access. In addition the inclusion criteria were that the participants had a diagnosis of AD according to NINCDS-ADRDA international standard diagnostic criteria and an MMSE score of 10-20 (moderately severe AD) or >20 (mild AD) and controls were required to be age-matched cognitively intact individuals.

At the clinic the patients were approached by either the doctor or nurse to determine if they were willing to participate in the study. In addition to ensuring that the patient met the inclusion criteria the doctor or nurse also assessed whether the patient was able to give consent themselves. If they were not, the carer accompanying the patient would be asked to act as a consultee. They were asked to read through the information sheets to make sure they knew what the study was about and were given an opportunity to ask any questions about the study or the procedure so they could make an informed decision. If they were happy for the patient to participate in the study they were asked to sign the consultee consent form. If the patient was deemed able to give consent themselves and was willing to partake in the study they were required to sign the patient consent form.

Age-matched controls were mainly recruited from the patient's environment for example the carer of the patient. These people were aware of the study as information sheets had been sent to the patients before their appointments, there were also posters displayed on the walls of the clinic asking for controls to participate. Therefore anyone visiting Hurstwood Park Neurological Centre could volunteer to be part of the study. If either people visiting the clinic or the centre were willing to participate they could approach either the doctor or the nurses involved in the study. They were then able to read through the information sheets, ask any questions and could sign the consent forms.

If patients or controls were willing to participate in the study but were unable to do it on the day of their appointment, they were also given the option of returning at a later date that suited them better or at their next appointment. In addition, when consent was taken each patient and control was also asked if they would be willing to donate another blood sample if the study required it.

Once consent had been taken the blood samples could be collected. This was done by venepuncture of the antecubital fossa by a trained nurse or doctor. Approximately 35ml of blood was taken in a variety of blood tubes: 3 sodium heparin tubes, 2 serum tubes and 1 EDTA tube. Each of the blood tubes was labelled with the patient code, all patient codes followed the same template e.g. HP_PTM_mm/dd/yy_AAABB_001, where the date is when the sample was taken, AAA are the first three letters of the participants surname, BB are the first two letters of the participants first name and 001 is the number of the sample (this increases with each new participant).

All the blood tubes were taken to the microbiology department of PRUH and transported to the virology department at RSCH using hospital sample transportation. The virology department at RSCH took one of the serum tubes to test whether patients/control were CMV positive and the remaining tubes were collected for experiments investigating the immune response to Aβ peptides.

3. The aggregation of amyloid-beta and variants resulting from post-translational modifications

3.1 Introduction

The build-up of the peptide Aβ into insoluble plaques in the brain is one of the main hallmarks of AD [319, 320] and is thought to play a role in its pathogenesis [22]. However, Aβ is not only found in disease it is also produced in healthy individuals [24] and Aβ monomers have been proposed to play a role in synaptic plasticity and neuronal survival [70, 71]. Initially it was proposed that the abnormal build-up of Aβ in the brains of people with AD leads to the neurodegeneration observed in the disease, however more recent studies have shown an improved correlation with cognitive decline and earlier intermediates, oligomers, of the Aβ peptide [28, 95, 114, 321, 322].

The plaques found in AD are composed mainly of insoluble fibrils of Aβ, however the peptide also exists in several different states of aggregation including monomers, oligomers and protofibrils all of which are soluble [85, 323-327]. In addition to the variety of morphologies of Aβ there are also a number of different isotypes determined by the amino acid position at which the peptide is cleaved [96, 328]. The most common forms range from 38 to 43 amino acids, however the isotype most implicated in AD is Aβ42 [51, 194, 329, 330]. This form has been observed to be the most amyloidogenic, most neurotoxic and in AD the amount of Aβ42 produced increases [101, 195, 196, 331].

Several studies have investigated how Aβ peptides are able to aggregate. It has been reported that different sections of the amino acid sequence may be

important in promoting aggregation [85, 332]. For example, a five amino acid sequence from residues 16-20, KLVFF, has been described as the hydrophobic core of the peptide and a number of studies have suggested its importance in the aggregation of Aβ [333-336]. Furthermore, elongating the C-terminal end of Aβ increases the propensity of the peptide to aggregate. This can be seen by the increase in aggregation of Aβ42 compared with Aβ40 [196, 337].
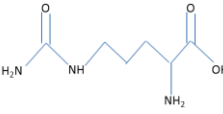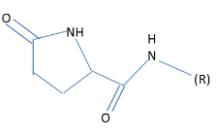
Furthermore, there are a number of post-translational modifications that exist in Aβ. One of these PTMs is a pyro-glutamate modification at amino acid position 3 (AβpE3) [59, 63, 72, 217, 224]. Aβ with this modification is found in high abundance in AD plaques and studies have proposed that it has an increased rate of aggregation when compared with unmodified Aβ42 [72, 76]. A second modification that is found in several proteins, including Aβ42 as well as others such as prion protein, is citrullination [60, 61, 338]. Less is known about the effect this modification has on the properties of Aβ42, however it is possible that there will be differences in the aggregation of Aβ as well as structure and its effects in AD, for example responses of the immune system.

The aim of these experiments was to determine the best protocol and condition to produce a range of Aβ species. In addition the differences in the aggregation of the Aβ peptides with post-translational modifications was investigated and transmission electron microscopy was used to determine whether the morphology is affected. Further to this, the aggregation of short length peptides, containing 10 amino acids were also investigated to examine which if any are able to aggregate.

3.2 Results

Four peptides were investigated, the unmodified Aβ1-42, and three PTM

variants:

Table 3 . 1: Full length Aβ peptides

Aβ 1-42	Unmodified peptide	1-DAEFRHDSGYEVHHQKLVFFAEDVGSN KGAIIGLMVGGVVIA-42
Aβcit5 1-42	Citrullination – Amino acid 5	1-DAEF-citr-HDSGYEVHHQKLVFFAEDVGSNKGAIIG LMVGGVVIA-42
AβpE3 3-42	Pyro-glutamate – Amino acid 3	pE-FRHDSGYEVHHQKLVFFAEDVGSNK GAIIGLMVGGVVIA-42
AβpE3cit 5 3-42	Citrullination – amino acid 5 and Pyro-glutamate – amino acid 3	pE-F-citr-HDSGYEVHHQKLVFFAEDVGSNKGAIIG LMVGGVVIA-42

To investigate the role of Aβ and its PTM variants in immune responses we

first had to determine the optimum conditions in which to produce fibrils of

peptides as well as intermediate species. In these experiments the formation of

different morphologies of Aβ was investigated, the four structures defined

here are oligomers, protofibrils, 'associated protofibrils' and fibrils.

Oligomers appeared as spherical structures in the images whereas protofibrils

were elongated and often curved. There were areas of 'associated protofibrils'

where the short protofibrils have continued to elongate and associate. The final structure observed were the long unbranched mature fibrils [339]

3.2.1 Preparation with protocol 1

For the first experiments preparation protocol 1 was used [315] to prepare all four of the Aβ peptides. This protocol has been proposed as a successful method for preparing peptide to form a range of Aβ morphologies resulting in the final formation of the mature, insoluble fibrils. Peptides were dissolved in HFIP to a concentration of 1mM, and then sonicated and vortexed for 1 minute each. The HFIP was evaporated and the remaining film was dried using vacuum desiccation. The peptide was dissolved in dry DMSO to a concentration of 1 mM. The DMSO was removed using desalt spin columns equilibrated with 50 mM PB (pH7.4). The final peptide was dissolved in PB and the concentration was measured using a spectrophotometer. TEM grids were produced immediately or after 24h incubation. The least aggregated form of the peptide that was generated from these experiments were oligomers which could be seen in images from all four of the peptides. The images were analysed qualitatively, visually determining the structures that were formed, as well as quantitatively, using ImageJ to measure the area of oligomers and the area of associated protofibrils as well as the length of the small individual protofibrils.

One initial observation of the TEM images, immediately after preparation, was that all four peptides had a largely non-aggregated morphology, however there were some differences between the different peptides. Aβ 1-42, AβpE3

3-42 and AβpE3cit5 3-42 all displayed a variety of different structures in their images but Aβcit5 1-42 displayed only an oligomeric morphology.

All four of the peptides showed oligomers in the TEM images at this timepoint. In addition both via visual analysis and measuring the oligomeric area suggested that the size of the majority of oligomers was consistent between all of the peptides. The general range observed was between 200nm^2 and 400nm^2, however for all of the peptides there were some outside this range but the distribution of areas was very similar in all cases (Fig 3.1).

This is where the similarities between Aβcit5 1-42 and the other peptides end. In addition to oligomers there was also evidence of oligomers associating and protofibrils being present. Furthermore, there are some areas where the protofibrils have appeared to associate, especially in the AβpE3 3-42 images. The individual protofibrils from the Aβ 1-42 and AβpE3cit5 3-42 images show the main distribution between 50nm and 100nm, while the AβpE3 3-42 peptide appeared to have a number of protofibrils of this length there were also several that had elongated up to 200nm. As mentioned before, there was also evidence of protofibril association which was most obvious in the AβpE3 3-42 images although the AβpE3cit5 3-42 peptide also showed evidence of this. All the areas that were visible were small regardless of the peptide.

The results obtained in these experiments show that the preparation protocol was successful in producing non-fibrillar structures of Aβ peptide from the unmodified as well as the PTM variants (Fig3.1) although it appeared that this particular protocol was more successful at forming the smaller structures in some peptides (Aβcit5 1-42) compared with others (AβpE3 3-42).
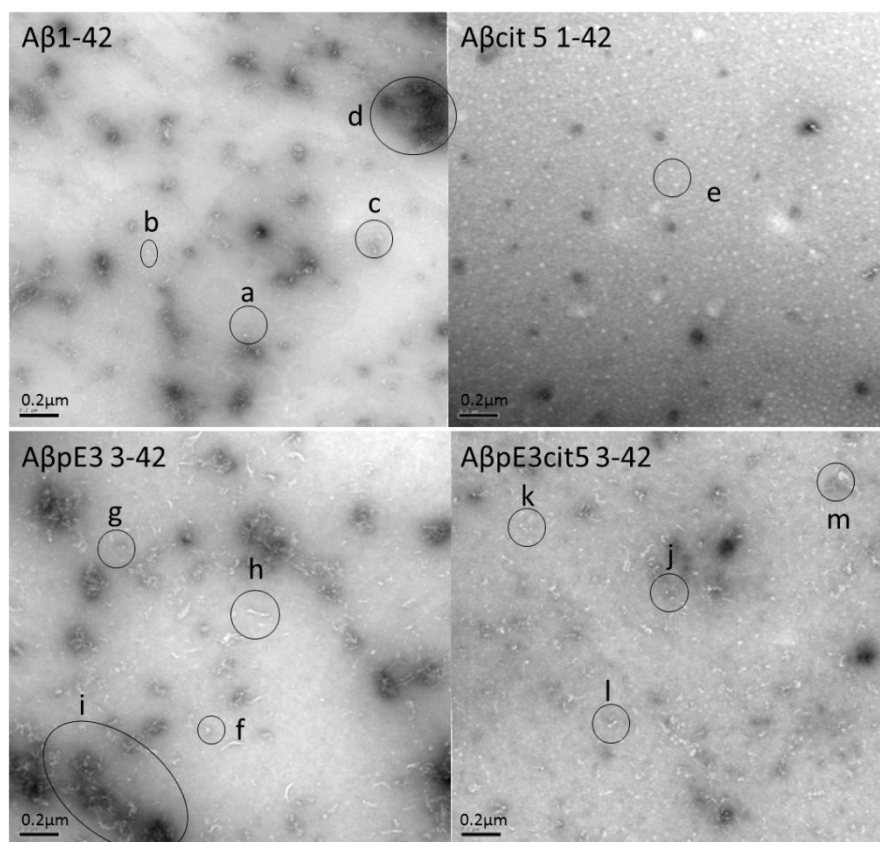
Figure 3 . 1: Immediately after preparation unmodified Aβ peptide and PTM variants all display an unaggregated morphology.

All peptides were prepared using protocol 1, peptides were disaggregated and dissolved in dry DMSO. DMSO was removed, peptides were diluted in 50mM PB and TEM grids were made immediately. The black circles highlight the different structures observed in the images. Individual oligomers shown by Aβ.a, Aβcit5.e, AβpE3.f and AβpE3cit5.j. Aligned oligomers in Aβ.b, AβpE3.g and AβpE3cit5.k. Single small protofibrils displayed in Aβ.c, AβpE3.h and AβpE3cit5.l. The final structures shown, areas of associated protofibrils, in Aβ.d, AβpE3.j and AβpE3cit5.m. Peptides were all incubated at 50μM.

Grids were also made from the same preparation of Aβ peptides after incubation at room temperature for 24h. When looking at these images there were clear differences between the peptides. Aβ 1-42 still maintained a mixed morphology and Aβcit5 1-42 had formed a very similar morphology, AβpE3 3-42 was composed almost entirely of areas of associated protofibrils and AβpE3cit5 3-42 had only large oligomers.

Aβ 1-42 still had a mixed morphology, however there were almost no visible oligomers. The main morphology observed were protofibrils the average length and distribution of which had not changed from the 0h timepoint. However, there were several areas where the protofibrils had elongated and associated to form clusters (Fig 3.2.A2). Aβcit51-42 had the most similar morphology to Aβ, although in addition to protofibrils and associate areas there were still oligomers present, many of which had aligned and begun to associate (Fig 3.2.Aβcit5 1-42). In addition it appeared that the associated areas of protofibrils were larger than the areas from the Aβ 1-42 peptide although there were no differences in the length of the individual protofibrils between Aβ 1-42 and Aβcit5 1-42.

The AβpE3 3-42 peptide showed the greatest amount of aggregation, there were no longer any visible oligomers or individual protofibrils, only areas where the peptide had elongated and associated. As the protofibrils had all associated it was not possible to measure the length as it was not clear where the protofibrils started or ended. However, visual analysis of all the AβpE3 3-42 images showed that the length of the protofibrils was far greater than in any other image from any other peptide. Furthermore, the areas where the

protofibrils had associated were far larger than any other peptide, when the areas were measured the size of the AβpE3 3-42 24h incubated peptide were almost all over 10 fold higher than any other areas observed (Fig 3.2.AβpE3 3-42).

The morphology of AβpE3cit53-42 was different to any of the other peptides, only oligomers were observed, however these were larger than any other oligomer observed previously from any of the peptides. Further to this the peptide appeared to associate to cover large areas of the TEM grid without forming a fibrillar morphology (Fig 3.2.AβpE3cit5 3-42).

This data suggested that 24h was not long enough to produce fibrils from any of the peptides after protocol 1 was used to prepare the peptide, although the AβpE3 3-42 peptide had aggregated to a greater extent than any of the other peptides.
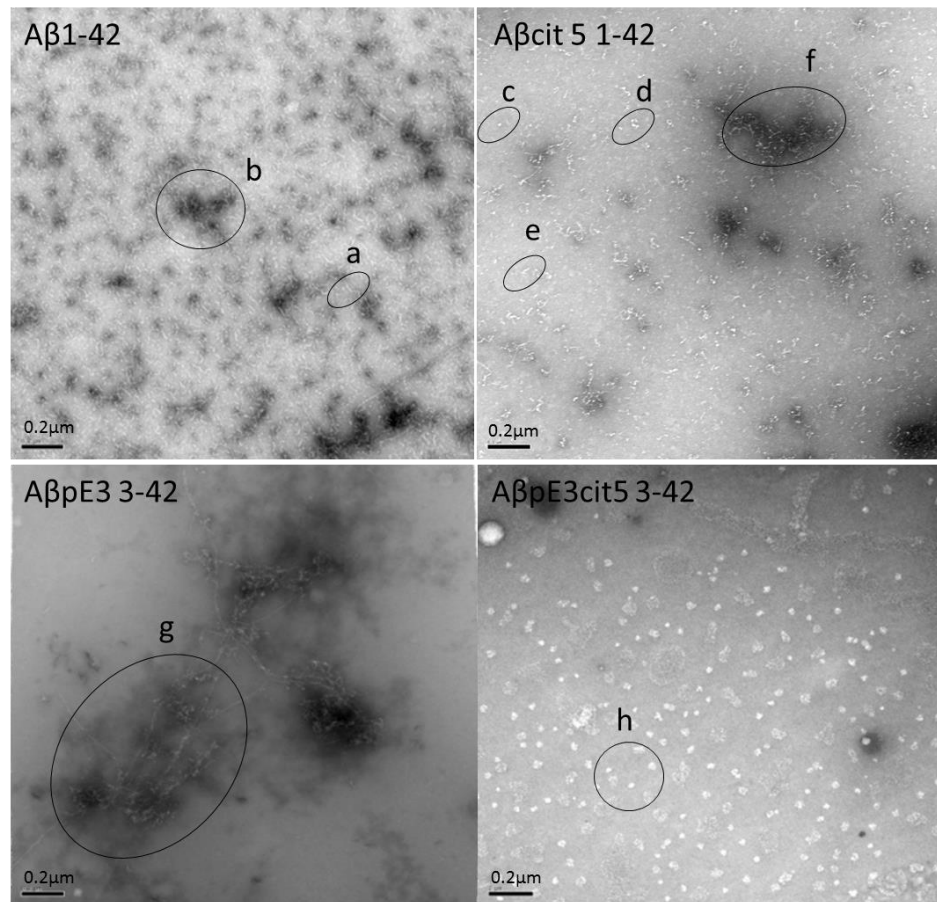
Figure 3 . 2: Only peptide with a pyro-glutamate modification formed large protofibrils after 24h incubation**.**

Peptides were prepares using protocol 1, peptides were disaggregated and dissolved in DMSO which was removed and peptides were dissolved in 50mM PB. They were allowed to aggregate for 24h and TEM grids were made. The important structures are shown by black rings. Aβ 1-42.a, and Aβcit5 1-42.e display individual protofibrils. Aβcit5 1-42.c and AβpE3cit5 3-42.h show single oligomers and Aβcit5.d shows aligned oligomers. Aβ 1-42.b, Aβcit5 1-42.f and AβpE3 3-42.g all highlight areas of associated protofibrils. Peptides were incubated at 50μM at room temperature.

As 24h was not a sufficient time to form fibrils of Aβ therefore to investigate whether increasing the incubation time would produce fibrils the Aβ peptides was incubated for 0h, 24h and 72h at room temperature. It had been prepared in exactly the same way as in previous experiments and TEM grids were made at each of the timepoints to monitor any changes in the peptide aggregation.

Several images were taken from the grids for Aβ 1-42 at each timepoint and a representative image is displayed in figure 3.3 At 0h and 24h the morphology of the peptide was as described previously, briefly there was a mixed morphology of oligomers and protofibrils at 0h and a mixture of protofibrils and areas of protofibrils at 24h. Despite the lack of oligomers at 24h the length of protofibrils was the same at both timepoints in addition a greater number of areas of protofibrils with a greater size were also observed at 24h (Fig 3.3.A,B).

The overall morphology of Aβ 1-42 after 72h of incubation is very similar to that seen at previous time-points. There was evidence of a small number of oligomers but the main morphology was protofibrils and associated areas. The lengths of the protofibrils had not increased at 72h compared with 0h or 24h however there were more areas of associated protofibrils that were larger in size (Fig 3.3.C). Although there was evidence of some aggregation by the peptides using this protocol it was not possible to detect any fibrils of any of the peptides, therefore a new protocol was investigated.
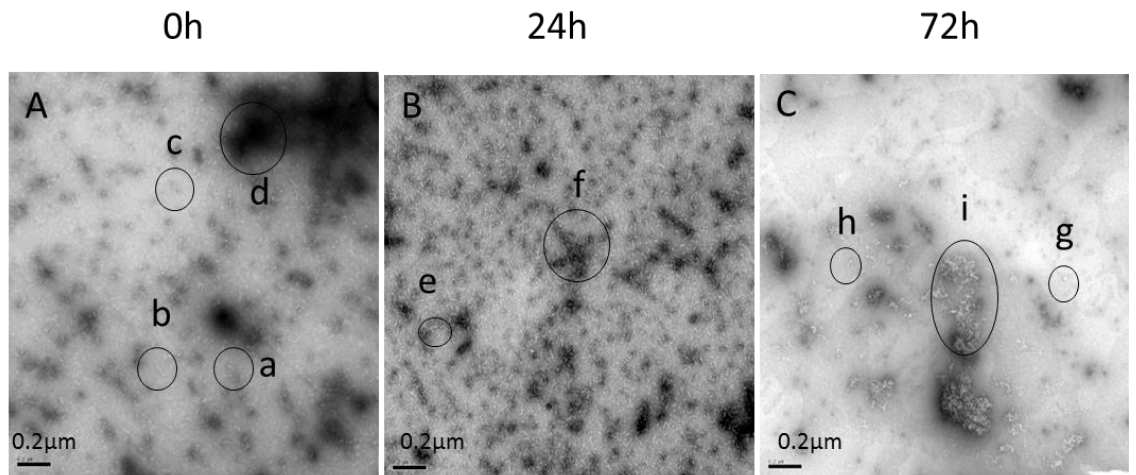
Figure 3 . 3: Unmodified Aβ 1-42 peptide does not form fibrils after 72h incubation at room temperature

Unmodified peptide was prepared using protocol 1, peptide was disaggregated and dissolved in DMSO. The DMSO was removed and diluted in 50mM PB. A TEM grid was prepared and an image of the peptide was taken immediately after preparation (A), or after aggregation for 24h (B) or 72h (C) at room temperature. The black rings highlight different structures observed in each image. Oligomers are shown by a and g, the oligomers had aligned in b. c, e and h all display individual protofibrils and d, f and and i show areas of associated protofibrils.

3.2.2 Preparation with protocol 2

The peptide was prepared using the method described by Dahlgren et al [316].

To form mature fibrils of the peptide the lyophilised peptide was initially

dissolved in HFIP which was immediately removed using vacuum

desiccation. The remaining film was dissolved in dry DMSO and then diluted

in HCl and stored at 37°C for 24h. TEM was performed on the peptide to

determine whether this method produced fibrils. Fig 3.4 shows that the

peptide was able to form fibrils under these conditions.
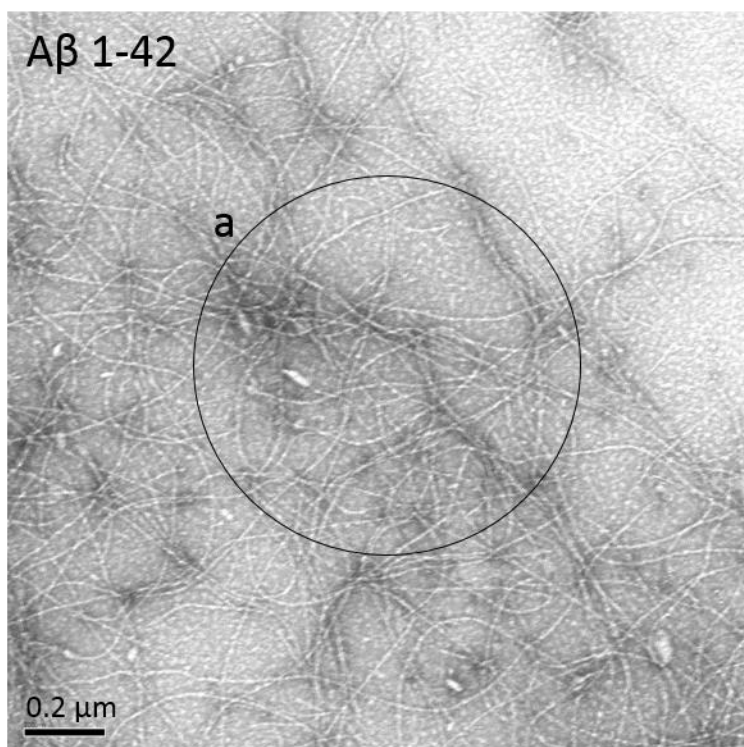
Figure 3 . 4 : Aβ 1-42 prepared using protocol 2 produces fibrils.

Aβ 1-42 was prepared was disaggregated with HFIP, the HFIP was removed
and the peptide was dissolved in dry DMSO before further dilution in HCl. It
was then incubated for 24h at 37°C. Black ring (a) shows an example of
mature fibrils.

3.2.2 Preparation with protocol 3

As the first protocol was unsuccessful in producing mature fibrils of Aβ peptide a new protocol was used that had been shown both to produce fibrils and to induce a response in similar immune response experiments as those performed in this thesis. This new protocol initially used only the unmodified peptide, Aβ, and followed the same initial steps as used previously in that the lyophilised peptide was weighed and diluted in HFIP. The HFIP was removed using vacuum desiccation and the resulting film was dissolved in water or in PB. Once the concentration of the peptide had been calculated using its absorbance at 280nm the peptide was incubated at a concentration of 100μM at 4°C for 24 to 240h.

A TEM grid was produced at several time-points throughout the incubation and the different structures were identified at each of these points. The first grid was produced 24h after preparation, and the main structure observed in the TEM images was oligomers, regardless of what the peptide had been incubated in. No other structure was observed in any of the PB incubated peptide (Fig 3.5.A1) but a small number of protofibrils were observed in some images of peptide incubated in water (Fig 3.5A1,A2). In addition there were some small areas of associated protofibrils, although there were very few of them (Fig 5d). After 96h when the second set of TEM images were taken the overall morphology of the PB incubated peptide remains in an oligomeric morphology, the size of the oligomers remained the same as at 24h and there was no evidence of any other structure in these images (Fig 3.6B).

However, the water incubated peptide showed some evidence of aggregation. Oligomers were still visible in the images (Fig 3.5e,f) but there were also several protofibrils (Fig 3.4g) as well as areas of associated protofibrils (Fig 3.5h), most of these were small areas although some had associated into larger areas (Fig 3.5B.1). The length of the protofibrils was larger than those seen at 24h and many of them have begun to elongate more extensively than seen before in the Aβ peptide.

The third time-point was after 168h of incubation. The peptide incubated in water had begun to form a fibrillar morphology (Fig 3.5C). The fibres appeared longer than in previous images and were observed to extend between areas of associated protofibrils, connecting them. There were no longer any small protofibrils or areas of associated protofibrils that were not connected to other areas, this was the most extensive aggregation seen in any TEM image from any peptide up to this point. In contrast the PB incubated peptide still remained in an oligomeric morphology.

The final images were taken 240h after peptide preparation. The PB incubated peptide still had an oligomeric morphology (Fig 6C), there was no evidence that protofibrils of any kind had developed during incubation suggesting that no aggregation had occurred. However the water incubated peptide was in a fully fibrillar morphology (Fig 5D). Additionally there were very few areas within the images that did not display the same fibrillar morphology suggesting that all the peptide has associated to form fibrils.
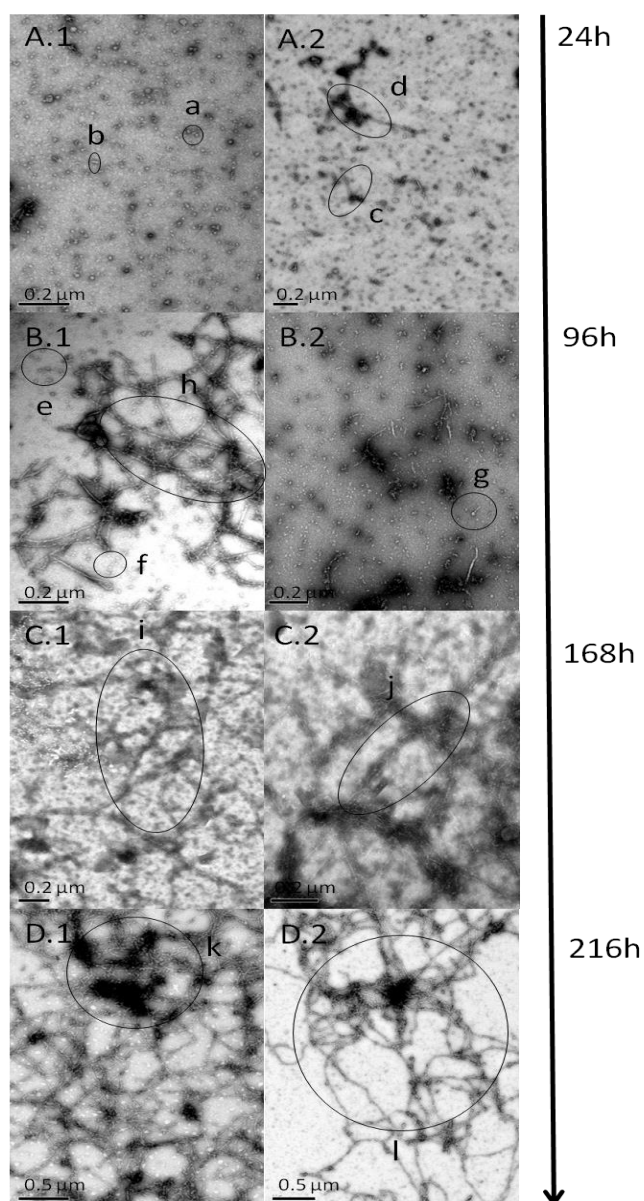
Figure 3 . 5: Unmodified Aβ 1-42 peptide aggregates to a fibrillar morphology after incubation in water for 240h

Peptide was prepared using preparation protocol 3. Peptide was disaggregated in water then dissolved in water. The peptide was aggregated at 4°C for 24h to 216h and TEM grids were prepared at four timepoints. Black rings highlight the important structures observed at each timepoint. Two images are shown from timepoint: 24h timepoint (A.1 and A.2), 96h timepoint (B.1 and B.2), 168h timepoint (C.1 and C.2) and 216h timepoint (D.1 and D.2). Oligomers shown by a and e and have aligned in b and f. individual protofibrils are displayed by c and g. Associated areas of protofibrils are shown by d, h, i, j, k and l. All peptides were incubated at 100μM. The length of the scale bar displayed on each image.
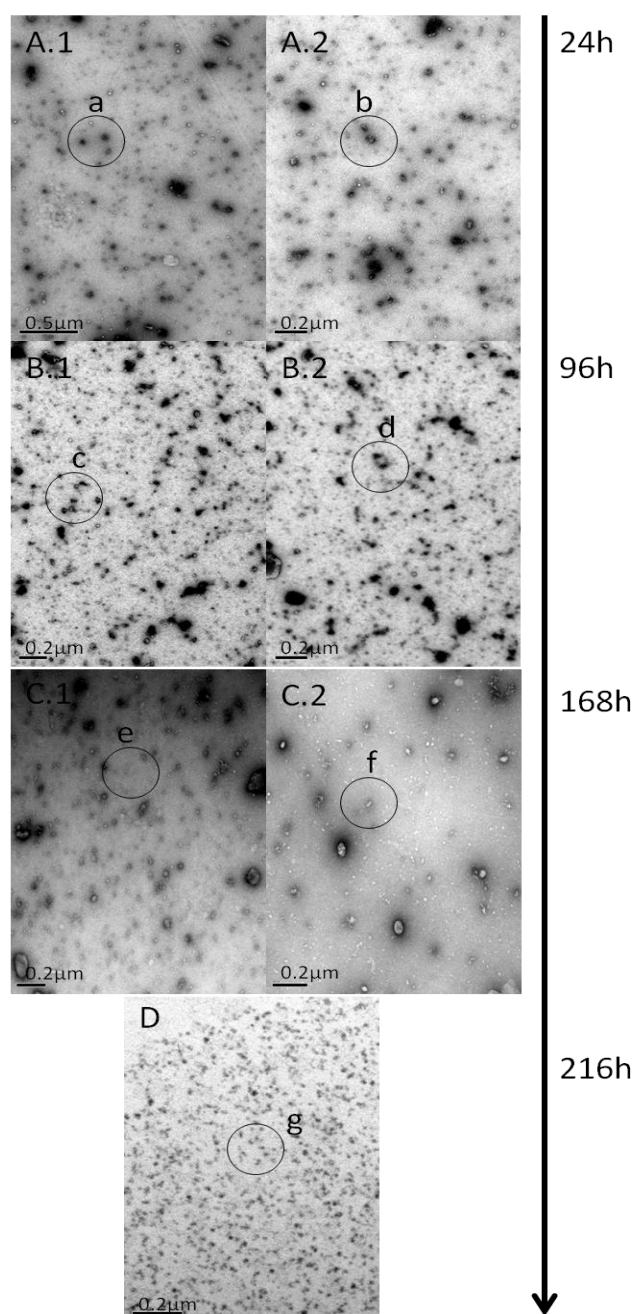
Figure 3 . 6: Unmodified Aβ 1-42 peptide does not aggregate when incubated in PB

Preparation protocol 3 was used to prepare the peptide. Peptide was disaggregated then dissolved in PB and left to aggregate at 4°C for 24 to 216h. The black rings surround the structures of interest in each image. Two images are shown from each timepoint: 24h timepoint (A.1 and A.2), 96h timepoint (B.1 and B.2), 168h timepoint (C.1 and C.2) and 216h timepoint (D.1 and D.2). Only one structures was observed a-e all show oligomeric peptide. All peptide was incubated at 100μM for the specified time and a TEM grid was produced at three timepoints between 24h and 240h.

The next set of experiments used the same peptide preparation protocol but was also performed on the PTM variants as well as the unmodified Aβ 1-42 peptide. Initially the peptide was incubated for 240h in water to determine whether all four of the peptides were able to form fibrils and to investigate whether the structure of those fibrils appeared to be the same. The TEM images produced showed that as well as the unmodified peptide the PTM variants were also able to produce fibrils and there are no differences in the fibrils formed. All the peptides had very long fibrils that had all associated so there were no areas of the pictures that did not display a fibrillar morphology (Fig 3.7B, D, F, and H).

In addition to the 216h timepoint, a TEM grid was produced after 96h to examine whether the intermediates formed by this timepoint were the same for the unmodified peptide as for the PTM variants (Fig 3.7A,C,E,G), and the images showed there were a number of differences in morphology. Aβ 1-42 had a mixed morphology, there were very few oligomers observed (Fig 3.7a) and the ones that could be seen had aligned with other oligomers. The most abundant structures were small protofibrils, most of which measured under 100nm in length (Fig 3.7b), which had in a number of areas begun to show evidence of elongation and had started to associate although these areas were still small. The Aβcit5 1-42 and AβpE3cit5 3-42 peptides had the most similar morphology, both peptides displayed a more aggregated morphology than the Aβ peptide although Aβcit5 1-42 showed more evidence of protofibril association than AβpE3cit5 3-42. There were oligomers in the images from both peptides, the majority of which had aligned and begun to fuse, in addition the Aβcit5 1-42 peptide also showed evidence of short protofibrils

which were only present in a very small number of AβpE3cit5 3-42 images. The main morphology in both of these peptides were elongated protofibrils that had associated.

Finally AβpE3 3-42 showed the most aggregated morphology of all the peptides. The images showed structures very similar to those seen after 240h from all the peptides meaning that it already had a fully fibrillar morphology after 96h (Fig 3.7j). There were no oligomers, individual protofibrils or associated areas as all the peptide was part of the overall fibrillar structure.
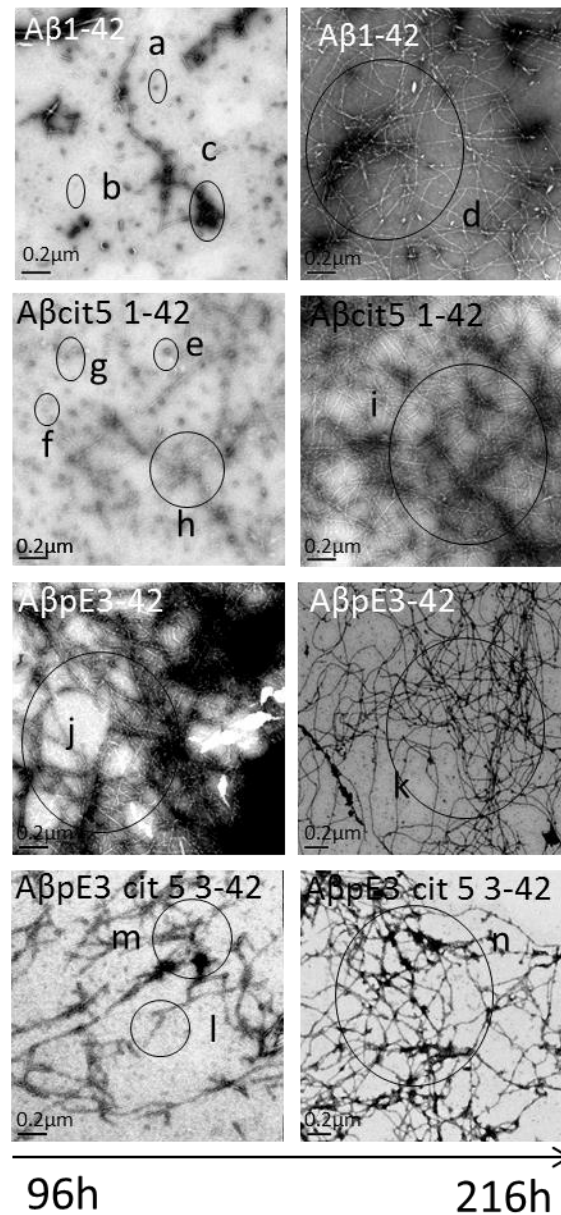
Figure 3 . 7: PTM variants of Aβ appear to aggregate faster than the unmodified form but all aggregate to the same fibrillar morphology by 240h

All peptides were made using preparation protocol 3. Peptides were disaggregated then dissolved in water and peptides were aggregated for 96h and 216h in water at 4°C. A TEM grid of each peptide was prepared at the two timepoints and the black rings show the important structures of peptide in the images. Oligomers can be seen in a and e and aligned oligomers in f. Circles b, g and l show single protofibrils and h and m show areas of associated protofibrils. A full fibrillar morphology is displayed in j, d, I, k and n.

As incubating Aβ 1-42 peptide in water at 4°C does not model well how Aβ 1-42 would aggregate in the body, and the literature shows that Aβ 1-42 is able to aggregate in PB, the same experiment was performed incubating the peptide in PB at 37°C. This would investigate whether increasing the temperature will produce fibrils incubated in PB. The TEM images showed that after 72h of incubation under these conditions although there is still some evidence of small protofibrils, the unmodified peptide has a largely fibrillar morphology similar to that seen in water, at 4°C for 240h (Fig 3.8).
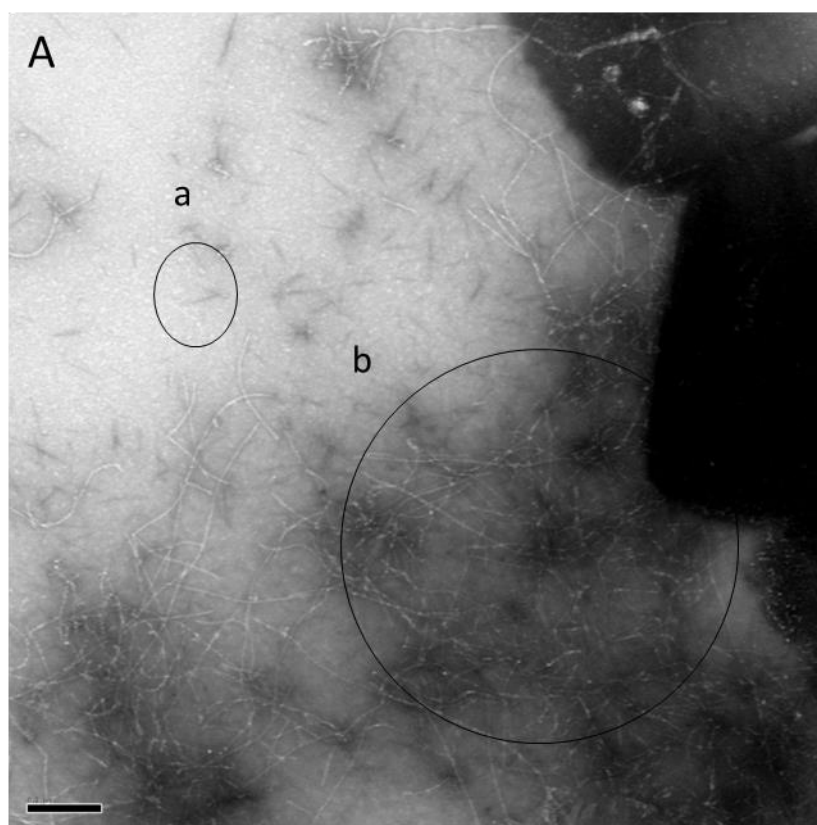
Figure 3 . 8: At 37°C in PB, unmodified Aβ is able to form fibrils

The peptide was prepared using protocol 3. The peptide was disaggregated, dissolved in PB and was left to aggregated at 37°C. A TEM grid was produced of the peptide after 72h incubation. The structures of interest are ringed in black. Small protofibrils are shown in a and areas of a fibrillar morphology are shown in b. The peptide was incubated at a concentration of 100μM and image is representative of all the images taken.

3.2.3 Aggregation of Aβ fragments

A further investigation was performed to determine whether shorter amino acid chains of the unmodified Aβ peptide were also able to form fibrils. These peptides were used in experiments investigating T-cell responses, so the ability of each of the peptides to aggregate was useful to know. Each peptide was 15 amino acids in length and overlapped every 5 amino acids. Peptide preparation 3 was used in these experiments, as it was the protocol that had previously produced fibrils, the peptides were all incubated for 240h and a TEM grid was produced of each of the peptides.

Table 3 . 2: Short Aβ peptide fragments

Name of peptide	Amino acid chain
Aβ 1-15	1-DAEFRHDSGYEVHHQ-15
Aβ 5-19	5-RHDSGYEVHHQKLVF-19
Aβ 9-23	9-GYEVHHQKLVFFAED-24
Aβ 13-27	13-HHQKLVFFAEDVGSN-27
Aβ 17-31	17-LVFFAEDVGSNKGAI-31
Aβ 21-35	21-AEDVGSNKGAIIGLM-35
Aβ 25-39	25-GSNKGAIIGLMVGGV-39
Aβ 28-42	28-KGAIIGLMVGGVVIA-42

There were several differences in the morphology of the peptides: Aβ 1-15 had formed small protofibrils, which was the only structure visible in the images. Aβ 5-19 showed evidence of more aggregation and areas of associated protofibrils were visible, although the protofibrils were less defined than in other images. The Aβ 9-23 and Aβ 13-27 peptides had both aggregated to form mature fibrils and there was very little evidence of any other structure. In addition to these two peptides, Aβ 25-39 and Aβ 28-42 had also formed mature fibrils seen by the straight un-branched structure of the peptides. The Aβ 17-31 peptide had only formed oligomers and the Aβ 21-35 peptide showed a mixture of oligomers and small protofibrils (Fig 3.9). These results suggest that the protocol used is able to produce fibrils but that the structure formed depended on the fragment of the peptide.
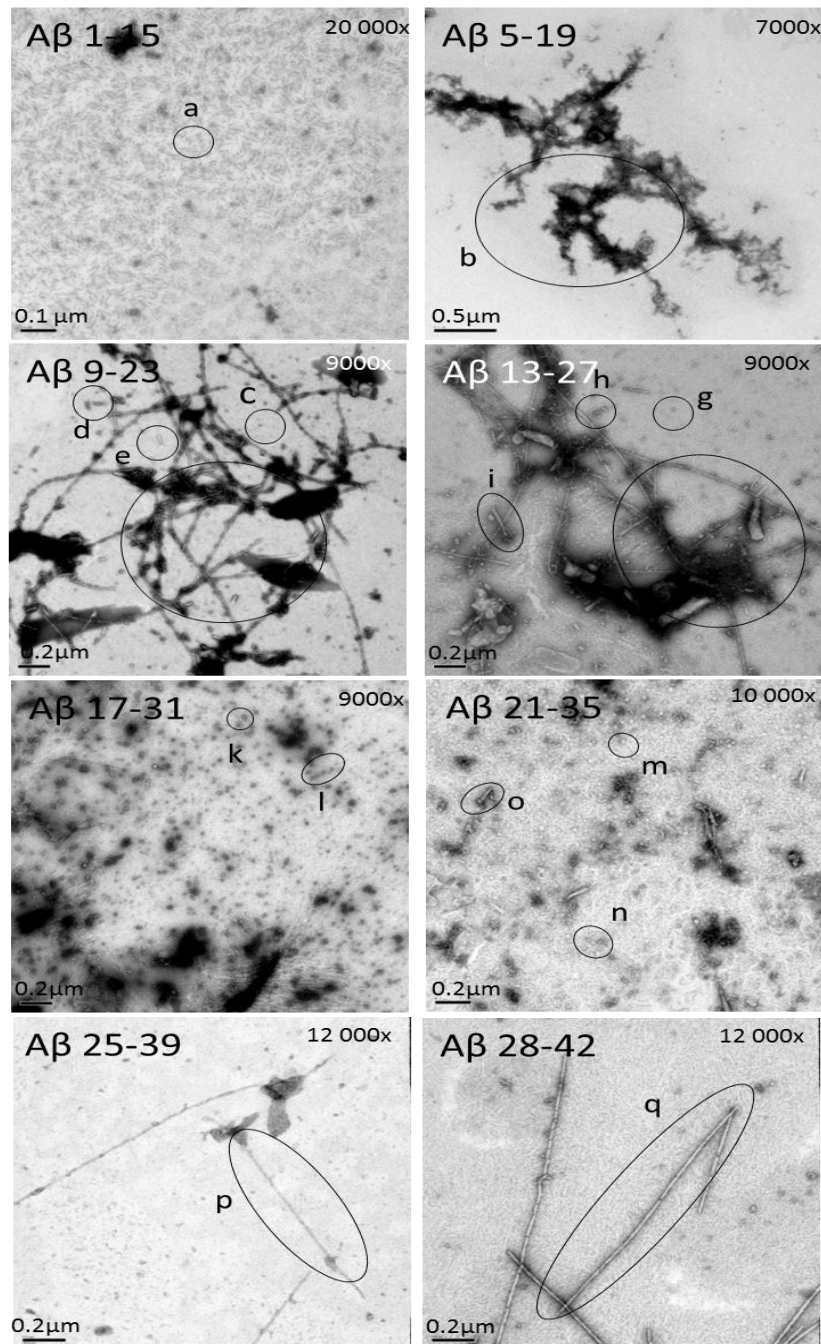
Figure 3 . 9**:** Only some Aβ peptide fragments are able to aggregate

Peptides were prepared using protocol 3. Peptide was disaggregated then dissolved in water and aggregated at 4°C. TEM grids were produced after 216h incubation. Oligomers of Aβ peptides are seen in c, g, k and m and could be observed to be aligned in d, h, l and n. Protofibrils can be seen circled by a, e, I, o, p and q. Associated areas of and fibres can be seen in f and j. All peptides were incubated at 50μM until grids were produced.

3.3 Discussion

The Aβ peptide can be found in a number of different morphologies some of which are believed to play a physiological role and others of which are thought to be detrimental. The important property that causes the production of the different species of Aβ is its ability to aggregate.

During the aggregation process several different structures are formed, immediately after the processing of APP Aβ is found in a monomeric form; the only morphology of the peptide believed to have a physiological role [51, 71, 96]. The most toxic form of the peptide is then observed, oligomers, which are usually characterised as small spherical structures followed by the protofibrils and areas where the protofibrils elongate and associate to form connected areas. The final structure seen in Aβ aggregation is insoluble fibrils that are thought to be main component of the extracellular plaques in AD [85, 102, 339, 340] It has been proposed that it is the aggregated forms of Aβ, oligomers onwards, that are responsible for the damage and symptoms observed in AD. Although there is still some debate over which species of the peptide are responsible for which aspects of the disease it is much more widely believed that the intermediate species of Aβ are the toxic structures and correlate much better than fibrillar Aβ with the symptoms of Aβ [28, 89, 95, 114, 321], while the fibrillar Aβ is thought to be more involved in the inflammation observed in the disease [268, 341-343].

The overall aim of this study was to look at immune responses to Aβ peptides it was therefore important that different structures of the peptide were investigated at different timepoints to determine if these differences could

explain differences in the responses seen from different immune cells. This part of the study was to find a protocol that would produce a range of peptide species as well as to look at differences in Aβ and three of its PTM variants.

One observation that was made in this study was that fibrils of Aβ were only seen after preparation using protocol 3 and incubating the peptide in sterile water. There were some differences in the protocols used to prepare the peptides and perhaps the most important were the length of time the peptides were incubated for and the temperature they were incubated at. However, the solvent the peptide was incubated in may also have affected the aggregation of the peptides.

Previous studies have shown that Aβ is able to aggregate when incubated in PB [100], although in this study when incubated at room temperature for up to 72h, despite this being reported to be long enough to produce fibrils of Aβ [3, 97], or at 4°C for up to 10 days it was not possible to see any fibrils. On the other hand when the peptide was incubated at 37°C for 72h it was possible to see the formation of fibrils. This suggests that the temperature of the incubation was having more of an effect than the solvent the peptide was dissolved in. To further support this, the peptide had remained in an oligomeric morphology when incubated at 4°C but had shown some evidence of aggregation to protofibrils/area of associated protofibrils when incubated at room temperature. The literature has shown that at low temperatures, such as 4°C, Aβ can be unable to form the β-sheet structure needed for the formation of fibrils but will form stable oligomers that remain as oligomers for extended periods of time [11, 101], furthermore, the growth of Aβ with β-sheet

structure has been observed to slow down when temperatures were decreased [11]. In these experiments aggregation of the peptide in water at 4°C did produce fibrils of Aβ as well as a range of different intermediate structures including oligomers and protofibrils. It has been suggested that the hydrophobicity of the peptide and its ability to form hydrogen bonds are key for the formation of fibrils of amyloid peptides [344]: fibril formation may depend upon the ability of the main chain of Aβ to form hydrogen bonds suggesting that water may increase the ability of water to form fibrils [345] and in hydrophobic sequences, like in Aβ, water has been shown to accelerate fibril formation [346]. This may explain why fibril formation was seen in water even at 4°C.

It was important for later experiments that the peptide was able to form mature fibrils and to know whether the PTM variants of Aβ were also able to aggregate in the same way. To compare the effects of these different peptides on the immune system it was also important to look at whether the intermediate species that were observed were the same as this could explain any potential cellular response differences in later experiments.

Although it is monomers of Aβ that are produced immediately after APP processing, it has often been observed that the smallest structure that can be detected after preparation of Aβ are oligomers [3, 100]. This is most likely due to the aggregation properties of the peptide, as soon as the peptide is dissolved in solution it will begin to aggregate, although this may occur more in different solvents, it is therefore possible that Aβ may begin to aggregate throughout the preparation process. In addition it has also been observed that HFIP may be able to cause the self-association of Aβ [104, 347], suggesting

that it may not be possible to prepare Aβ in monomeric form using either of these protocols.

Even though only protocol 3 using water was able to produce fibrils in this study, there were several similarities in the aggregation of the peptides, regardless of the protocol used. There is now a generally accepted order of aggregation based on the appearance of different structures of Aβ at different times and it is believed that these are the precursors to fibril formation [1, 15, 85, 339, 348]. As in a number of other studies, the first forms of Aβ that were observed were the small globular structures believed to be oligomers [3, 85, 102, 339, 340]. These have been proposed to form after a conformational shift in monomers from an α-helix to a β-sheet conformation [85] and it is this β-sheet conformation that allows the further aggregation of the Aβ peptide [84, 85, 349].

In several of the TEM images studied, in addition to the presence of globular oligomers there were also areas where they had lined up, often several in a row. Further to them lining up there was also evidence of them associating and fusing, often giving a beaded appearance where the 'fusion' is occurring. The association of oligomers has been proposed as a mechanism by which oligomers are able to form protofibrils [3]. As seen in the literature, the next structure of the Aβ observed were protofibrils, the next structure of Aβ thought to form during aggregation [85, 339, 340]. This was seen in the images from protocol 3 incubation in water, initially only the globular oligomeric structures were observed however as the peptide was allowed to incubate protofibrils appeared, furthermore in the images from protocol 1

although there was evidence of protofibrils even in the earliest images, after incubation for 24h or 72h there was more evidence of protofibrils and less evidence of oligomers. The belief that Aβ oligomers are able to form protofibrils explains why there is an increase in protofibrils and a decrease in oligomers, it has often been proposed that oligomers and protofibrils are in equilibrium with each other [105, 350]

It is thought that after protofibrils have formed they are able to elongate and associate to form areas that are connected [3, 103, 347, 351]. In this study in all Aβ preparations that produce some aggregation there are differing lengths of protofibrils that appear to increase in length the longer the peptide was incubated for. There is still some debate over how this elongation occurs but popular theories are that is occurs by lateral association [3, 347] or by the addition of smaller species and other protofibrils to the ends of the peptides [103, 351].

As the peptides were incubated for longer, especially in regards to the protocol 3 prepared peptides, the protofibrils continued to elongate and associate to form denser areas of the peptides and eventually took on the structure associated with mature fibrils, long, smooth and un-branched [85, 339, 352, 353]. The final TEM taken at 240h of incubation in water showed that there was almost no evidence of other structures of the peptide by this point suggesting that most, if not all, of the peptide had formed fibrils.

The above description of the aggregation of the Aβ in this study is based on the unmodified Aβ1-42 peptide, as is the majority of the literature cited. However, as there are a number of Aβ peptides found in the brains of people

with AD that contain PTMs, examples of this were also examined. AβpE3 3-42, the peptide with an AβpE3 modification has been fairly extensively researched in terms of aggregation and structure, however the other two peptides also used in this study have been much less studied. Aβcit5 1-42 has a Cit5 modification and AβpE3cit5 3-42 contained both modifications.

The ability of AβpE3 3-42 to aggregate and form fibrils is supported by a fairly large body of research; the results from this study suggest that the peptide is able to aggregate in the same manner as the unmodified peptide. The first similarity between AβpE3 3-42 and unmodified Aβ 1-42 was that the same intermediate species of Aβ were seen during the aggregation of both peptides; oligomers, protofibrils and associated areas of protofibrils. Although the same structures were not always observed at the same timepoints as the unmodified Aβ 1-42 each of the intermediate structures did appear in the same order. Studies into the aggregation of modified Aβ peptides have proposed that the AβpE3 3-42 modified peptide is able to form the β-sheet structure necessary for the aggregation of Aβ, furthermore several groups have shown that AβpE3 3-42 is able to form oligomers, which are found in AD brains, as well as a high number of fibrils in the insoluble plaques in the disease.

This published data adds support to the findings here that the AβpE3 3-42 peptide and the unmodified Aβ 1-42 form the same intermediate species and fibrillar species as well as containing the same β-sheet structure.

The aggregation of Aβcit5 1-42 also appeared to follow the same aggregation as the unmodified and AβpE3 3-42 peptides. Again, the same intermediate

species were observed in the same order although as with AβpE3 3-42 there were differences in the structures seen at different timepoints. In contrast to AβpE3 3-42, there is very little published data reporting on citrulline Aβ peptides, although recent research has implicated citrullinated Aβ in AD [60, 61]. Results from studies of the effect of citrullination on other proteins with amyloid properties, such as a propensity to aggregate have suggested that citrullination may increase β-sheet structure of the peptide suggesting, like the AβpE3 3-42 peptide, the Cit5 Aβ may aggregate in the same way as the unmodified peptide [338].

Finally there is no research into Aβ peptide with both of these modifications but the results from this study have suggested that even with two PTMs there is no change in the way that this form of Aβ aggregates. Particularly when looking at the protocol 3 prepared peptide there was very little difference in the aggregation of the AβpE3cit5 3-42 peptide compared with any of the others.

Further to the appearance of intermediate structures being consistent between peptides, it was also true for their ability to form mature fibrils. As is proposed in the literature the insoluble plaques observed in AD are composed of fibrils of Aβ with and without PTMs. The most abundant of these is the AβpE3 3-42 peptide although there are a number of other variants that have been shown to be in the plaques such as the Cit5 Aβ [60, 61]. The fact that these two peptides are found alongside the unmodified peptide, in fibrillar form, in the plaques supports the evidence that all of the peptides in these experiments were able to form fibrils.

Combined, all the data suggests that the modifications to Aβ used in these experiments do not prevent the formation of fibrils and do not change how the peptide aggregates. In addition to this, unpublished data using X-ray fibre diffraction to investigate the structure of these four peptides (from the same batch) has shown that all four of the peptides have the same structure.

Despite the process of aggregation appearing to be the same for all the peptide used in these experiments, there were differences in when the different intermediate species were visible depending on the peptide. One of the first observations made was that the AβpE3 3-42 peptide appeared to have a more aggregated morphology at the earlier timepoints than any of the other peptides. For example, after preparation with protocol 1, even immediately after preparation there was evidence that AβpE3 3-42 had begun to aggregate more than the other peptides in that there were already a number of protofibrils and areas where protofibrils had associated. Furthermore by the 24h timepoint AβpE3 3-42 had formed large areas where the protofibrils had elongated and associated which was not the case for any of the other peptides. Similar was seen after the use of protocol 3, at the 96h timepoint AβpE3 3-42 had a fibrillar morphology in comparison to the three remaining peptides which had a mixture of oligomeric and protofibrillar morphology. These results suggest that the AβpE3 3-42 peptide is able to form aggregates earlier than the unmodified peptide as well as the Aβcit5 1-42 and AβpE3cit5 3-42 peptides.

As stated before, there has been a variety of research into the aggregation of AβpE3 3-42, the majority of which has shown an increased propensity for

aggregation compared with the unmodified peptide [59, 63, 74, 108]. This could explain why AβpE3 3-42 appears to have aggregated earlier than the other peptides in these experiments. This increase is thought to be due, in part, to the increased hydrophobicity and the accompanying decrease in solubility. It has been proposed that the loss of charges, from the modification, that results in the increase in hydrophobicity is the 'driving force' for increased aggregation [74]. In addition to the increased hydrophobicity it has also been suggested that the reduced lag-phase of AβpE3 3-42 causes earlier nucleation or 'seeding' of the peptide from which protofibrils and mature fibrils are able to elongate [74, 108, 198] (McColl 2009). These changes in properties of AβpE3 3-42 may explain the earlier appearance of aggregated AβpE3 3-42 peptide.

There is very little, if any research into the aggregation of the Aβcit5 1-42 peptide, as it has fairly recently been identified as a potential target in AD [60, 61]. In this study the results from the TEM images suggested that at the timepoints looked at, the Aβcit5 1-42 peptide has aggregated more than the unmodified peptide. For example, after use of protocol 3 at the 96h timepoint, it appears that the Aβcit5 1-42 peptide has more associated, longer protofibrils than the unmodified form, although it has not aggregated to the extent of the AβpE3 3-42 peptide. Although the Cit5 peptide has been identified in AD there are no studies into its aggregation [61], however studies into the effect of citrulline on other amyloid peptides has been investigated. One study has shown that citrullinating cellular prion protein (PrP) leads to an increase in β-sheet content and a brighter congo-red fluorescence. As the congo-red is used as a stain for amyloid structures and β-

sheet content is known to be required for the aggregation of amyloid-proteins, the results suggested that citrullination may increase aggregation in PrP [338]. As PrP and Aβ share their amyloid properties it is therefore possible that citrulline could have a similar effect on Aβ 1-42.

In addition to the β-sheet structure, another property of Aβ 1-42 that allows it to aggregate is its hydrophobicity [101, 354, 355]. Citrullination or deimination involves the substitution of an arginine for a citrulline [356-358]. Arginine is a positively charged hydrophilic amino acid, exchanging this for an uncharged citrulline results in the loss of charge from the peptide which will increase its hydrophobicity [356, 359], therefore potentially increasing its propensity to aggregate. These two properties could explain why the Aβcit5 1-42 peptide appeared to be aggregated earlier than the unmodified form.

Finally, the AβpE3cit5 3-42 peptide containing both the AβpE3 3-42 and Cit5 1-42 modifications when prepared using protocol 3 also appeared to have aggregated more than the unmodified Aβ at the 96h timepoint. There is no literature on the effect both of these modifications would have on a peptide but considering that the PTMs change the charge of the peptide [59, 63, 75, 356, 357, 359, 360] as well truncating the peptide [59, 63, 361] it is very possible that it would alter the properties of the peptide including its propensity to aggregate.

The last part of the study involved the investigation of fragments of Aβ 1-42. This was done as the epitopes of the peptides were used to investigate T-cell activation so it was useful to know whether the peptides would aggregate as it could affect these responses. Each fragment contained 15 amino acids and

overlapped every 5. It was observed that after the incubation time not all of the peptides had aggregated to the same extent. There are certain areas of the Aβ peptide that are believed to have greater ability to aggregate for example the hydrophobic core is thought to be residues 16-20. This particular cluster is present in the Aβ 9-23 and Aβ 13-27 peptides and may therefore be responsible for the ability of these two peptides to aggregate [85, 351, 362, 363]. The Aβ 5-19 peptide, which also appeared to show some aggregation, although not the extent of Aβ 9-23 or Aβ 13-27, contains residues 16-19 which could explain why there was some level of aggregation but not fibril formation [364]. In addition to residues 16-20 there is also evidence that the C-terminal end of the peptide, specifically residues 28-42 also have a high probability of forming β-sheet structures and therefore aggregating [85]. The Aβ 28-42 peptide used in this experiment is composed of this region suggesting why it was able to form mature fibrils, in addition the Aβ 25-39 peptide which contained the majority of this region also appeared to be able to form fibrils. The remaining three peptides, Aβ 1-15, Aβ 17-31 and Aβ 21-35, although showed some level of aggregation i.e. oligomers or protofibrils, did not aggregate to the extent of the other peptides perhaps suggesting they did not contain any or enough of the regions that increase the ability of the peptide to aggregate. A summary of all peptides, preparations and aggregation species is shown in table 3.3 below.

These experiments have confirmed that unmodified Aβ 1-42 is able to form fibrils, although the protocol used to prepare the peptide can alter the aggregation of the peptide. Furthermore all three of the PTM variants of Aβ have also been shown to form fibrils and that the intermediates formed during

aggregation appear to be the same as those of the unmodified peptide, suggesting that the chosen modifications do not prevent the formation of aggregated forms of Aβ or the aggregation pathway. In addition the TEM has suggested that modifications to the Aβ peptide have affected how early intermediates and fibrils of the peptide were visible using these particular preparation protocols, suggesting that the modification may affect the propensity of the peptide to aggregate.

Table 3 . 3: Summary of Aβ peptides, protocols used and morphology observed

Peptide Designation	Preparation protocol	Incubation temperature	Incubation time	Peptide species present
Aβ 1-42	1	RT	0h	Oligomers Small protofibrils
	1	RT	24h	Small protofibrils Small areas of associated protofibrils
	1	RT	72h	Protofibrils Areas of associated protofibrils
Aβcit5 1-42	1	RT	0h	Oligomers
	1	RT	24h	Oligomers, Small protofibrils Areas of associated protofibrils
AβpE3 3-42	1	RT	0h	Oligomers Small protofibrils Small areas of associated protofibrils
	1	RT	24h	Large areas of associated protofibrils
AβpE3cit5 3-42	1	RT	0h	Oligomers Small protofibrils Small associated areas of protofibrils
	1	RT	24h	Oligomers
Aβ 1-42	3 water	4°C	24h	Oligomers
	3 water	4°C	96h	Oligomers Small protofibrils Associated protofibrils (Small)
	3 water	4°C	216h	Fibrils
	3 PB	4°C	24h	Oligomers
	3 PB	4°C	96h	Oligomers
	3 PB	4°C	216h	Oligomers
Aβcit5 1-42	3 water	4°C	96h	Elongated protofibrils Associated areas
	3 water	4°C	216h	Fibrils
AβpE3 3-42	3 water	4°C	96h	Fibrils
	3 water	4°C	216h	Fibrils
AβpE3cit5 3-42	3 water	4°C	96h	Elongated protofibril
	3 water	4°C	216h	Fibrils
Aβ 1-15	3 water	4°C	216h	Small protofibrils
Aβ 5-19	3 water	4°C	216h	Associated areas (small)
Aβ 9-23	3 water	4°C	216h	Fibrils
Aβ 13-27	3 water	4°C	216h	Fibrils
Aβ 17-31	3 water	4°C	216h	Oligomers
Aβ 21-35	3 water	4°C	216h	Oligomers Small protofibrils
Aβ 25-39	3 water	4°C	216h	Fibrils
Aβ 28-42	3 water	4°C	216h	Fibrils

4. The responses of TLR2 and TLR4 to Amyloid-beta peptides and post-translationally modified variants

4.1 Introduction

It has been proposed, and is now widely accepted, that inflammation occurs in AD, although the role of inflammation in disease progression is still debated [284]. For example it is unclear whether inflammation in AD has neuroprotective and beneficial effects in the disease or whether it contributes to degeneration [115, 365-367]. Studies have shown that there are higher numbers of active microglia and higher levels of inflammatory mediators such as cytokines, chemokines and complement factors in people with AD [23, 115, 284]. However clinical trials using anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) have been largely unsuccessful [368]. Despite the lack of success, work is still ongoing to investigate the mechanism by which inflammation in AD occurs.

$A\beta$ peptides, the main component of the AD plaques, have been suggested as an inducer of neuroinflammation in AD. There is a wide range of evidence supporting the role of $A\beta$ in inflammation, including: the clustering of active microglia around $A\beta$ plaques [265-269], the activation of the complement system by $A\beta$ [369-375] the involvement of the co-stimulatory molecule CD14 (involved in TLR4 activation) [280, 376] and the inflammatory processes observed in transgenic mouse models that over produce $A\beta$ [282, 377, 378]. It has been hypothesised that inflammation could occur through a number of different mechanisms, one of which is through activation of PRRs [268, 379].

PRRs are expressed in high levels by microglia, the resident immune cells of the CNS, and detect a variety of PAMPs and DAMPs [380-382]. The most widely researched PRR are the TLR family and in AD TLR2 and TLR4 are thought to be involved in neuroinflammation [279, 283, 383].

TLR2 and 4 have been observed to be upregulated in plaque material compared with plaque free tissue in APP23 transgenic mouse model of AD [384]. Additionally, transgenic mouse models AD have also shown an increase in transcription levels of TLR2, TLR7 and CD14, age-related induction of TLR2, TLR4, TLR7 and TLR9 transcripts in transgenic mouse brains and overexpression of TLR4 in APP transgenic mouse brains when compared with wild type mice [276, 385].

Furthermore, post-mortem studies of human brains have shown co-localisation of TLR2 and CD14 with microglia [386]. Patients with AD display an enhanced expression of CD14 in parenchymal microglia from the frontal cortex and hippocampus [376] and over expressed TLR4 on glial cells has been observed to associate with Aβ plaques in the entorhinal cortex [276].

In addition to an increase in the expression of TLRs in the brains of both transgenic mice and humans with AD, it has also been shown that Aβ causes the release of pro-inflammatory mediators through its interaction with TLR2 and TLR4. The stimulation of THP-1 cells (a human monocytic cell line) with fibrillar Aβ leads to the release of TNFα and the addition of antibodies to neutralise either TLR2 or TLR4 reduces the amount of TNFα detected [274]. Fibrillar Aβ has been shown to bind to THP-1 cells, which is inhibited by pre-treatment with neutralising antibodies for TLR2, TLR4 and CD14 [281].

HEK293 cells expressing either TLR2 or a TLR4-MD2-CD14 complex have been shown to release IL-8 after stimulation with fibrillar Aβ [275, 276]. In addition, Stewart et al used HEK293 cells to show the importance of the complex TLR4-TLR6-CD36 in Aβ recognition [277]. Murine microglia and human monocytes have both been observed to release IL-6 and nitric oxide (NO) in response to Aβ. The production of both NO and IL-6 was higher from microglia isolated from wild-type mice than from TLR4 mutant mice and there was a reduction in IL-6 released from human monocytes after treatment with anti-TLR4 antibody (αTLR4) [276]. Reed-Geaghan et al suggested that TLR2, TLR4 and CD14 form a complex that is required for a response to be observed to fibrillar Aβ [281]. Although there are some conflicting results the vast majority of studies point towards the involvement of TLR2 and TLR4 in Aβ recognition.

There is some debate whether the morphology of the peptide affects whether Aβ is able to activate TLRs [274, 275, 279, 284]. However, the majority of studies now agree that Aβ peptides need to have a fibrillar morphology in order to produce a response from TLRs [274, 276, 279].

This study used a well-known model for TLR activation HEK293, primary human monocytes and peripheral blood mononuclear cells (PBMCs) obtained from patients with AD and age-matched controls to investigate whether Aβ peptides could activate TLR2 and TLR4. We also investigated whether Aβ peptides with PTMs were able to produce a response and whether this is different from the unmodified peptide.

4.2 Results

4.2.1 Aβ stimulation of HEK293 cells expressing TLR2 or TLR4MD2CD14

The initial experiment used HEK293 cells expressing TLR2 to investigate its role in the recognition of Aβ 1-42 and its PTM variants. The peptides were all prepared using preparation protocol 1, peptides were dissolved in HFIP, the alcohol was removed, peptides were dissolved in dry DMSO which was removed using a desalt column and the final peptide was diluted in PB. They were used either immediately after preparation or were allowed to aggregate for 24h. The HEK293 cells expressing TLR2 were stimulated with either 2.5μM or 10μM peptide, these are concentrations that have been shown to produce a response in HEK293 cells in published results [275], for approximately 18h and an ELISA was used to measure IL-8 in the supernatants.

The results obtained from the ELISA showed that no IL-8 could be detected from HEK293-TLR2 cells. No differences were observed in IL-8 response when peptide was incubated for different lengths of time or when the concentration of the peptide was changed. In addition, no response was seen after the use of unmodified or PTM variants of Aβ, although the cells responded to the positive controls suggesting that the cells were responding normally (Fig 4.1).
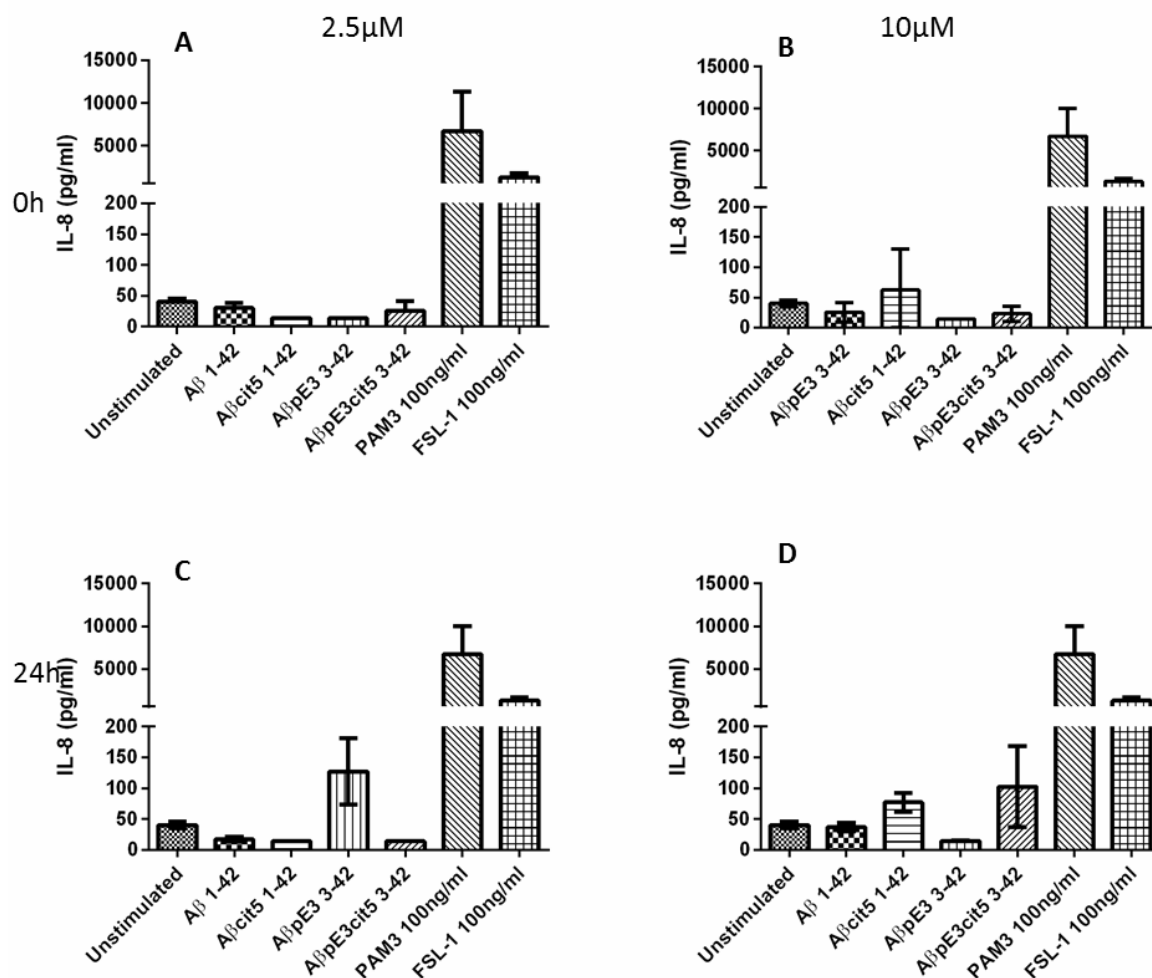
Figure 4 . 1: HEK293 cells expressing TLR2 do not respond to Aβ peptides or its PTM variants when prepared using protocol 1.

Synthetic Aβ 1-42 peptide and its three PTM variants Aβcit5 1-42, AβpE3 3-42 and AβpE3cit5 3-42 were disaggregated and diluted in DMSO. The DMSO was removed and final peptide was diluted in 50mM PB. The peptides were used to stimulate HEK293 cells expressing TLR2. Peptides were left to aggregate for 0h or 24h and used at a concentration of 2.5μM or 10μM. Activation of TLR2 was measured by IL-8 ELISA of cell supernatant after 18h of incubation. A) 2.5μM concentration after 0h peptide aggregation B) 0h peptide aggregation, 10μM concentration C) 2.5μM peptide aggregated for 0h D) 24h peptide aggregation, 10μM. 100ng/ml PAM3 and FSL-1 were used as positive controls for TLR1/2 and TLR2/6 and unstimulated cells were used as the negative control. n=2.

In order to investigate whether the concentration of the peptide was sufficient to produce a response through TLR2, a titration of the peptide was performed. Only the unmodified peptide was used, prepared using protocol 1 and incubated for 24h, at a concentration of 2.5, 5, 10 and 20µM and cells were stimulated for 18h. An IL-8 ELISA was performed on the supernatants of the cells and a viability assay (MTT assay) was carried out to ensure there was no cell death.

There was no response to the peptide at any concentration, although the cells responded to their natural ligands (Fig 4.2A). Cell viability was calculated as the cellular activity of stimulated cells as a percentage of cellular activity of the untreated cells. The results showed that there was an increase in cellular activity of almost all stimulated cells when compared with the untreated samples (Fig 4.2B), suggesting that cell death was not responsible for the lack of IL-8 production.

IL-8 ELISA

A

MTT Assay

B
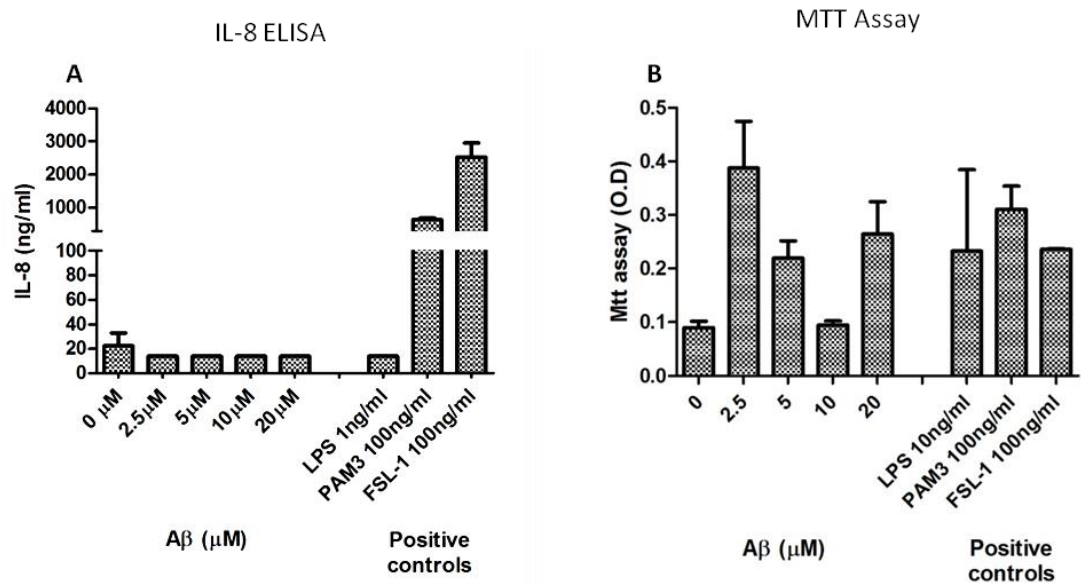
Figure 4 . 2: TLR2 expressing HEK293 cells do not respond to Aβ 1-42

Amyloid beta peptides were disaggregated, dissolved in DMSO which was removed using Desalt columns. The final peptide was diluted in 50mM PB and left to aggregate for 24h before stimulating HEK293 TLR2 cells. The peptide was used at a concentration of 2.5μM, 5μM, 10μM and 20μM. They were also stimulated with 1mg/ml LPS, 100ng/mlPAM3 and 100ng/ml FSL-1. All cells were stimulated for 18h and supernatants were collected for an IL-8 ELISA (A). Cell viability was checked with an MTT assay on the same cells, mean absorbance, of duplicates, at 580nm is plotted ± SD (B). n=1

As no IL-8 had been observed in response to the any of the peptides, TEM analysis was performed on the peptides to determine whether a fibrillar morphology was observed. As none of the peptides had formed fibrils, a longer aggregation time was selected. The peptides were initially prepared the same way but were left for 72h to aggregate. For the next experiments HEK293 cells expressing TLR2 and cells expressing the TLR4-MD2-CD14 complex were used. In order for optimal TLR4 activation TLR4 requires the co-receptors CD14 and MD2, therefore cells stably expressing both of these co-receptors were used [180, 387]. For these experiments only the unmodified peptide was used and was prepared using protocol 1 to further address whether TLR2 can be activated by Aβ and investigate whether a response can be seen through TLR4.

As there were no differences observed in the HEK293 TLR2 titration experiments and previous research has shown activation of HEK293 cells with 10μM Aβ peptide, this was the concentration used to stimulate cells for 18h, an IL-8 ELISA was performed on the cell supernatants. No response was seen to the peptide from either cell type, although they both responded to the positive controls for TLR2 or TLR4 (Fig 4.3). In addition, an MTT assay was performed to confirm there was no reduction in cell viability and the results showed no evidence of reduced cell viability in either cell type (Fig 4.4).
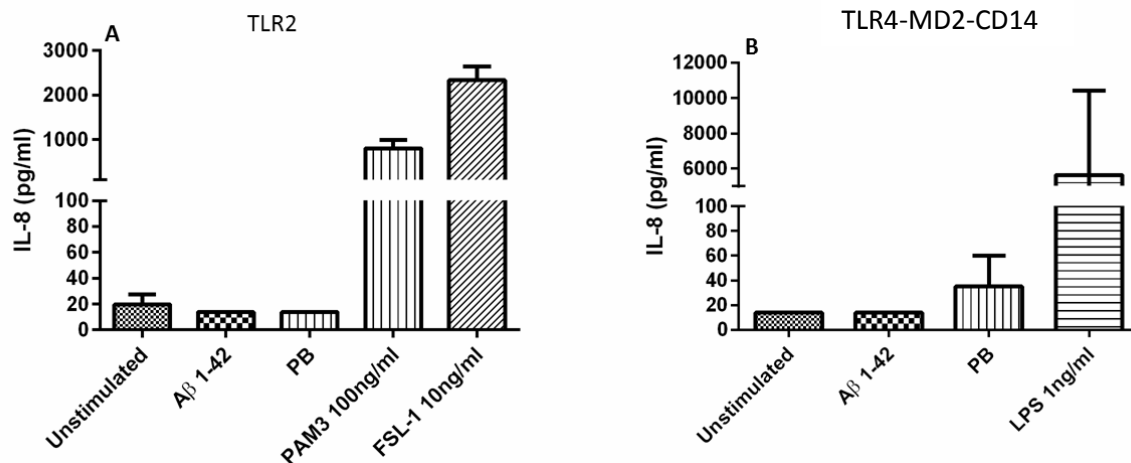
Figure 4 . 3: Unmodified peptide prepared using protocol 1 does not cause the activation of HEK293 cells expressing TLR2 or TLR4-MD2-CD14

Unmodified peptide was disaggregated then diluted in DMSO. DMSO was removed and peptides were diluted in 50mM PB and allowed to aggregate for 72h at room temperature. HEK293 cells expressing TLR2 or TLR4-MD2-CD14 were stimulated with 10μM peptide for 18h and supernatants were used for IL-8 ELISA. A) Peptide stimulated TLR2 expressing cells B) Cells expressing TLR4-MD2-CD14 stimulated with peptides. PB (diluted in media) and unstimulated cells were used as negative controls, 100ng/ml PAM3 and 10ng/ml FSL-1 were used as the positive controls for TLR2 and 1ng/ml LPS as the positive control for TLR4. The means of triplicates from three experiments was calculated, displayed is the overall mean from those experiments ± SD. n=3. $P<0.05 = *$, $P<0.01 = **$, $P<0.001 = ***$
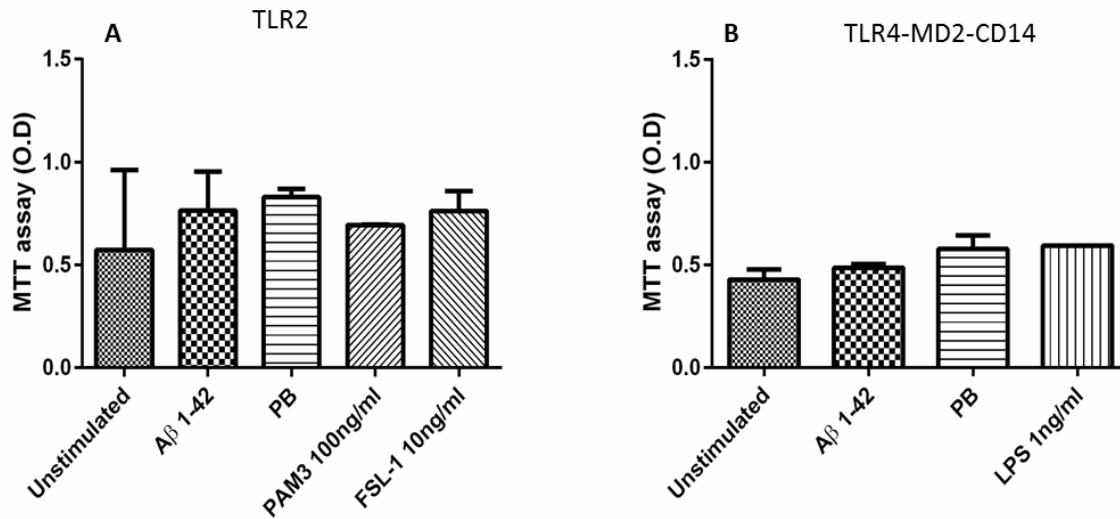
Figure 4 . 4: Aβ 1-42 peptide prepared using protocol 1 does not reduce cell viability in HEK293 cells expressing TLR2 or TLR4

HEK293 cells expressing TLR2 or TLR4-MD2-CD14 were stimulated with 10μM Aβ 1-42 for 18h. The peptide was disaggregated and diluted in DMSO, which was removed. The final peptides was dissolved in 50mM PB and left to aggregate at room temperature for 72h. An MTT assay was performed on the cells. A) HEK293 TLR2 B) HEK293 TLR4-MD2-CD14. Mean absorbance, of duplicates, at 580nm is plotted ± SD. n=1

The next experiments explored whether the preparation of the peptide could affect the activation of TLR2 or TLR4. For these experiments only the unmodified peptide was used and was prepared using preparation protocol 2 (dissolved in HFIP, vacuum desiccated, dissolved in DMSO, diluted in media or HCl overnight at 4°C or 37°C respectively). TEM images were also obtained to confirm the morphology of the peptide. After incubation at either 4°C or 37°C, 10μM peptide was used to stimulate HEK293 cells expressing either TLR2 or TLR4-MD2-CD14 and an IL-8 ELISA was performed on supernatants. The cell viability after peptide stimulation was checked using an MTT assay and displayed as the percentage of cellular activity of the untreated control.

 Despite both cell types responding to its positive control, changing the preparation of the peptide did not result in the production of IL-8 from either cell type (Fig 4.5), however it did affect the cell viability. The cellular activity had more than halved in the TLR2 expressing HEK293 cells after stimulation with both the 4°C and 37°C Aβ 1-42 peptide (Fig 4.6A). The proportion of cells that were viable after stimulation of the TLR4 expressing HEK293 cells was reduced to only 15% after stimulation with the 37°C peptide, but was over 70% after stimulation with the 4°C peptide (Fig 4.6B). The reduction in viability shows that the peptide was toxic to the cells, under certain condition and would therefore reduce or prevent any cytokine production in response to the peptides.
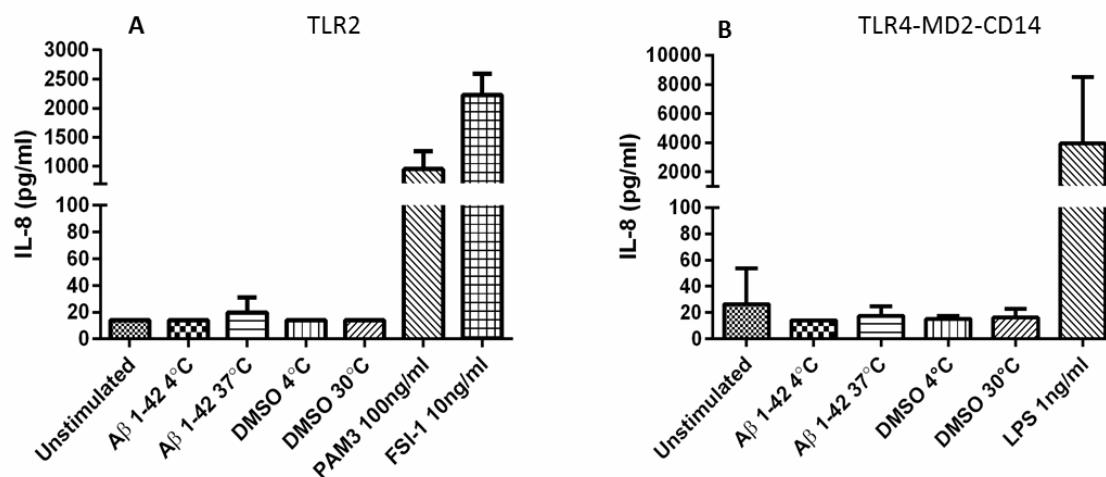
Figure 4 . 5: Protocol 2 does not cause a response from HEK293 cells expressing TLR2 or TLR4-MD2-CD14

Unmodified Aβ 1-42 peptide was disaggregated then dissolved in DMSO. DMSO was further diluted in either media or HCL and left to aggregate at either 4°C or 37°C for 24h. The peptide was used to stimulate HEK293 cells expressing TLR2 or TLR4-MD2-CD14 for 18h, IL-8 ELISA was performed on the cell supernatants. A) TLR2 expressing cells stimulated with peptides B) TLR2 expressing cells stimulated with positive controls C) TLR4-MD2-CD14 expressing cells stimulated with peptide D) TLR4-MD2-CD14 expressing cells stimulated with LPS. The negative controls were DMSO (diluted in media) at 4°C and DMSO (diluted in media) at 37°C and media alone, the positive controls were 100ng/ml PAM3, 10ng/ml LPS for TLR2 and 1ng/ml LPS for TLR4. The means of triplicates from three experiments was calculated, displayed is the overall mean from those experiments ± SD. n=3. p<0.05 *, p<0.01 **, p<0.001 ***.
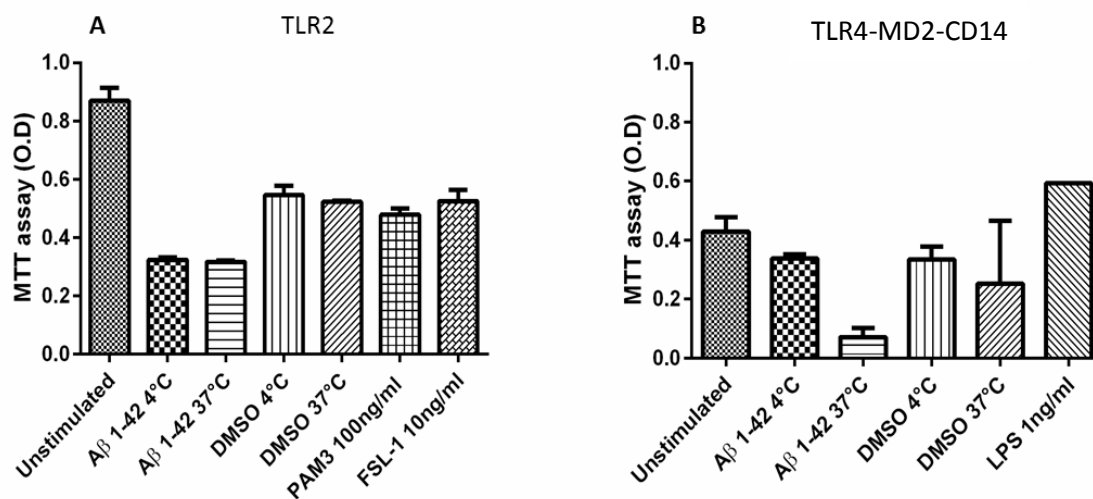
Figure 4 . 6: Aβ 1-42 peptide prepared using protocol 2 reduces cell viability in HEK293 cells expressing TLR2 or TLR4

HEK293 cells expressing TLR2 or TLR4-MD2-CD14 were stimulated with 10μM Aβ 1-42 for 18h. Peptide was disaggregated and dissolved in DMSO. It was then further diluted in either media or HCl and allowed to aggregate at either 4°C or 37°C to produce oligomers or fibrils. An MTT assay was performed on the cells. Mean absorbance, of duplicates, at 580nm is plotted ± SD. A) HEK293 TLR2 B) HEK293 TLR4-MD2-CD14.

In order to ensure the HEK293 cells were expressing the receptors being investigated, flow cytometry was used to measure the proportion of cells expressing TLR2 or TLR4. The cells were stained with an anti-TLR2 antibody, or an anti-TLR4 antibody. In order to gate the HEK293 cells a singlet gate was used first, a TLR2 vs TLR4 quadrant was added to the single cells which showed the proportion of cells expressing each type of receptor (Fig 4.7).

Each type of HEK293 cell was expected to express one TLR, either TLR2 or TLR4. The flow cytometry results showed that the cells were expressing the expected TLR; one the background staining had been removed, 60% of the HEK293 TLR2 cells were expressing TLR2 and 40% of TLR4 cells expressed TLR4 (Fig 4.7). These results suggest that the receptors being investigated were present on the majority of cells they were expected to be on, so it is unlikely that a lack of receptors is responsible for no IL-8 production in response to the Aβ peptides.
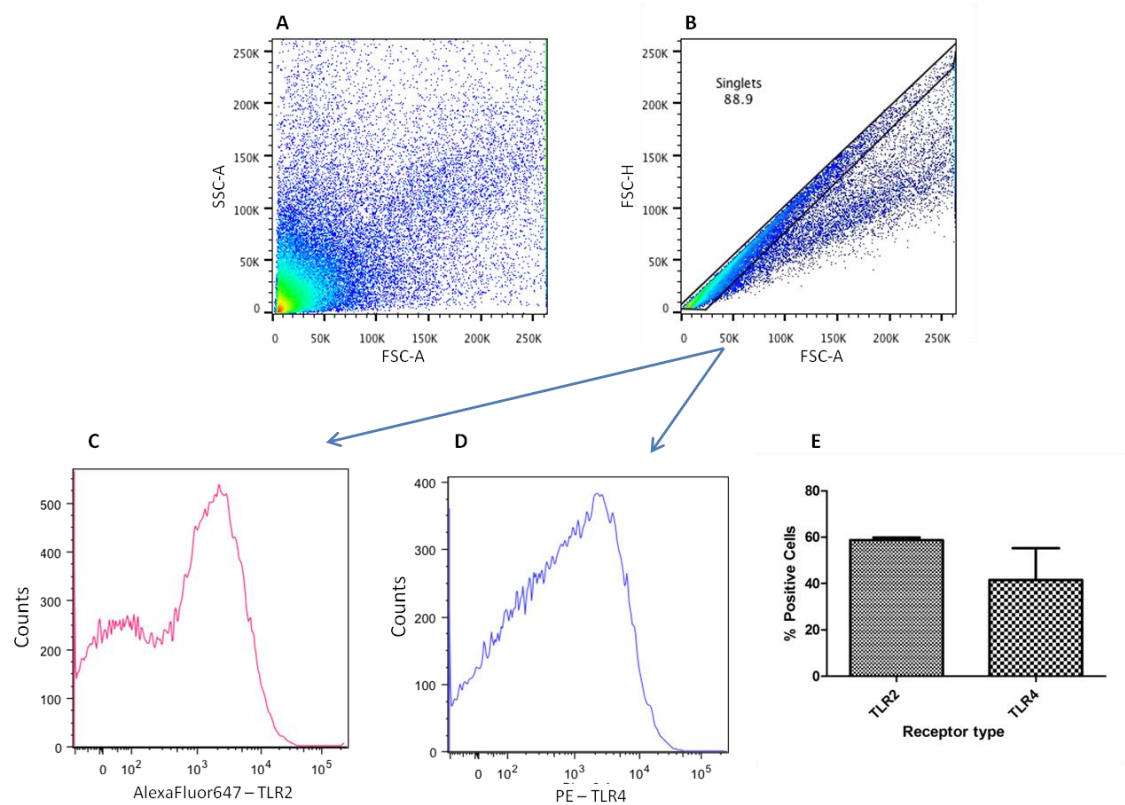
Figure 4 . 7: TLR expression on HEK293 cells

HEK293 cells expressing TLR2 or TLR4-MD2-CD14 were stained with antibodies for TLR2 or TLR4. A) Ungated cells B) singlets C) Histogram showing TLR2 expressing cells E) histogram showing TLR4-MD2-CD14 expressing cells. D) Proportion of TLR2 and TLR4-MD2-CD14 HEK cells expressing TLR2 or TLR4. Mean from duplicates minus background staining ± SD.

4.2.2 Aβ stimulation of primary human monocytes

As no response was seen from the HEK293 cell line to the synthetic Aβ peptides, primary human monocytes were used to determine if a response could be seen in a different cell type. As these were primary cells they would also express any co-receptors or factors that are not present on HEK293 cells that may be needed for Aβ to activate TLRs. The peptide was prepared by diluting in DMSO and incubated for 72h, a concentration of 10μM and 25μM was used to stimulate the cells for 18h. The supernatants were collected and an IL-1β and TNFα ELISA were both performed. In addition to the use of the synthetic peptides; a recombinant Aβ 1-42 peptide was also used alongside the synthetic peptides, to investigate whether a peptide from a different source would produce a different response. The cell viability was also checked to ensure that any cell death was not affecting the results.

Although there was a significant increase in both TNFα and IL-1β in response to the ligands for TLR2 and TLR4, there was no production of either cytokine in response to synthetic peptides. Furthermore, altering the concentration from 10μM to 25μM had no effect on cytokine production (Fig 4.8). In addition the MTT assay showed no reduction in cell viability in response to the peptides at either concentration or the positive controls, suggesting there was no cell death (Fig 4.9).
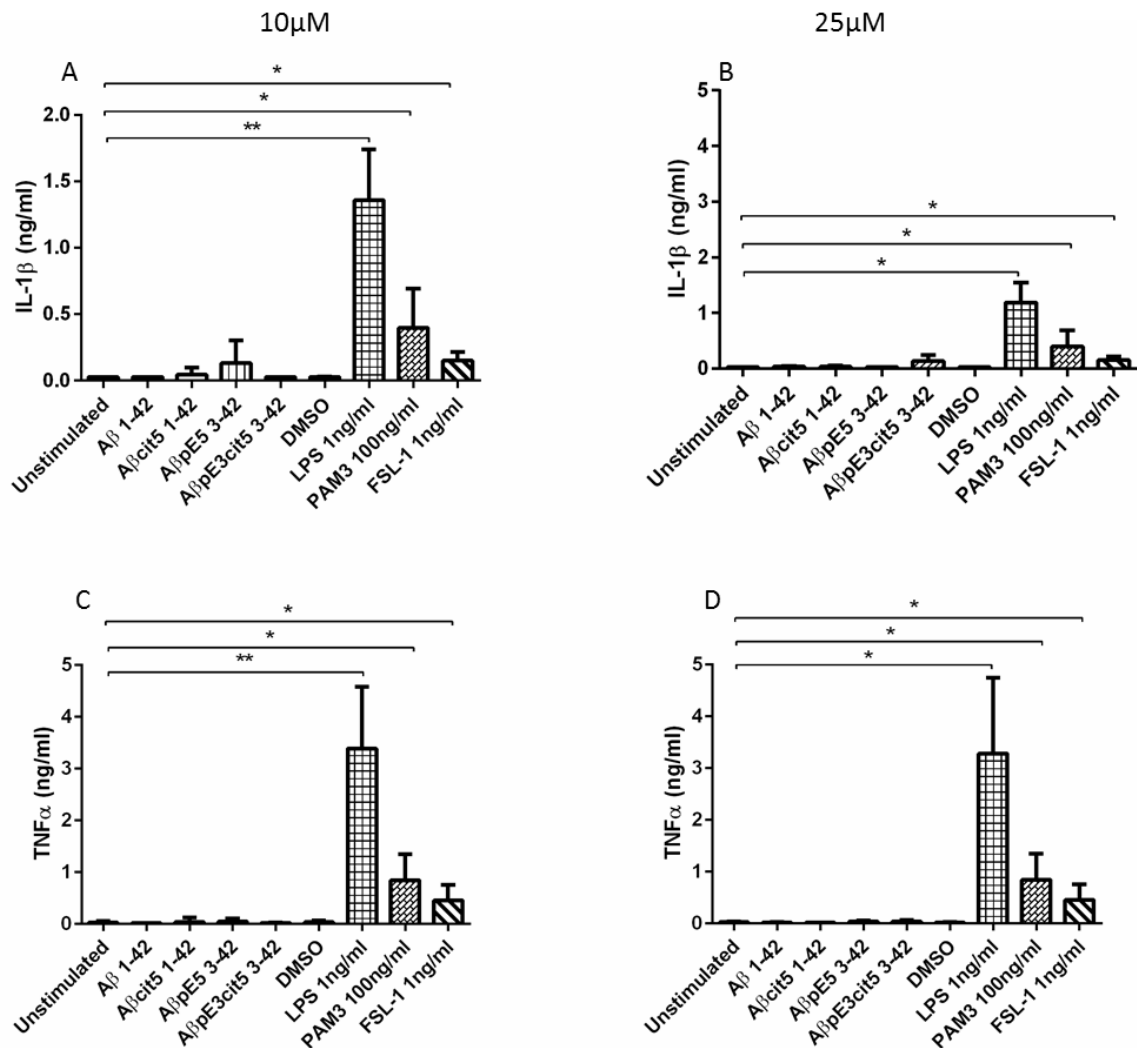
Figure 4 . 8: Primary human monocytes do not respond to Aβ 1-42 or PTM variants

Cryopreserved human monocytes were thawed and stimulated with synthetic Aβ peptides for 18h. The supernatants were collected and IL-1β and TNFα ELISAs were performed. A) Stimulation with 10μM peptide, IL-1β output B) stimulation with 25μM, IL-1β measured C) cells stimulated with 10μM, TNFα measured D) 10μM peptide used for stimulation TNFα output. 10ng/ml LPS, 100ng/ml PAM3 and 10ng/ml FSL-1 used as positive controls. Media and DMSO used as negative controls. P<0.05 *, p<0.01 **, p<0.001 ***. The means of triplicates from each experiment were calculated, the overall mean form all the experiments is plotted ± SD. n=3.
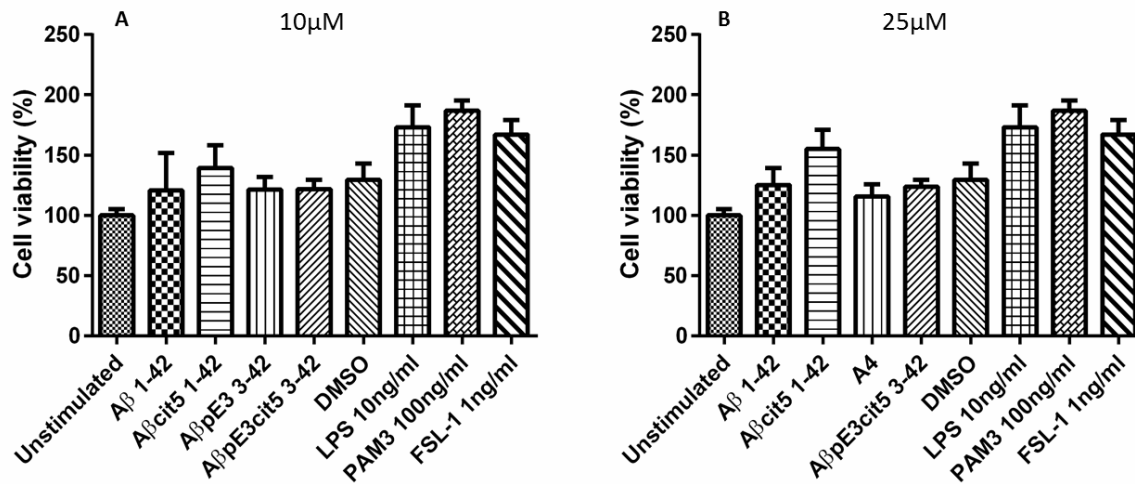
Figure 4 . 9: There is no reduction in cell viability in primary human monocytes in response to Aβ or its PTM variants

Primary human monocytes were stimulated with Aβ, Aβcit5, AβpE3 or AβpE3cit5 at a concentration of 10μM (A) or 25 μM (B), for 18h. An MTT assay was performed on the cells. Mean absorbance, of duplicates from different experiments was calculated, at 580nm. The overall mean from all the experiments is plotted ± SD. n=2

When the same experiments were performed with the recombinant Aβ 1-42, however, there was an increase in the amounts of IL-1β and TNFα produced. The amounts of IL-1β and TNFα were significantly higher after stimulation with the peptide than the untreated control; furthermore the amount of both of the cytokines produced was also much larger than after stimulation with all of the positive controls (Fig 4.10). An MTT assay was also performed to check the viability of the cells and showed that the peptide at both concentrations did not reduce cell viability (Fig 4.10C)
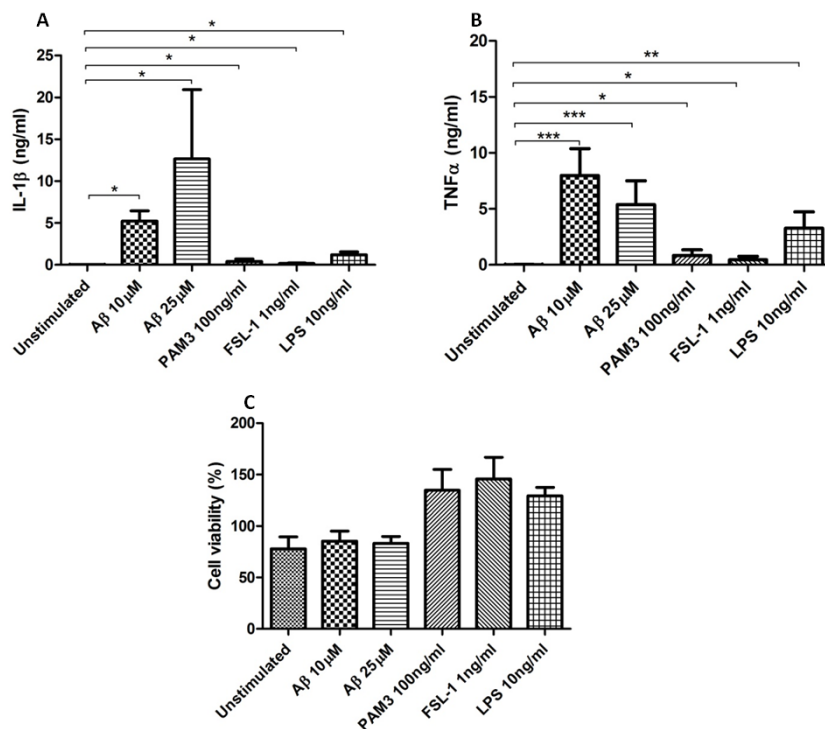
Figure 4 . 10: Recombinant Aβ 1-42 peptide produces an IL-1β and TNFα response in human monocytes

Recombinant Aβ 1-42 peptide was left to aggregate in DMSO for 72h and cryopreserved monocytes were stimulated with 10μM and 25μM for 18h. The supernatants were collected and an IL-1β and TNFα ELISA were performed. Cell viability was checked using an MTT assay. A) IL-1β B) TNFα C) MTT assay. 10ng/ml LPS, 100ng/ml PAM3 and 1ng/ml FSL-1 were positive controls. Unstimulated cells were used as the negative control. The mean of the triplicates from each experiment were calculated and plotted, bars show the overall mean ± SD. n = 3. p<0.05 *, p<0.01 **, p<0.001 ***

Due to the Aβ 1-42 being recombinant and therefore produced in *E.coli* it was necessary to check that the response that was observed was due to the peptide and not a contamination from the bacteria. To do this, the experiment was performed exactly as before but each stimulation condition was performed with and without polymyxin B (PMB). In addition to the PMB the concentration of the peptide used was also changed to 0.1μM, 1μM and 10μM. This was because such a large production of the cytokines had been seen previously, if LPS was present in the peptide it could saturate the PMB meaning it would not be possible to tell if the response was due to the peptide or to LPS. The IL-1β and TNFα ELISAs were performed as before and the response with and without PMB were compared.

As seen previously the recombinant Aβ 1-42 produced a significant TNFα and IL-1β response from the human monocytes. The amount of TNFα produced increased in dose-dependent manner: at 0.1μM 6.78ng/ml was measured, at 1μM 7.43ng/ml was measured and at 10μM 5.9g/ml was detected. The effect of PMB on TNFα production was also dependent on the concentration used to stimulate the cells. PMB had no effect on the highest concentration of peptide used but caused a decrease to 1.282ng/ml at the lowest concentration and a small decrease to 5.88 at the middle concentration. This suggests that PMB was saturated with LPS at the higher concentration (Fig 4.11C). The peptide also caused the release of IL-1β, the amount went from 15.2ng/ml to over 20ng/ml as the peptide concentration was increased from 0.1μM to 10μM. However, in contrast to TNFα, PMB did nothing to reduce the IL-1β signal when stimulated with any concentration of Aβ 1-42, in fact after stimulation with 0.1μM and 1μM Aβ 1-42 IL-1β production increased (Fig 4.11A). The experiment was then repeated twice more to see if the same pattern occurred. In these experiments there was less IL-1β produced when PMB was added to the lowest

concentration but not the two highest concentrations of peptide (Fig 4.11B). The

amount of IL-1β released went from 8.14ng/ml without PMB to 2.48ng/ml when

PMB was included. This suggests that the cytokines detected after stimulation with

recombinant Aβ1-42 from rPeptide was due to a contamination in the peptide.
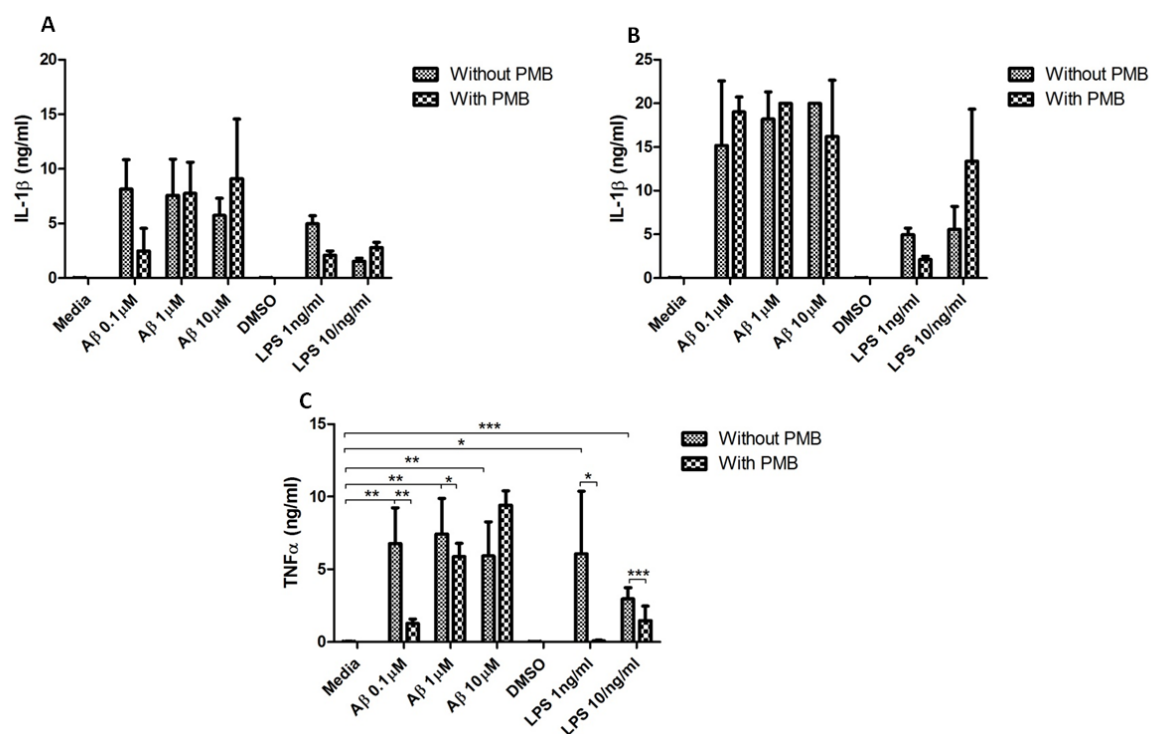
Figure 4 . 11: PMB reduces TNFα and IL-1β produced in response to recombinant Aβ 1-42 peptide

Recombinant peptide was dissolved in DMSO and left to aggregate for 72h. Cryopreserved monocytes were stimulated with 0.1, 1 or 10μM peptide for 18h. IL-1β and TNFα ELISAs were carried out on the supernatants. A) IL-1β results from first experiment, n=1 B) IL-1β results from second and third experiment, n=2. C) TNFα, n=3. 1ng/ml and 10ng/ml LPS were used as positive controls and unstimulated cells were the negative controls. The mean of the triplicates from each experiment were calculated and plotted, bars show the overall mean ± SD.

4.2.3. Alternative Aβ preparation: Testing peptide aggregation

Due to the lack of a positive response being observed with first two protocols for peptide preparation a final preparation protocol was used that had been used in similar experiments. Previous peptide preparations and peptide incubation times were based on published data showing the production of fibrils. However, TEM analysis of these images showed that the peptide was not able to produce fibrils after the use of protocol 1 and after protocol 2 the peptide reduced cell viability. Peptide preparation 3 was shown to produce fibrils and was subsequently used to stimulate HEK293 cells to investigate whether a response could be observed.

Initially only the unmodified peptide was used, HEK293 TLR2 and TLR4-MD2-CD14 cells were stimulated. The HEK293 cells were plated and incubated until ready to use and were then stimulated with 15μM peptide for 18h. As before the supernatants were collected and an IL-8 ELISA was performed. This experiment examined the peptide at a number of different timepoints: 24h, 96h (4d), 168h (7d) and 216h (9d) and at each of these points an IL-8 ELISA was performed and a TEM grid was prepared.

Peptide left to aggregate in PB did not show any evidence of aggregation in the TEM images. In addition at each of the time points there was no response to the peptide from either the HEK293-TLR2 or HEK293-TLR4-MD2-CD14 cells, although both cell types responded to the positive controls (Fig 4.12).

Similarly, the peptide aggregated in water for 24h also showed no evidence of aggregation and did not induce a response from either of the cell types. After 96h, the peptide had begun to aggregate but did not possess a fibrillar morphology (Fig 4.13C). There was also no evidence of IL-8 production from either TLR2 or TLR4-

MD2-CD14 expressing HEK293 cells after stimulation with the peptide at this timepoint (Fig 13A, B).

At the 168h timepoint the peptide had a fibrillar morphology, however it was unable to induce the production of IL-8 from TLR4-MD2-CD14 expressing HEK293s (Fig 4.13B). In contrast, there was an increase of 182.6pg/ml IL-8 above the unstimulated background after stimulation of TLR2 expressing HEK293 cells (Fig 4.13A). Further to this, after stimulation with the peptide aggregated for 216h, which also had a fibrillar morphology, there was an increase of 206ng/ml IL-8 from the TLR2 expressing cells (Fig 4.13A). No response was seen from the TLR4-MD2-CD14 expressing cells, although a response was observed after stimulation with LPS.
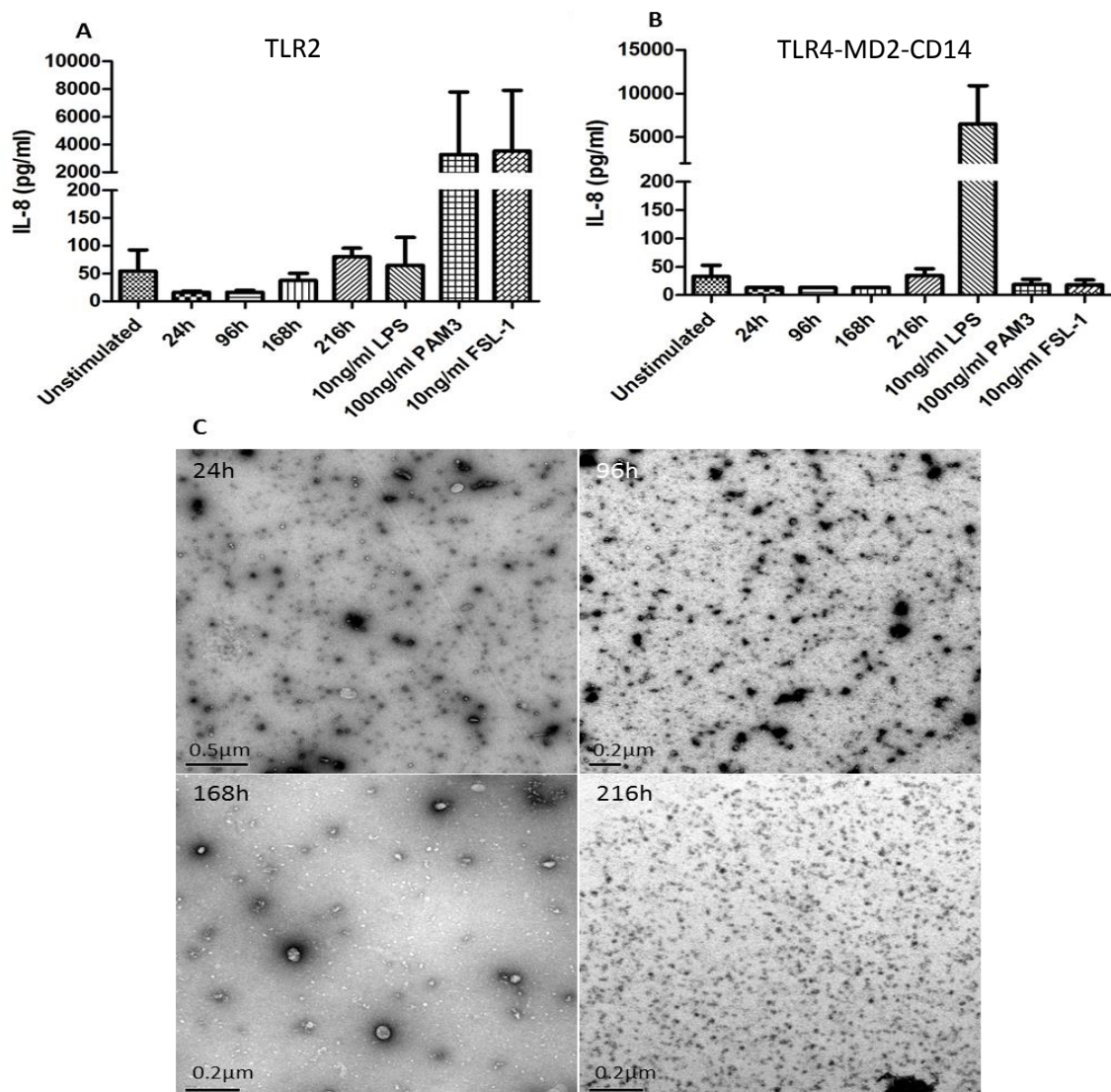
Figure 4 . 12: Aβ 1-42 peptide incubated in PB does not cause HEK293 cells expressing TLR2 or TLR4-MD2-CD14 to release IL-8

Aβ 1-42 peptide was disaggregated and diluted in 50mM sterile PB and was left to aggregate at 4°C. The peptide was aggregated for different lengths of time and HEK293 cells expressing TLR2 or TLR4-MD2-CD14 were stimulated with 25μM Aβ 1-42 peptide for 18h. The supernatants were collected for an IL-8 ELISA. A) TLR2 expressing HEK293 cells, B) TLR4-MD2-CD14 expressing HEK293 cells. C) TEM images of Aβ 1-42 peptide at four different timepoints 24h, 96h, 216h, 216h. 10ng/ml LPS was used as the positive control for TLR4, 100ng/ml PAM3 and 10ng/ml FSL-1 were used as positive controls for TLR2, unstimulated cells were used as negative control. Bars show the mean of triplicates ± SD. n=1
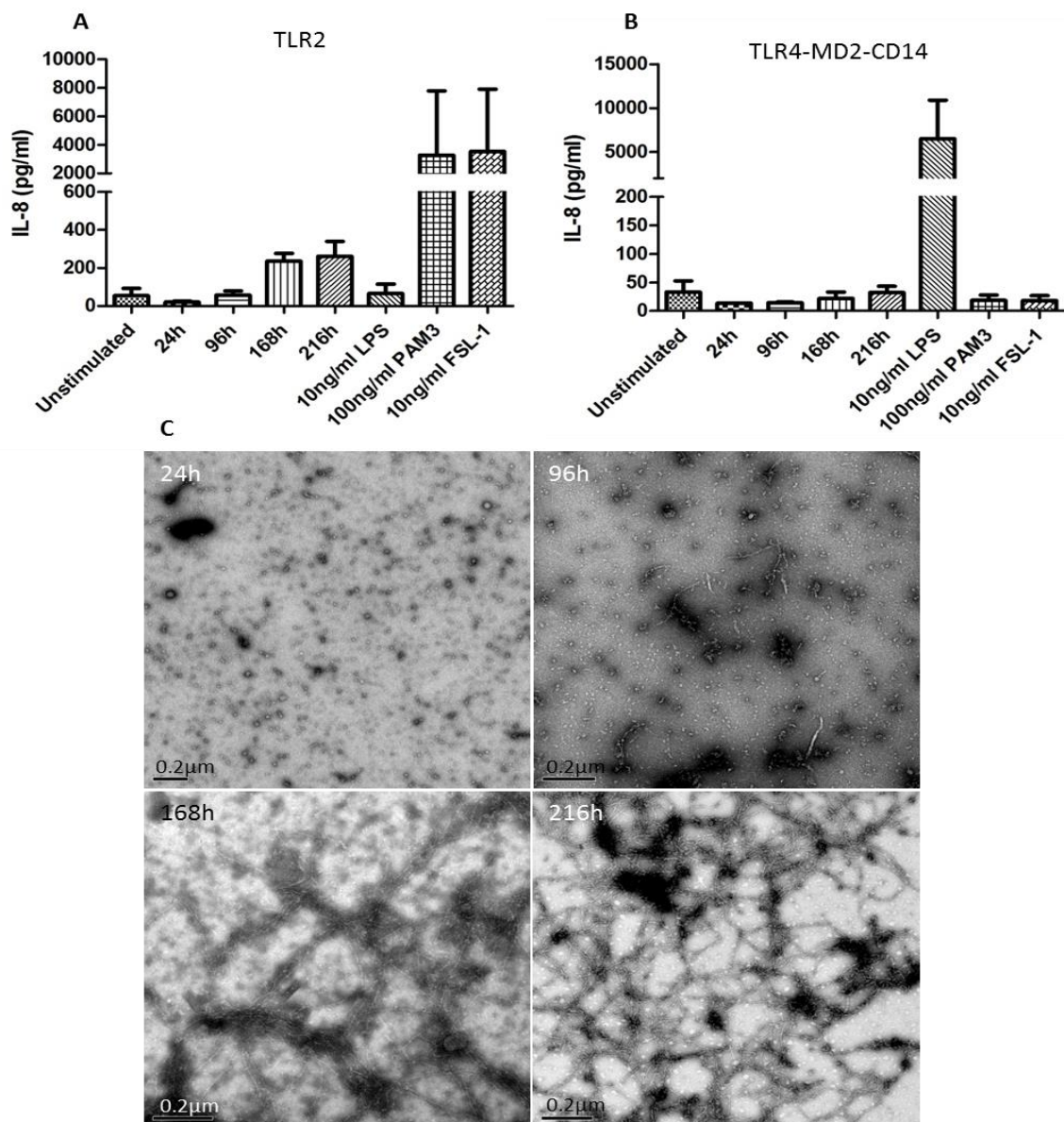
Figure 4 . 13: Aβ 1-42 peptide incubated in water for 168h or longer causes HEK293 cells expressing TLR2 but not TLR4-MD2-CD14 to release IL-8

Aβ 1-42 peptide was disaggregated and diluted in sterile water and was left to aggregate at 4°C. The peptide was aggregated for different lengths of time and HEK293 cells expressing TLR2 or TLR4-MD2-CD14 were stimulated with 25μM Aβ 1-42 peptide for 18h. The supernatants were collected for an IL-8 ELISA. A) TLR2 expressing HEK293 cells, B) TLR4-MD2-CD14 expressing HEK293 cells. C) TEM images of Aβ 1-42 peptide at four different timepoints 24h, 96h, 216h, 216h. 10ng/ml LPS was used as the positive control for TLR4, 100ng/ml PAM3 and 10ng/ml FSL-1 were used as positive controls for TLR2, unstimulated cells were used as negative control. Bars show the mean of triplicates ± SD. n=1

After these experiments a titration was performed with the unmodified peptide to find the best concentration of peptide to use. The peptide was prepared as before and only incubated in water at a concentration of 15μM, 30μM or 50μM was used. The IL-8 ELISA showed an increase in production between the 15μM and the 30μM stimulation, however there was very little difference between the 30μM and the 50μM stimulations. Therefore the 30μM concentration was used for the remaining experiments (Fig 4.14A). In contrast there was no production of IL-8 from the HEK293 cells expressing TLR4 when any of the peptide concentrations were used (Fig 4.14B).
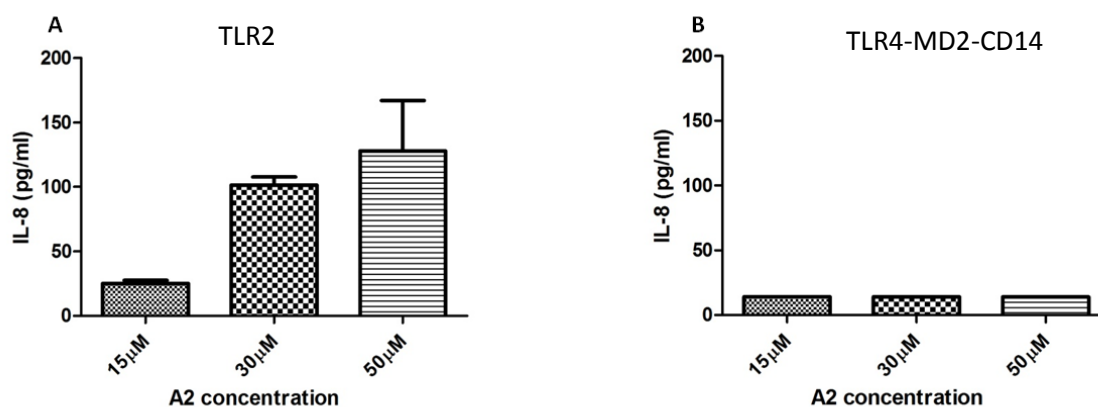
Figure 4 . 14: Titration of unmodified Aβ 1-42 peptide

Unmodified peptide was were disaggregated and dissolved in water before being allowed to aggregate for 216h at 4°C. HEK293 cells expressing TLR2 (A) or TLR4-MD2-CD14 (B) were stimulated with 15, 30 and 50μM for 18h, the supernatants were collected for IL-8 ELISA. Bars show mean of triplicates ± SD.

The experiments were repeated using only the unmodified Aβ 1-42 peptide to ensure the response was seen consistently. The IL-8 ELISA showed that there was a significant increase in the amount of IL-8 produced from TLR2 expressing HEK293 cells when compared to the untreated control (Fig 4.15A) but there was no production of IL-8 from TLR4 expressing cells (Fig 4.15B)
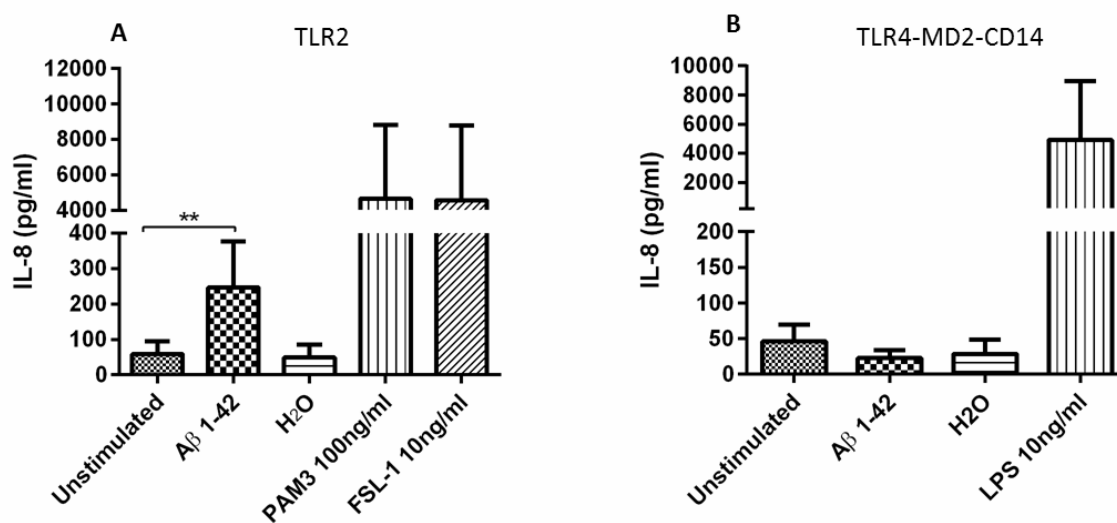
Figure 4 . 15: HEK293 cells expressing TLR2 but not TLR4 are activated by Aβ 1-42 peptide

Unmodified Aβ 1-42 peptide was disaggregated and dissolved in water before being allowed to aggregate for 216h. HEK293 cells expressing either TLR2 (A, B) or TLR4-MD2-CD14 (C, D) were stimulated with peptides, negative and positive controls for 18h. The supernatants were collected and an IL-8 ELISA was performed. 10ng/ml was the positive control for TLR4, 100ng/ml PAM3 and 10ng/ml FSL-1 were the positive controls for TLR2. Unstimulated cells and HFIP/water were the negative controls. $P<0.05 = *$, $P<0.01 = **$, $P<0.001 = ***$. The mean of the triplicates from each experiment were calculated and plotted, bars show the overall mean ± SD n=3.

As a response was seen by the HEK293-TLR2 cells to the unmodified peptide, the next step was to investigate whether the PTM variants of the peptide had the same response. All four peptides were used to stimulate the TLR2 and TLR4-MD2-CD14 expressing cells. The peptides were all prepared using protocol 3 and were all incubated in water for 216h. A TEM grid was also prepared at this timepoint to ensure that the peptides had a fibrillar morphology (Fig 4.16E). The peptide was used at a concentration of 30μM and the cells were stimulated for approximately 18h before an IL-8 ELISA was performed.

There was no evidence of IL-8 production from HEK293 TLR4-MD2-CD14 cells to any of the peptides with or without PTMs (Fig 4.16C). However, all four of the peptides induced a significant response from the TLR2 expressing cells. The amount of IL-8 measured was very similar for Aβ 1-42, Aβcit5 1-42 and AβpE3cit53-42 and although the mean amount of IL-8 released in response to AβpE3 appeared to be higher there was no significant difference between AβpE3 3-42 and the other peptides (Fig 4.16).
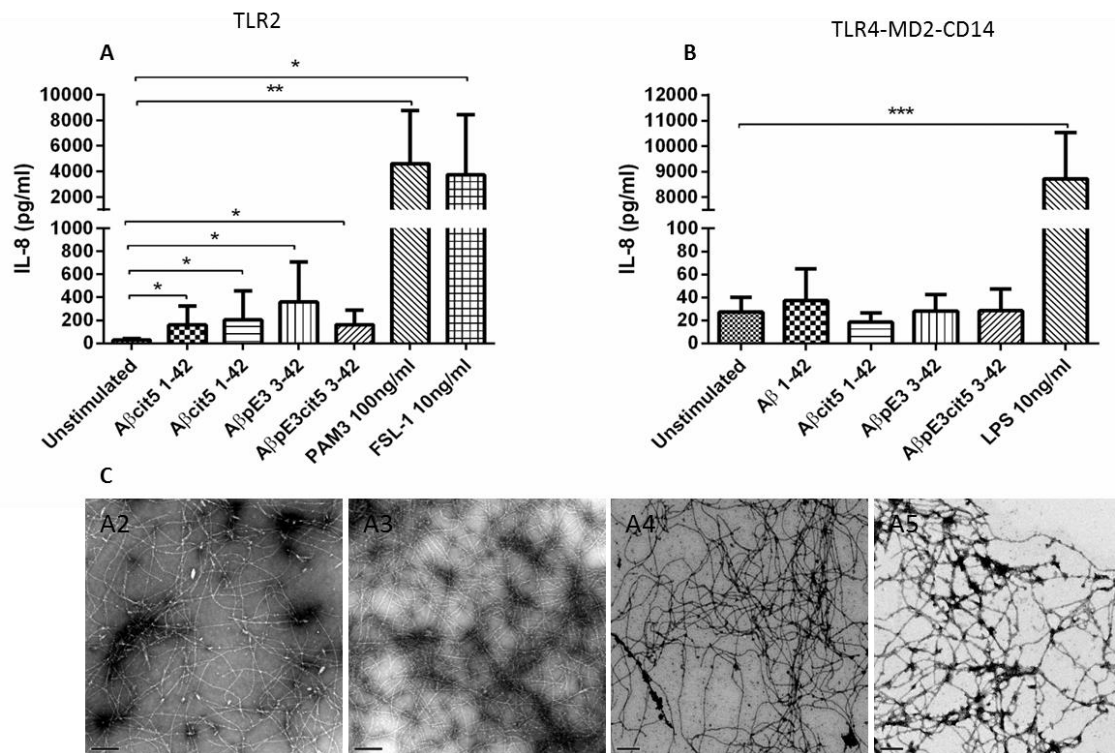
Figure 4 . 16: Unmodified and PTM variants of Aβ activated HEK293 expressing TLR2 but not TLR4

Unmodified Aβ 1-42 peptide and its PTM variants were disaggregated and dissolved in water and allowed to aggregate at 4°C for 216h. A) TLR2 expressing HEK293 cells were stimulated with 30μM Aβ 1-42, Aβcit5 1-42, AβpE3 3-42 or AβpE3cit5 3-42 and negative and positive controls for 18h, an IL-8 ELISA was performed on the supernatants. B) The same peptides and experiments were performed on HEK293 cells expressing TLR4-MD2-CD14. C) TEM images of peptide morphology. 10ng/ml LPS was the positive control for TLR4, 100ng/ml PAM3 and 10ng/ml FSL-1 were the positive controls for TLR2. The negative control was unstimulated cells. $P<0.05 = *$, $P<0.01 = **$, $P<0.001 = ***$. The mean of the triplicates from each experiment were calculated and plotted, bars show the overall mean $\pm$ SD. n=3.

To further investigate the effects of these peptides on TLR2 and TLR4, primary human monocytes were also tested. These cells were defrosted from frozen stocks and then treated with 30μM of each of the peptides for 18h. The supernatants were collected and an IL-1β and TNFα ELISA were performed. The peptides were incubated for either 72h or 216h to examine whether monocytes responded differently to fibrillar and non-fibrillar Aβ peptides.

Although the monocytes significantly responded to the ligands for TLR2 and TLR4 they failed to respond to the any of the peptides from either timepoint. There was no significant difference in either IL-1β or TNFα production when comparing the peptides to the negative control although they all appeared to be significantly lower than all three of the positive controls (Fig 4.17).

In addition to cryopreserved monocytes, fresh monocytes were also used to ensure that freezing the cells had no effect on peptide stimulation. They were treated exactly as the cryopreserved monocytes, although only Aβcit5 1-42 and AβpE3 3-42 were used in a fibrillar morphology. No response was seen after stimulation with any of the peptides but the cells did produce both cytokines in response to LPS and PAM3 (Fig 4.18).
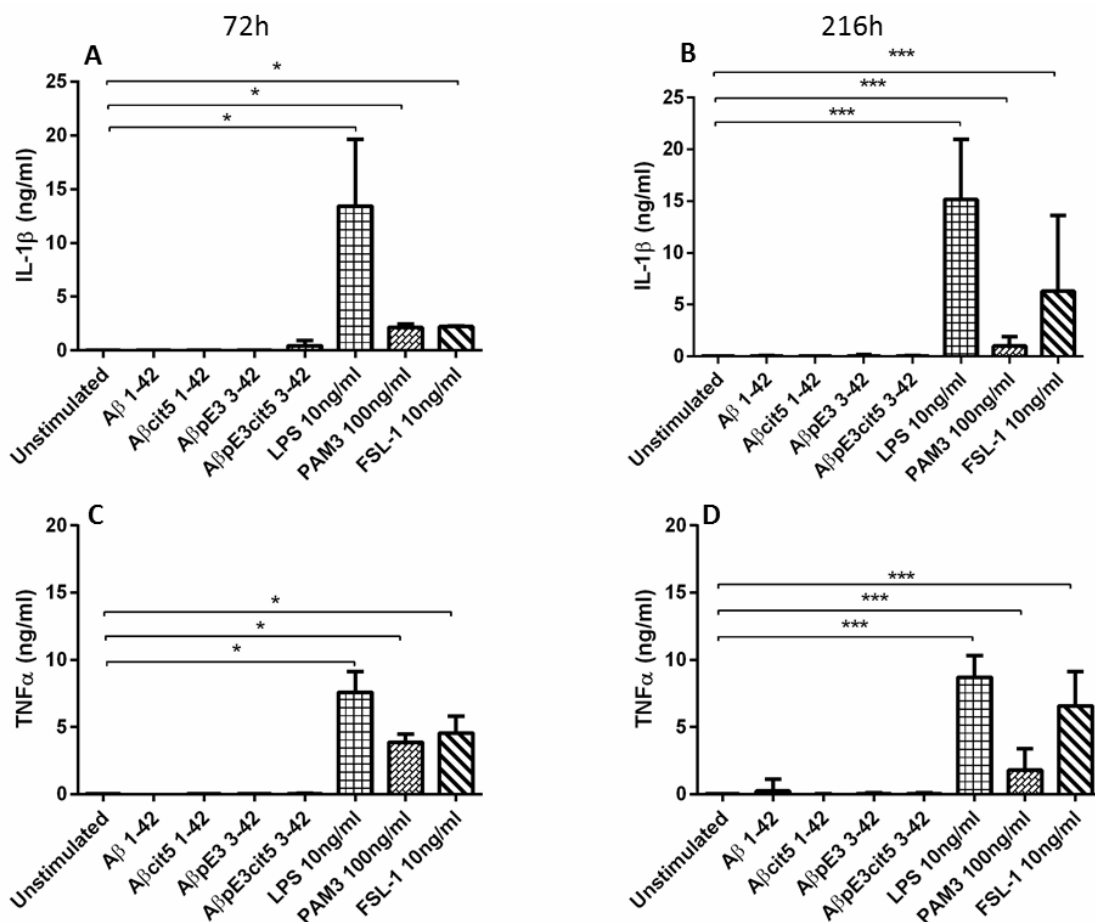
Figure 4 . 17: Cryopreserved monocytes are not activated by Aβ peptides or its PTM variants using protocol 3.

Unmodified Aβ 1-42 and PTM variants were disaggregated and dissolved in sterile water and left to aggregate at 4°C for 72h or 216h. Cryopreserved primary human monocytes were stimulated with 30μM of all four peptides for 18h and supernatants were collected for IL-1β and TNFα ELISA. IL-1β produced after stimulation with peptides incubated for 72h (A) or 216h (B). TNFα produced after stimulation with peptide incubated for 72h (C) or 216h (D). 10ng/ml LPS, 100ng/ PAM3 and 10ng/ml FSL-1 were used as positive controls for TLR4 or TLR2 Unstimulated cells were used as the negative control $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***. The mean of the triplicates from each experiment were calculated and plotted, bars show the overall mean ± SD. n=3.
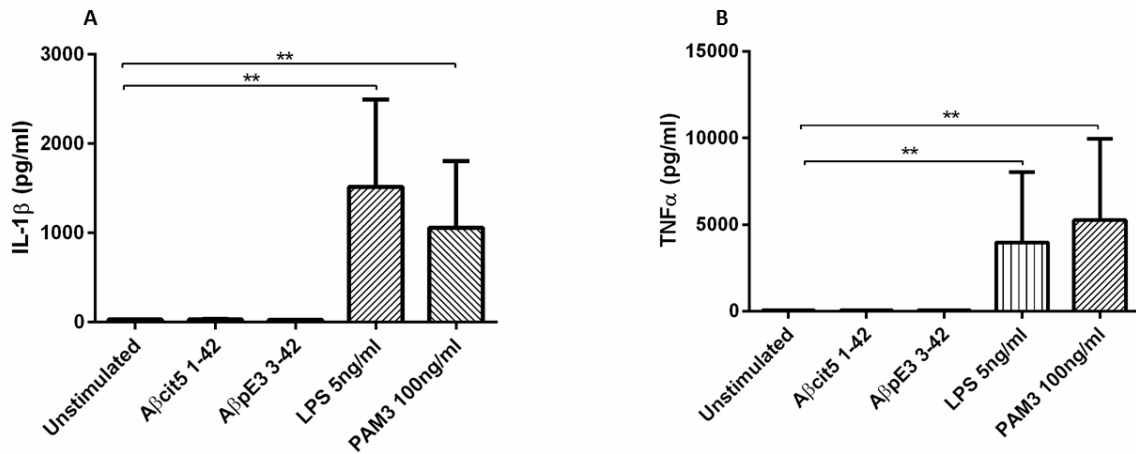
Figure 4 . 18: Aβ peptides are unable to cause the release of IL-1β or TNFα

Fresh monocytes were isolated and plated. They were stimulated with 30μM Aβcit5 1-42 or AβpE3 3-42 peptide or 5ng/ml LPS or 100ng/ml PAM3 for 18h. An IL-1β and TNFα was performed on the cell supernatants. A) IL-1β, B) TNFα. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. The mean of the triplicates from each experiment were calculated and plotted, bars show the overall mean ± SD. n=3.

4.2.4 Aβ induced responses in PBMCs

As there had been a response observed to Aβ peptides from HEK293 cells expressing TLR2 and TLR4, the response of patients with AD and age-matched controls were also investigated despite the lack of monocyte response from primary monocytes. For these experiments, PBMCs were isolated from heparinised blood obtained from patients and controls and were plated in 96-well plates. The peptide had been left to aggregate for 216h to ensure it had a fibrillar morphology. They were then stimulated with all four of the peptides at a concentration of 30μM for 18h. The supernatants were collected and IL-1β and TNFα ELISAs were performed.

The results described here were performed using the same batch of peptides that had been used for all previous experiments, however a number of subsequent experiments were performed using a second batch of the peptides. But, the results seen from the two batches were very different. Results obtained using the second batch are described in a later chapter.

The responses from patients and controls were compared in order to determine if there were differences between the two groups. All control PBMCs produced a significantly higher amount of IL-1β but not TNFα in response to all of the peptides when compared to unstimulated control. This was also true for the positive control, LPS. Similarly, none of the patient samples produced an increased level of TNFα after stimulation with any of the peptides (Fig 4.20), however half of the patients produced more IL-1β than the untreated control and half of them did not respond to any of the peptides (Fig 4.19). All of the patients also produced both TNFα and IL-1β in response to LPS. Despite the differences in patient responses there was still a

significant increase in the mean production of IL-1β when all the patients were

combined (Fig 4.19).
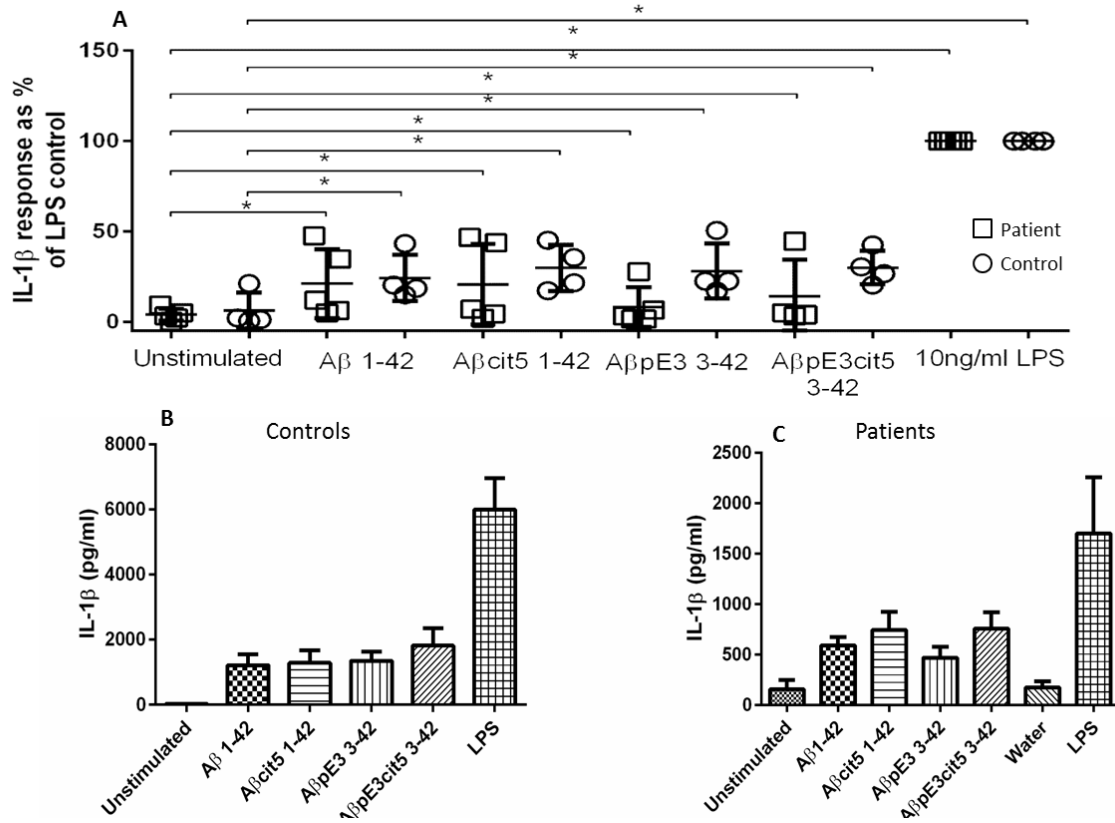
Figure 4 . 19: PBMCs from patient and control samples produce IL-1β in response to Aβ peptide and its PTM variants

PBMCs were stimulated with Aβ peptides which were disaggregated and dissolved in water before being allowed to aggregate for 216h. PBMCs were stimulated with 30μM peptide for 18h and an IL-1β ELISA was performed on cell supernatants. A) IL-1β production from patients (squares) and age-matched controls (circles) B) Representative experiment for age-matched control, C) Representative experiment for patient with AD. p<0.05 *, p<0.01 **, p<0.001 ***. Each point represents the mean of duplicates from one participant, the lines show the overall mean ± SD. n=6 patients, n=5 age-matched controls

180
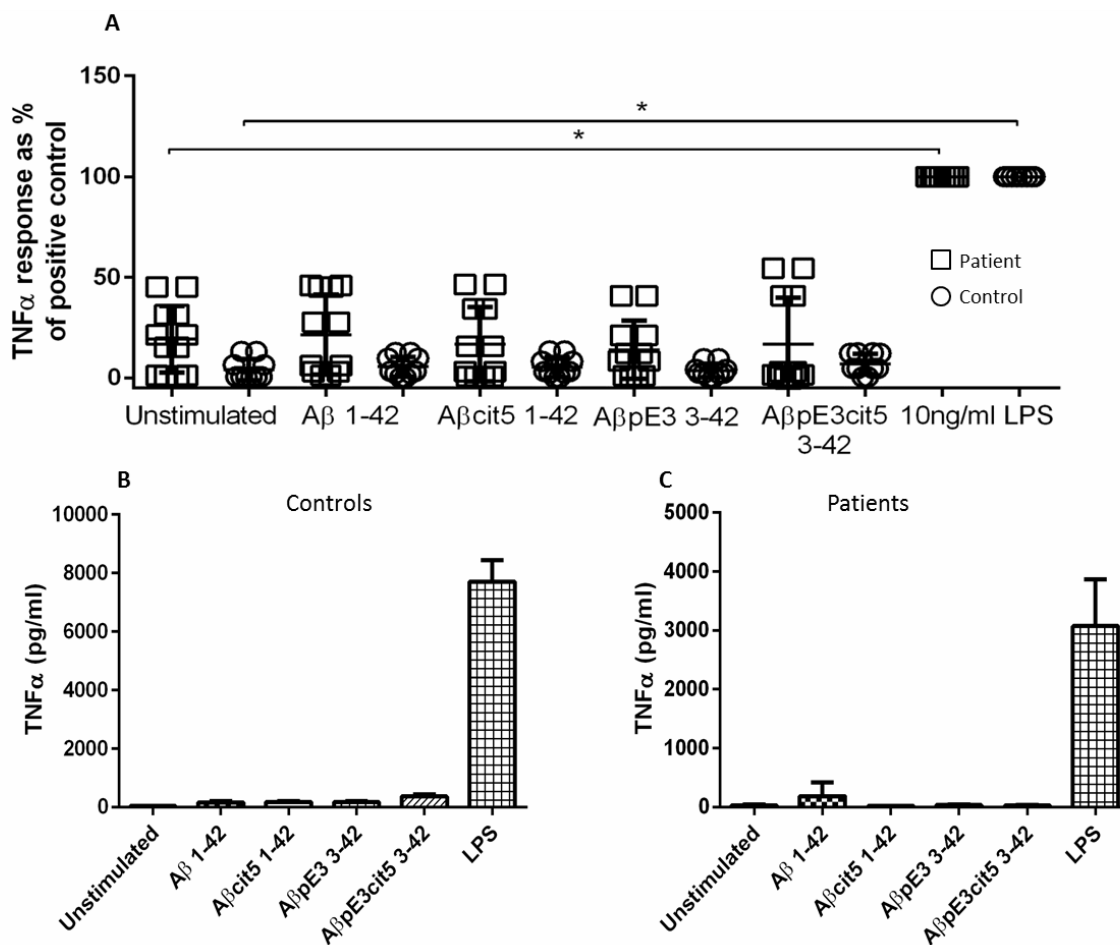
Figure 4 . 20: PBMCs from patient and control samples do not produce TNFα in response to Aβ peptide or its PTM variants

PBMCs were stimulated with Aβ peptides which were disaggregated and dissolved in water before being allowed to aggregate for 216h. PBMCs were stimulated with 30μM peptide for 18h and a TNFα ELISA was performed on cell supernatants. A) TNFα production from patients (squares) and age-matched control (circles), B) Representative experiment for age-matched control, C) Representative experiment for patient with AD. p<0.05 *, p<0.01 **, p<0.001 ***. Each point represents the mean of duplicates from one participant, the lines show the overall mean ± SD. n=6 patients, n=5 age-matched controls.

In order to investigate which cell type was producing cytokines in response to the Aβ peptides, intracellular staining of PBMCs was used. In PBMC cultures containing, T-cells, B-cells, NK-cells and monocytes, TLRs would be predominantly expressed in the highest levels on monocytes [388], furthermore monocytes and macrophages are considered the main source of IL-1β [389]. Therefore, the surface markers HLA-DR and CD14 were used to gate monocytes. In addition, to remove any T-cells from the gated population antibody for CD3 was also included. Figure 4.21 shows the gating strategy.

The percentage of cells with intracellular IL-1β and TNFα was measured in response to all four of the peptides as well as PAM3 and LPS. PBMCs were isolated as before from the heparinised blood of healthy controls and stimulated with 30μM peptide for 18h and antibody staining was performed. In addition, neutralising antibodies for TLR2 and TLR4 were added to the experiment to determine whether the peptide was acting through either of these receptors.

The monocytes were initially gated as single cells then the population positive for HLA-DR were selected. The final cells were selected as CD14$^+$CD3$^-$ and the proportion with intracellular IL-1β, TNFα or both was measured. The gating strategy can be seen in figure 4.22. In addition to this a previous experiment investigated the percentage of CD14$^+$CD3$^-$ that expressed TLR2 and TLR4. A representation of the distribution of these cells is shown in Fig 4.22, it was observed that 89.48% and 90.72% patient and control monocytes respectively expressed both TLR2 and TLR4. In addition 8.7% of patient monocytes expressed TLR4 alone and 9.5% of healthy control monocytes, whereas less than 1% of both patient and human monocytes expressed TLR2 alone.
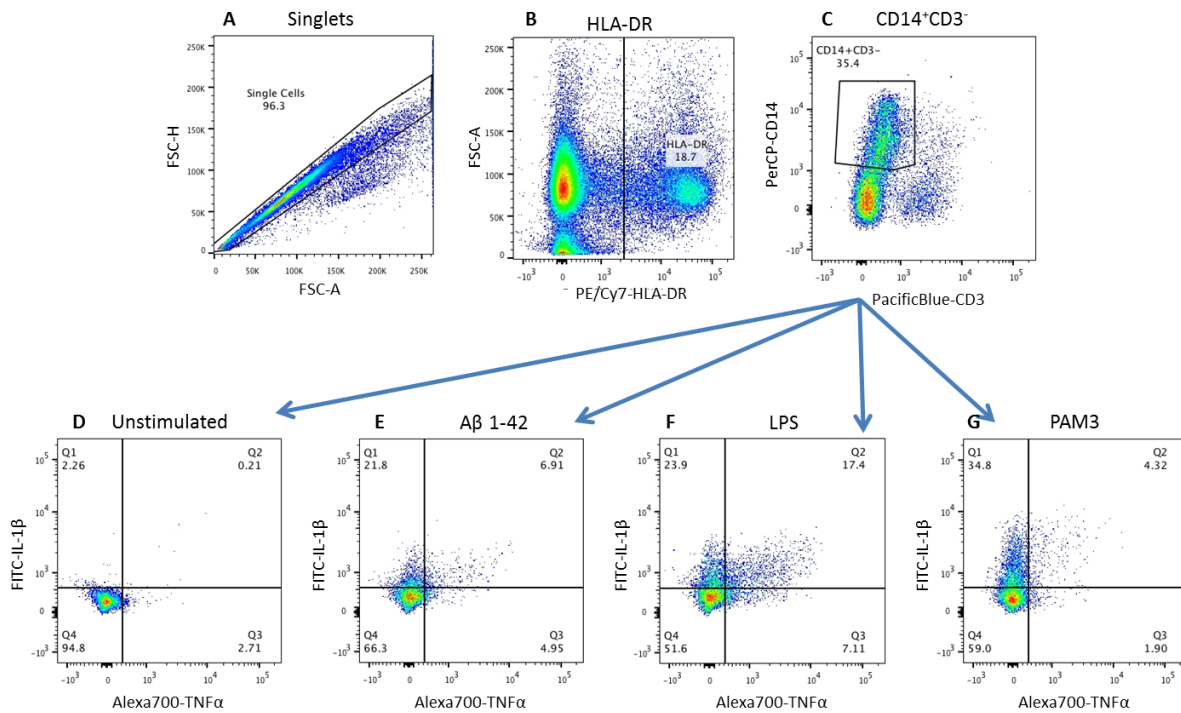
Figure 4 . 21: Gating strategy for monocytes

PBMCs were acquired using LSR-II flow cytometer. A) Single cells, D) HLA-DR$^+$ population, E) CD14$^+$CD3$^-$. F) Negative control, E) Aβ stimulated cells F) LPS stimulated cells G) PAM3 stimulated cells.
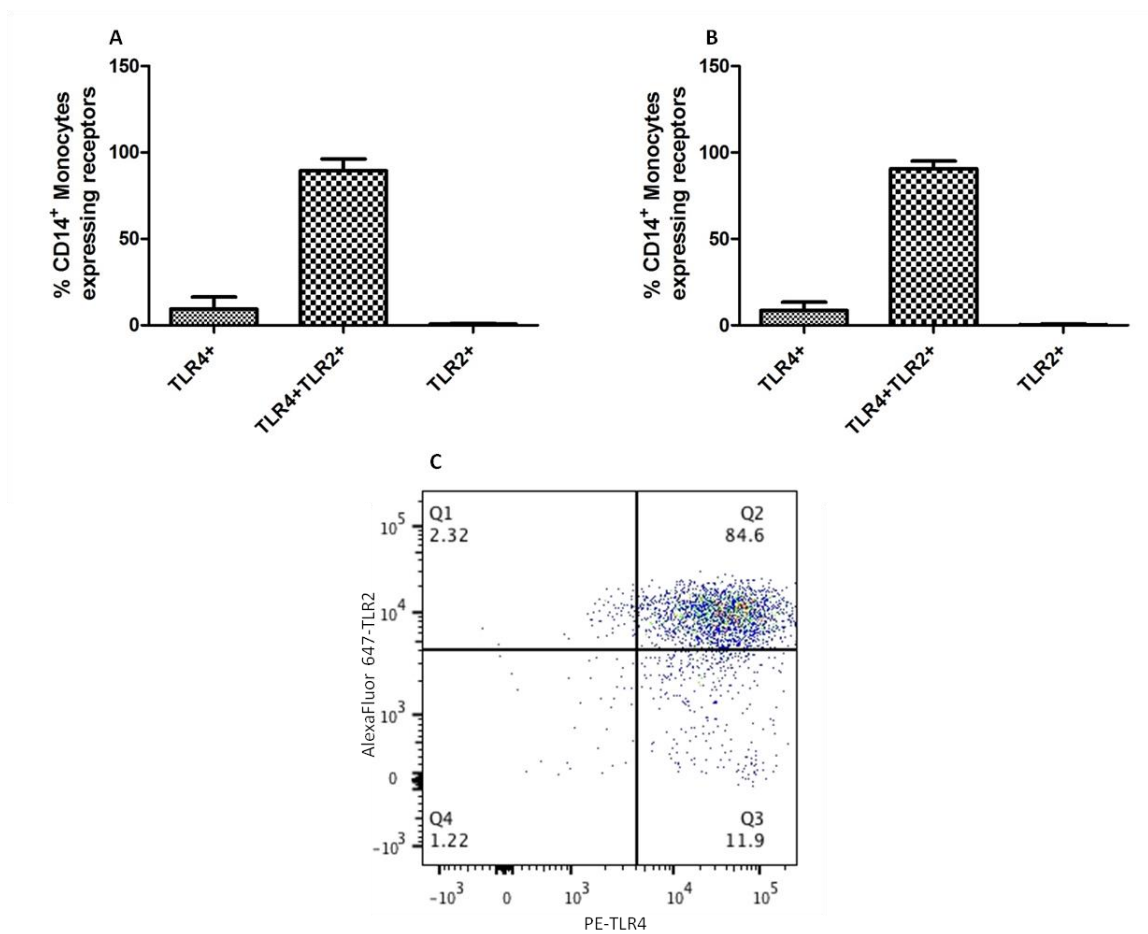
Figure 4 . 22: Expression of TLR2 and TLR4 on CD14⁺ monocytes

PBMCs were isolated and stained with antibodies for HLA-DR, CD14, CD3, TLR2 and TLR4. Cells were gated to show the expression of TLR2 and TLR4 on HLA-DR⁺CD14⁺CD3⁻. A) Proportion of cells expressing TLR2, TLR4 or TLR2 and TLR4 on patient samples. B) Proportion of cells expressing TLR2, TLR4 or TLR2 and TLR4 on healthy controls. C) Representative image scatter of CD14⁺ monocytes. The means from duplicates was calculated and plotted, bars show the overall mean ± SD. n=3

The intracellular staining showed that in response to the four peptides as well as the positive controls there was an increase in the proportion of cells expressing IL-1β and it was shown that it was the monocytes that were producing IL-1β in response to the peptides. Aβ 1-42, Aβcit5 1-42 and AβpE3 3-42 all caused an increase in the proportion of cells expressing IL-1β as well as LPS and PAM3. There was an increase from 1% of cells expressing IL-1β in the unstimulated control which increased to approximately 17% after Aβ stimulation, 23% after Aβcit5 1-42 and 10% after AβpE3 3-42 stimulation. LPS, PAM3 and the peptides all showed a very similar proportion of cells expressing IL-1β (Fig 4.23A).

In addition, monocytes also showed a small increase in cells that were positive for both IL-1β and TNFα, although the increase was far smaller than for IL-1β alone. The unstimulated cells had less than 1% of cells expressing both cytokines whereas Aβ 1-42, Aβcit5 1-42 and AβpE3 3-42 as well as PAM3 all increased to approximately 4%. LPS had a greater effect and increased the proportion of cells to 10% (Fig 4.23B). Furthermore, there was no increase in cells expressing TNFα in response to any of the peptides or the two positive controls (Fig 4.23C). All experiments were performed in duplicate and the mean for each donor was calculated and plotted.
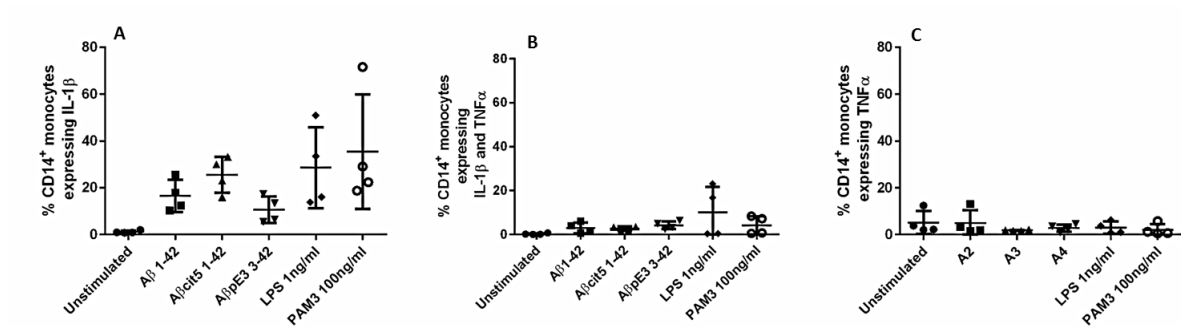
Figure 4 . 23: Aβ 1-42 and its PTM variants all increase proportion of cells with intracellular IL-1β

Unmodified Aβ 1-42 and its PTM variants were disaggregated and dissolved in water before being allowed to aggregate for 216h at 4°C. PBMCs from healthy controls were stimulated with the peptides for 2h before BFA was added for a further 14h. Cells were then stained with surface and intracellular antibodies and acquired using LSR-II flow cytometer. Cells were gated as HLA-DR^{+}CD14^{+}CD3^{-}, monocytes. A) Intracellular IL-1β, B) double positive cells, C) intracellular TNFα. 10ng/ml LPS and 100ng/ml PAM3 were used as positive controls for TLR4 and TLR2 and unstimulated cells were the negative control. Each point represents the mean of duplicates from one participant, the lines show the overall mean ± SD. n=4.

To determine whether these responses occur because of the peptides activating TLR2 the receptors were blocked with a neutralising antibody (αTLR2). The antibody was added to PBMCs before stimulation and then cells were treated exactly as before.

The effect the antibody had, was different depending on the individuals. αTLR2 reduced the proportion of cells expressing IL-1β in two samples, a difference of 10% was seen in both cases, but not in the other two. The response to Aβcit5 1-42 was reduced in three out of the four samples, one by 20% and two by 10%. The response to AβpE3 3-42 was reduced by 15% in one sample, 5% in a second sample but not at all in the remaining two.

For all three peptides, the same sample (donor 3) had a reduction in the proportion of cells expressing IL-1β after the addition of αTLR2, furthermore this donor also showed the greatest reduction in cells expressing IL-1β. However, for all other donors there was variation in which peptide responses were reduced. IL-1β expressing cells were reduced after PAM3 stimulation in all the samples (Fig 4.24).
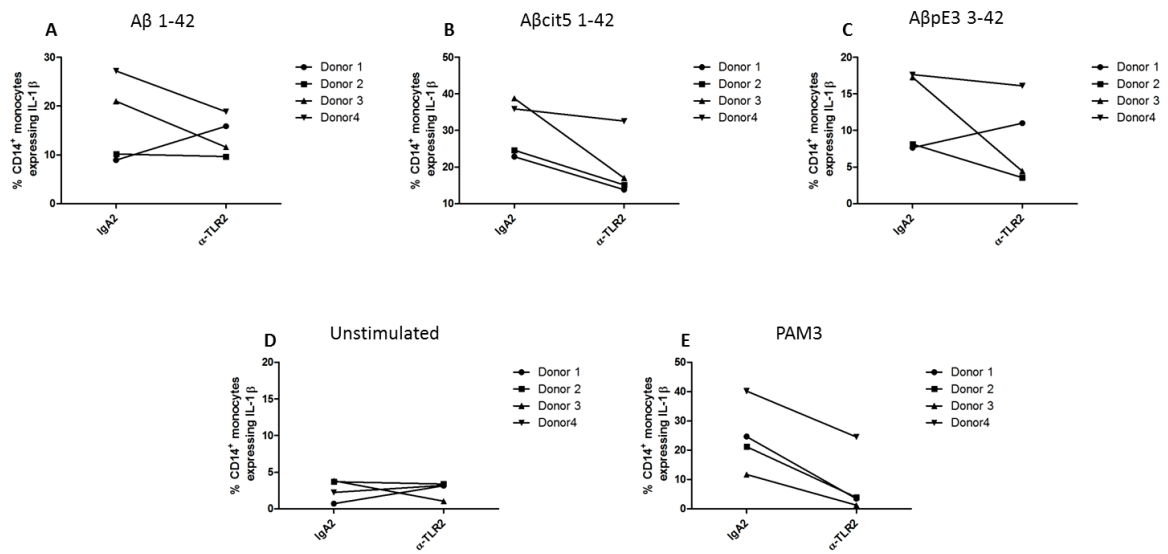
Figure 4 . 24: Neutralising TLR2 antibody reduces the proportion of cells expressing IL-1β in some donor samples but not in others

PBMCs were treated with either IgA2 isotype control or α-TLR2 antibody. The cells were then stimulated with Aβ 1-42, Aβcit5 1-42 or AβpE3 3-42 peptide or the positive control and incubated overnight. Cells were stained with surface antibodies CD3, CD14 and HLA-DR and intracellular antibodies IL-1β and TNFα. The proportion of cells expressing IL-1β were measured. A) Aβ, B) Aβcit5, C) AβpE3, D) Negative control and E) PAM3. Each point represents the mean of duplicates from one participant. n=4.

As there were some differences in the effect the αTLR2 antibody had on the expression of IL-1β, the composition of the PBMCs from each donor was examined to determine if there were any variations in the proportion of cell types that may explain why αTLR2 has a different effect on different donors.

Monocytes were of the most interest here as they express the greatest number of TLRs, this would mean that if there were fewer monocytes there would be fewer TLRs. Therefore the αTLR2 antibody may have more of an effect as it would be able to block a greater proportion of the receptors.

In general the proportion of each cell type was very similar for all four of the donors, however, there were some donors that contained a smaller proportion of specific cell types. B-cell and T-cell levels were within a few percent for each of the donors (Fig 4.25A, 4.25B) but for monocytes donor 3 showed approximately 3 fold less monocytes than all other donors (Fig 4.25C) and donor 2 had around half the proportion of NK-cells (Fig 4.25D). For both monocytes and NK-cells, all other donors showed very similar proportions of cells.
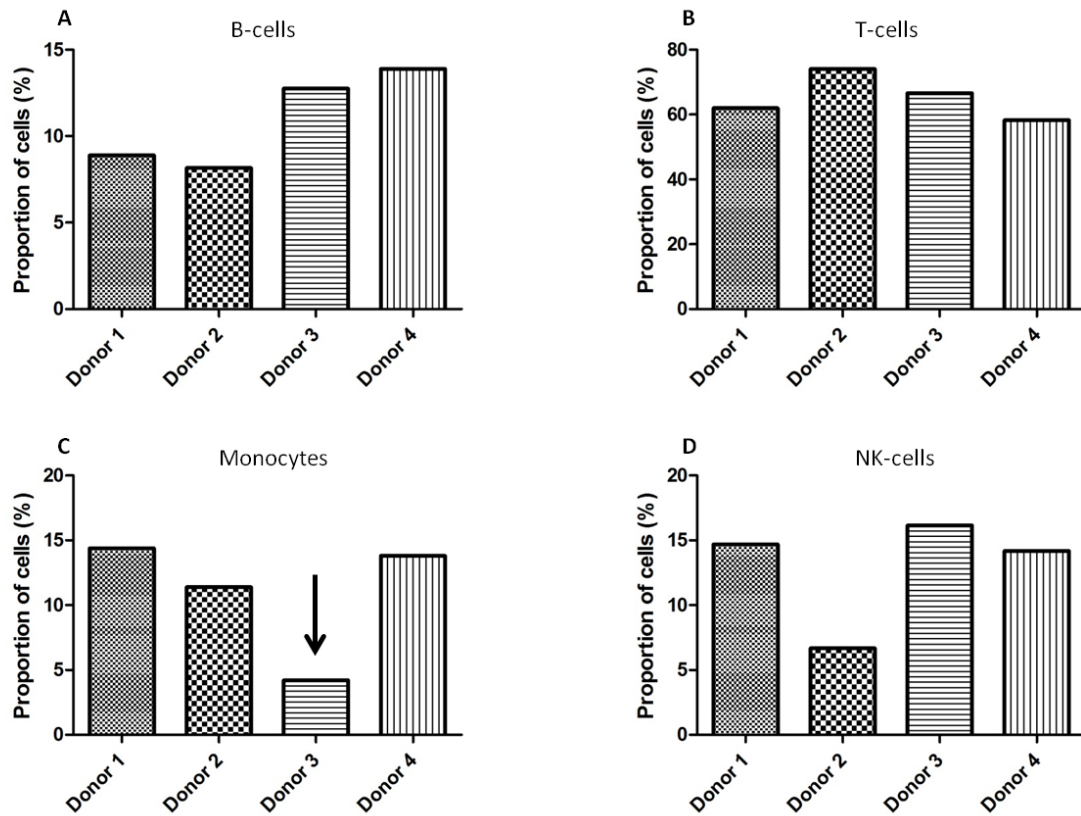
Figure 4 . 25: Composition of donor PBMCs

PBMCs were isolated from the heparinised blood of four donors and the cellular composition was determined using flow cytometry. The four main cells that make up PBMCs were calculated as a proportion of the whole number of cells A) B-cells, B) T-cells, C) Monocytes and D) NK-cells. The arrow indicates the donor with a low proportion of monocytes.

4.3 Discussion

Inflammation is one of the major hallmarks associated with AD and has been proposed to be involved in disease progression [116, 390]. It has been suggested that the action of resident immune cells of the brain, microglia, is responsible for this inflammation through their activation by the peptide Aβ. It is Aβ that is the main constituent of the insoluble plaques observed in the disease and many studies have reported that the association of microglia in the brain with the Aβ plaques causes inflammation and therefore contributes to the progression of the disease. In addition to the activation of CNS immune cells, it has also been suggested that Aβ is able to cause inflammation in the periphery through the activation of monocytes and macrophages, although it is unclear how. The induction of peripheral inflammation is also thought to contribute to AD [116, 118, 390-392]. Previous studies have proposed the role of the TLR2 and TLR4 receptors in Aβ induced inflammation in AD, however there has been no research into the role that PTM variants of the peptide play in the activation of these receptors. As the plaques in AD are composed partly of PTMs, some studies have suggested approximately 50% of the Aβ found in the plaques contains the pyroglutamate modification at amino acid 3 (AβpE3 3-42), the aim of this study was to investigate any differences in TLR2 and TLR4 response to the unmodified Aβ peptide and three PTM variants.

Initially, these experiments utilised a model that is extensively used to investigate the activation of TLRs. HEK293 cells stably expressing TLR2 were initially used and neither the unmodified Aβ 1-42 or the PTMs variants were able to induce a response in these cells, using protocol 1. There are a small number of studies that have used HEK293 cells expressing TLR2 or TLR4 to investigate their activation by Aβ: Liu et

al showed the release of IL-8 from TLR2 expressing HEK293 cells after stimulation with Aβ 1-42, Walter et al used HEK293 cells to highlight the importance of MD2 and CD14 in TLR4 activation by Aβ 1-42 and Stewart et al showed there was a large increase in the activation of TLR4 expressing HEK293 cells when it was in a complex with CD36 and TLR6. Due to the toxic nature of the Aβ peptides, in particular the oligomeric species, an assay looking at whether the peptide affected cell viability was performed [101, 393, 394]. However, there was no evidence of reduced cell viability after stimulation with the peptide suggesting that the peptide was not toxic to the cells. In addition, peptide concentration was increased to determine whether a higher concentration would induce a response, however, this had no effect on the activation of TLR2.

As TLR4 has also been implicated in AD, HEK293 cells expressing the TLR4-MD2-CD14 complex were also tested with the peptides. The peptide concentrations were chosen as all the published work read using HEK293 cells showed that 10μM would activate the receptors expressed on the cells but using this recommended concentration did not result in any activation of either cell type. The cell viability assays provided evidence that there was no cell death and the positive controls for TLR2 and TLR4 both caused a response, suggesting that the cells were functioning normally. In addition, there were high levels of expression of TLR2 or TLR4 on the cells, all of which point towards an inability of the peptide, prepared with protocol 1, to activate either TLR2 or TLR4. TEM analysis of the peptides prepared using protocol 1 showed that mature fibrils of the peptides had not formed during the aggregation period, despite published data suggesting this is sufficient time to produce fibrils [3, 364, 395]. All experiments carried out on HEK293 cells expressing TLR2 or TLR4 used peptide with a fibrillar morphology [275, 276, 281].

To investigate whether the preparation of the peptide could affect whether a response was observed from either cell type, a second preparation was tested. There are a number of different ways to prepare Aβ peptide and there is some debate over the best protocol to use [100, 274, 316, 396], as protocol 1 had not been used as the preparation protocol in any of the HEK293 experiments published a second protocol that had been, was tested [275].

After peptide stimulation of both TLR2 and TLR4 expressing cells no response was seen, however after assessing the viability of the cells it was observed that on all occasions the peptide was causing some decrease in cell viability in one or both of the HEK293 cell types. It is therefore unlikely that a response to the peptide would have been detected.

There are a variety of studies that have proposed that Aβ peptide has different effects when in different morphologies, for example monomers are believed to be non-toxic and potentially have beneficial effects [51, 71, 397, 398] whereas oligomers are thought to be the most toxic form of the peptide. Moreover, in order for the activation of TLRs to occur it has been hypothesised that the peptide requires a fibrillar morphology [275]. Both protocol 1 and 2 were designed to produce oligomers and fibrils of Aβ [85, 315, 316], however, upon examination of peptide prepared using protocol 1 it was observed that only oligomers and protofibrils had formed, suggesting the peptide might not have the necessary morphology to produce a response from TLR2 or TLR4. The peptide prepared using protocol 2, incubated at 37°C for 24h, was able to form fibrils, however it also caused a large reduction in cellular activity which could explain why no IL-8 was detected.

To look in more detail at whether a TLR response could be observed if the peptide had a more aggregated morphology a timecourse experiment was set up. This preparation had been shown to produce both fibrils of Aβ and TLR responses in similar experiments, as well as not causing cell death [274]. As suggested in the literature, a response was only observed after stimulation with peptide that had a fibrillar morphology. The peptide had been incubated in either water or in PB, at all of the timepoints after incubation in PB the peptide had an oligomeric morphology and did not produce a response from either cell type, in addition the early timepoints of water incubated peptide, which all had a non-fibrillar morphology also failed to produce a response. However, the peptides incubated for 168h or more, which had a mature fibrillar morphology were able to produce IL-8 from the TLR2 expressing cells although no response was observed from the TLR4 expressing cells, suggesting that fibrillar Aβ can activate TLR2 but not TLR4. As the viability of the cells was not affected by stimulation with the early aggregates of Aβ and the cells responded to their endogenous ligands we concluded that the unaggregated peptides were unable to activate either the TLR2 or the TLR4 expressing cells.

There are several studies that have looked at the activation of TLR2 or TLR4 by Aβ peptides, the majority of TLR4 studies have highlighted the necessity of TLR4 to be part of a complex in order to be activated by Aβ, in fact there is no response observed when TLR4 alone was stimulated [276, 277, 281]. In contrast, there are fewer studies suggesting that TLR2 requires complexes other than heterodimerisation with TLR1 and TLR6 [275, 279, 283]. One paper, Walter et al, suggested that TLR4 in a complex with MD2 and CD14 is able to release IL-8 in response to Aβ [276]. However, when experiments using the same conditions

described were tried in this study there was no production of IL-8 in response to the peptide in any of its morphologies.

According to a variety of other studies different complexes including TLR4 have been proposed to be needed in order to see the release of inflammatory cytokines. These papers have suggested different complexes including TLR6, CD36, TLR2, $\alpha_6\beta_1$ integrins and CD47 [399], .For example, Stewart et al found that a complex of TLR4-TLR6 and CD36 greatly increased the response of TLR4 to Aβ, the same experiments were performed using a TLR4-TLR6 and CD14 complex however this failed to have to same effect, the authors suggested that CD14 is not as important as other co-receptors for TLR4 response to Aβ [277]. Further investigations by Reed-Geaghan et al, proposed that TLR2-TLR4 and CD14 were required for the activation of the NF-κB pathway in response to Aβ [281].

Considering that the majority of TLR4 studies have been performed on THP-1 monocytes and murine microglia [96, 274, 282, 400-403] that would also express the co-receptors mentioned above and that experiments using HEK293 cells have looked at different receptor complexes, it would appear that TLR4 alone is not activated by Aβ. It also appears that there are some discrepancies about which co-receptors may be involved as some show that CD14 is needed while others suggest that a complex with the CD36 receptor is required for the release of inflammatory cytokines and CD14 is not. It is therefore possible that the reason that no activation was observed in HEK293 cells expressing TLR4-MD2-CD14 was that it was not the complex that is needed for TLR4 activation.

One further explanation for responses being observed in published data but not in these experiments could be due to the peptides themselves. It has been shown in this

study that different batches of peptide can produce different responses, even when the same experimental techniques are performed. Although there are only a few studies that have looked at differences in the effect the peptide has on responses, such as immune responses, there are some that have looked at differences in the aggregation properties of the peptides [82, 404-406] highlighting that batch-to-batch differences are fairly well known. It is therefore possible that the batch of peptide used for these experiments was not able to induce a response from HEK293-TLR4-MD2-CD14 cells.

As highlighted in this study the use of recombinant peptides can affect the responses observed. The production of Aβ peptide in *E.coli* can potentially lead to endotoxin contamination [407-409], as this is likely to be an LPS contamination it is possible that responses observed to these recombinant peptides are due to LPS activation of TLR4, not specifically the peptide. However, as not all studies use these peptides and the majority of them test for endotoxin contamination it is not likely that all the responses are due to contamination so the role of TLR4 cannot be ruled out [274, 277, 281, 400].

The need of Aβ to have a fibrillar morphology to elicit innate immune responses was also observed after stimulation of PBMCs, these were only able to produce IL-1β in response to fibrillar Aβ. When the Aβ 1-28 form of the peptide, which did not aggregate, was used no IL-1β was detected in the cell supernatants. Combined all the data agree with published results showing the importance of the peptide morphology in producing innate immune responses [274-276, 281].

In the brains of people with AD, in addition to the 'normal' Aβ peptide there are also a number of peptides containing PTMs [60, 72, 74, 198, 347, 410]. Due to the high

abundance of these peptides and their potential to alter immune responses a number of them were selected as they may play a potentially important role in inflammation in AD [66, 411, 412]. When each of the peptides was used in a fibrillar morphology, all of them were able to cause the release of IL-8 from TLR2 expressing HEK293 cells but not from TLR4 expressing cells. Furthermore all of the peptides also caused the release IL-1β but not TNFα from PBMCs. The type of response observed to each of the peptides was the same, all of them were able to activate TLR2 expressed on HEK293 cells and induce the release of IL-1β from PBMCs in addition to not inducing activation of isolated monocytes. Furthermore there were no significant differences in the size of the responses observed from any cell type.

To date there are no studies investigating the immune responses to these peptides however, there are a number of studies that have looked at the similarities and differences between unmodified Aβ 1-42 and variants with PTMs, in particular several studies have focused on the AβpE33-42 peptide used in this study. There is a variety of techniques that can be used to study the aggregation and structure of Aβ peptides, for example X-ray diffraction can be used to determine the structure of the fibrils showing whether they have the characteristic β-sheet structure associated with Aβ, in addition TEM can be used to determine the morphology of the peptide. Studies carried out to investigate the aggregation and structure of the peptide, have shown that it has an increased propensity to aggregate as well as being more stable. This has been proposed to be due to an increase in hydrophobicity and an increase in resistance to peptidases, resulting from a loss of charge during the modification process of the peptide. It has also been documented that the peptide has an increased tendency to form β-sheets. Despite the differences documented in the propensity of the AβpE3 3-42 to aggregate the investigations suggest the structure of the peptide is

very similar if not the same as the unmodified Aβ 1-42. The TEM images obtained in this study of all four of the peptides suggested that the mature fibrils formed, shared several similarities. In addition, unpublished X-ray diffraction analysis of the same four peptides (from the same batch) showed that all the peptides used had the same β-sheet structure.

In addition to Aβ other amyloid peptides, including superoxide dismutase 1(SOD1) and islet amyloid polypeptide (IAPP), which also share the same structure as Aβ are able to induce the release of inflammatory cytokines [413-415]. It has been suggested that the β-sheet structure of the aggregates of these peptides are essential for innate immune activation and therefore the release of inflammatory markers [416]. The observation that the fibrils of all four of the peptides have a β-sheet structure could explain why the aggregated form of the peptides were able to induce a response and the fact that all of the peptides have the same structure may be why there were no significant differences in the responses seen after stimulation with the peptides.

Despite monocytes not responding to the fibrillar preparations of Aβ 1-42 they did respond to a recombinant version of the peptide (rPEP). This peptide was used to investigate whether an Aβ 1-42 peptide from a different source would activate monocytes. The recombinant peptide caused a large increase in the amount of both IL-1β and TNFα produced in comparison to the negative controls and was in fact much higher than the positive controls included in the experiment. However, as the peptide had been produced in *E.coli* it was necessary to ensure that the response being measured was a result of the peptide not a contamination. Contamination from bacteria has been shown to be a problem in recombinant proteins and peptides

especially for cells such as monocytes and macrophages that have high expression of TLR4 [408, 409, 417, 418]. PMB was added to the experiments to reduce any signal that was a result of the presence of LPS. PMB works by binding to the lipid A portion of LPS preventing it from binding to the TLR [419]. However, if the concentration of LPS is too excessive it can saturate the PMB and therefore an LPS response would still be seen. As the lower concentration of recombinant Aβ 1-42 from rPEP would likely have a smaller amount of endotoxin contamination, PMB saturation would explain why there was a reduction in the amount of TNFα produced only when the lower concentrations of the peptide were used. A similar effect was seen in regards to the amount of IL-1β produced, in the majority of cases there was a reduction in IL-1β measured after the addition of PMB when the lower concentrations of peptide were used. However, in one of the experiments there was an increase in IL-1β produced after the addition of PMB, in previous studies it has been shown that under some conditions that PMB is able to induce the release of IL-1β [420, 421]. Both the results from TNFα and IL-1β suggested that there was a contamination with LPS in the recombinant Aβ 1-42 peptide from rPEP.

Despite observing a response to Aβ peptides in HEK293 TLR2 cells and PBMCs there was no response seen from purified monocytes. This would suggest that Aβ is not able to directly activate monocytes, however intracellular staining and flow cytometry showed that in PMBCs it was the monocytes that were producing the IL-1β, suggesting that monocytes can be activate by Aβ but it may require the presence of other cells types. In PBMCs there are three main cell populations in addition to monocytes: T-cells, B-cells and NK cells, it is therefore possible that one of these may influence the activation of the monocytes.

There are documented differences in the response of monocytes stimulated in isolated cultures compared with monocytes in PBMC cultures. Pinke et al 2013, showed that after stimulation with LPS the release of IL-1β was significantly higher from PBMC monocytes than from isolated monocytes. This effect was particularly prominent in elderly patients [422]. The authors propose that the reason for the increase in IL-1β release from PBMC monocytes is an interaction between monocytes and T-cells that results in the T-cells having an immunomodulatory effect on monocytes therefore increasing their production of IL-1β. The same effect was also seen for several other cytokines, including TNFα and IL-6 but the effect was more far more pronounced for IL-1β.

The majority of research into monocyte activation by Aβ peptide has been performed on the human monocytic cell line THP-1. These studies show that significant levels of cytokines, such as TNFα or IL-1β are released in response to Aβ [274, 423-425]. However, there are far fewer studies looking into the activation of primary monocytes by Aβ peptide and none looking at the activation by PTM variants of Aβ. Fiala et al, showed that only a small amount of IL-1β, 25pg/ml, could be detected after stimulation with Aβ. If this amount of IL-1β was released in response to the peptides in this study it would not have been detected [278]. In this study as IL-1β was detected from monocytes after PBMC stimulation but not isolated monocytes it may be because the amount of IL-1β released was too low to be detected in isolated monocytes but the signal was increased due to the effect of other cells, such as T-cells, in PBMCs. Furthermore, it has been shown that the presence of T-cells in glial cultures enhanced the release of pro-inflammatory cytokine after stimulation with Aβ peptide [426] further suggesting that T-cells may influence the inflammatory responses seen after Aβ stimulation.

There are a number of ways T-cells are able to influence the activation of monocytes, for example through the release of cytokines or by direct cell-to-cell contact between T-cells and monocytes. Studies have observed that direct T-cell – monocyte contact can induce the release of IL-1 from monocytes in the absence of any lymphokine release suggesting that contact alone may be enough to affect the activation of monocytes [262, 427]. It has been shown that leptin-stimulated T-cells and CD3-stimulated T-cells act as a stimulus for monocytes to release IL-1β [262, 428, 429]. Furthermore, the type of T-cell stimulus may affect the pattern of products produced by monocytes. Several surface receptors have been proposed to be involved in contact activation, including: CD40/CD40L, leukocyte function antigen (LFA)/intercellular adhesion molecule (ICAM)-1 and CD2/LFA3. However, although all of them have been shown to play some role in contact activation antibodies blocking these interactions do not abolish monocyte activation suggesting there are still mechanisms that need to be identified [262].

In addition, or instead of, contact activation it is possible that the activation of T-cells could lead to the release of IL-1β from monocytes. It has been proposed that interferon-gamma (IFN-γ) produced by β-specific Th1 cells promotes microglial activation (Browne 2013). In addition it has been reported that Aβ is able to induce the production of IFN-γ-inducible protein-10 (IP-10) [430-432]. IP-10 is able to induce the production of IFN-γ from T-cells and has been shown to increase monocyte responses to stimulants such as LPS [433]. Therefore one possible mechanism for the involvement of T-cells in monocyte activation could be that IP-10 released, along with other cytokines, by monocytes after Aβ stimulation results in the release of IFN-γ from T-cells, which then increases the release of cytokines such as IL-1β from monocytes.

The use of TLR2 neutralising antibody had inconclusive results, in some experiments there was the reduction of the proportion of cells expressing intracellular IL-1β, but in others there was not. For the Aβ 1-42 and AβpE3 3-42 peptide half were affected by the antibody and half were not, whereas for the Aβcit5 1-42 3 out of the 4 samples had a reduced proportion of cells expressing IL-1β after the addition of α-TLR2. This implies that TLR2 is involved in the activation of monocytes by Aβ peptide but also suggests that it is not the only mechanism involved [434]. To further support the role of TLR2 in Aβ recognition, it was observed that the αTLR2 antibody had more of an effect on one of the participant samples. This sample had a smaller proportion of monocytes than the other samples tested, this would imply that the overall expression of TLR2 in the sample would be smaller, therefore it is possible that the TLR2 neutralising antibody would block a greater proportion of TLR2 and could therefore have a greater effect. As only TLR2 was blocked in the experiments it is still possible that other TLRs may be involved [276, 277, 435-437] as well the activation of different receptors [268, 284, 438], furthermore as the effect T-cells have on monocytes is unlikely to be mediated through TLRs, neutralising TLR2 may not have a significant effect. Even if TLR2 is involved, if T-cells are increasing the responses observed the neutralising antibody may not be able to effectively reduce the signal, This could explain why αTLR2 was not always successful at significantly reducing the proportion of cells expressing IL-1β.

Despite the ambiguous results regarding monocyte activation, it still appears that Aβ peptide and its PTM variants were able to activate monocytes but that there are a number of mechanisms involved in the immune response to the peptide.

There were some differences in PBMC responses from patients and controls, all of the cells isolated from the age-matched controls responded to the peptides to produce IL-1β but in contrast the peptides only produced an IL-1β response in half of the patients. This suggests that people without AD respond more efficiently to the Aβ peptide than patients with AD.

In AD, as with many other age-related diseases, it has been reported that there is chronic inflammation, often referred to as inflammaging [438-441]. This chronic inflammation is thought to influence the ability of cells such as monocytes to respond to pathogens. It has been observed that in the elderly the immune system is less able to respond to infections and proposed that the increase in circulating cytokines may be responsible for this [442-444]. It has been hypothesized that in the brains of people with AD, inflammation induces an alternative activation state, also known as a deactivated phenotype, in microglia, which is potentially due to the presence of Aβ. This is thought to affect the inflammatory response and ability of the cells to phagocytose the peptide [123, 435, 445]. A decrease in the sensitivity of the cells to respond to stimulants may also affect other properties of the cells, such as its ability to produce cytokines, therefore altering the response to Aβ. As chronic inflammation is also observed in the periphery of patients with AD, immune cells such as monocytes may undergo a similar shift to an altered activation state and would therefore also be less reactive to the Aβ peptide. In the elderly, where chronic inflammation occurs, peripheral monocytes have shown a reduced response to LPS [446, 447]. The ELISA experiments looking at PBMC responses to Aβ peptides suggested that there was a higher level of background inflammation in patients compared with controls, which could be an indication of chronic inflammation. As patients with AD have a higher level of chronic inflammation, it is also possible that

they have an increased number of alternatively activated monocytes, this could explain why some of the patients appeared to have a reduced response to the Aβ peptides.

The results obtained in this chapter, while not providing conclusive evidence for the role of TLRs in AD, have suggested that TLR2 is involved in the recognition of Aβ and its PTM variants but there is also the involvement of other mechanisms that also contribute to the inflammatory responses seen. In addition, the response to the four peptides tested appears to be the same, supporting the idea that Aβ with PTMs also contribute to the inflammatory responses in AD and the similarities in response is likely to be due to the similarities in peptide structure.

5. The role of Aβ peptide in the activation of T-cells

5.1 Introduction

It is now widely accepted that the immune system is involved in AD and that there is a potential role for the peptide Aβ, the main constituent of the insoluble extracellular plaques seen in the disease [448-451]. Although it is thought that the immune cells of the CNS, microglia and astrocytes, are responsible for the widespread inflammation observed in the disease [116, 124, 343, 452-455] there is evidence to suggest that peripheral immune cells such as T-cells, which are not normally found in the brain, are also involved [303, 304, 434, 456].

Typically under physiological conditions the brain is considered an immunologically privileged area suggesting an absence of 'traditional' immune cells, such as T-cells, monocytes and macrophages, although the CNS has its own resident macrophages – microglia [268]. However, in a number of neurodegenerative disease, including AD and Parkinson's disease (PD), there is evidence of disruption to the blood brain barrier (BBB) allowing the entrance of peripheral immune cells into the brain [457, 458]. Furthermore, it is believed that activated T-cells are able to cross the blood brain barrier even when there is no damage to it [515]. Within the brain studies have observed the presence of T-cells in the brain parenchyma, however it has been proposed that these numbers may be underestimated as T-cells may enter the Virchow-Robins space and be more difficult to detect [285, 287, 459]. It is still largely unknown whether this is beneficial or detrimental to the brain in AD. In addition to T-cells entering the brain it has also been proposed that activation of circulating T-cells causing the release of inflammatory mediators in the periphery may affect the activation of microglia in the brain as well as monocytes in the

periphery [304]. There is strong evidence for the role of peripheral inflammation in AD [379, 390, 460].

There is some inconsistency in Aβ related T-cell research and a number of questions remain regarding the activation of T-cells by Aβ peptides. For example, it has been questioned whether Aβ is able to trigger a specific proliferative response [304], although some studies have provided evidence of Aβ-specific T-cells [289, 303, 434, 456]. Furthermore, there is debate over whether the response of T-cells to Aβ is enhanced or diminished in elderly people and people with AD [303, 461]. It has also been suggested that Aβ causes modulatory effects in the immune system, for example by enhancing proliferative responses to other antigens [304].

While some studies have looked at proliferation and cytokine production in response to Aβ as an indicator of T-cell activation others have looked at different mechanism in the activation of these cells. One example of this is the phosphorylation of specific protein kinase C (PKC) isotypes in response to Aβ [308, 309, 462]. PKCs are thought to be mediators in immune cell activation, for example PKC-δ is potentially involved in T-cell migration and PKC-ζ in the T-cell dependent immune response [309, 463-465]. It was shown that Aβ could induce the phosphorylation of these two isoforms of PKC in T-cells, providing more evidence that Aβ peptide can activate T-cells [308, 309, 462].

This study has looked into the ability of Aβ to cause specific T-cell proliferation and whether the phosphorylation of PKC-δ and PKC-ζ could be seen after stimulation with the peptide. Flow cytometry was used to determine whether Aβ induces these types of responses and was additionally used to measure intracellular cytokine production.

5.2 Results

5.2.1 T-cell proliferation

The activation of T-cells was measured in a number of ways, one of which was by proliferation in response to an antigen. In these experiments a PKH-lipid dye was used to measure the proliferative response of T-cells to Aβ peptide. The principle of this test is to stably incorporate a fluorescent dye into the lipid regions of the cell membrane so that the dye becomes more dilute as the cells proliferate.

In all experiments, flow cytometry was used to measure the proportion of proliferated cells. These were identified by reduced PKH-FITC staining.

The gating strategy chosen for these experiments was different to 'typical' lymphocyte gating because it was difficult to tell where the proliferated cells lay in the FSC-A versus SSC-A plot. Proliferated cells change size and granularity and there is variation with respect to these changes, so a 'typical' lymphocyte gate could miss proliferated cells. In order to overcome this problem, the cells gated first were $CD3^+$ cells so the whole population of T-cells was identified. The second gate selected only the single cells and the final gate measured the $CD3^+PKH^-$ population (Figure 5.1).
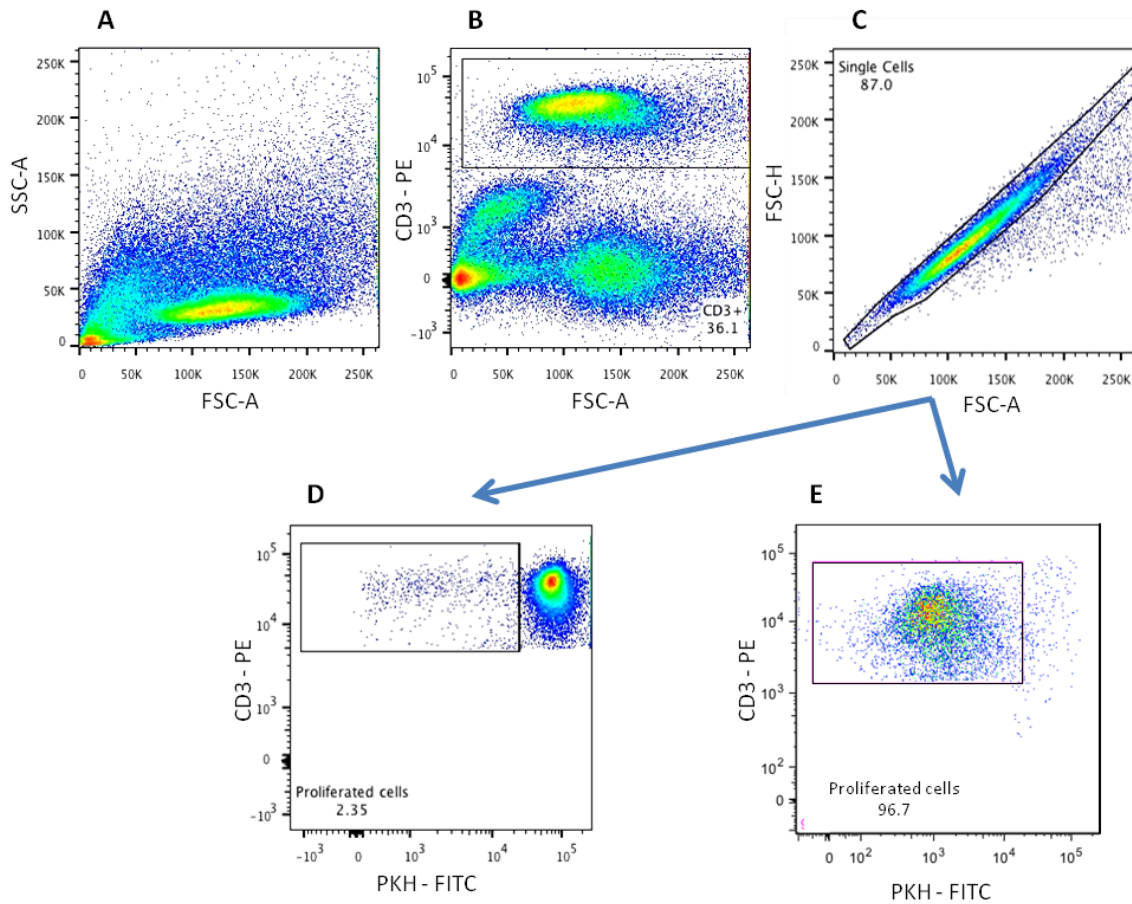
Figure 5 . 1: Gating strategy for CD3$^+$ T-cell proliferated

A) Ungated PBMCs showing forward scatter vs side scatter B) T-cells gated as CD3$^+$ cells C) Single cells D) proliferation of unstimulated CD3 expressing T-cells E) proliferation of PHA stimulated CD3 expressing T-cells.

The initial experiment used the unmodified peptide, Aβ 1-42, as well as the three PTM variants, Aβcit5 1-42, AβpE3 3-42 and AβpE3cit5 3-42. The peptides were used immediately after preparation to ensure the most unaggregated morphology possible and the PBMCs were stimulated for 5 days. An anti-CD3 antibody was used to identify T-cells and after the incubation period there was no evidence of differences between the proliferation of unstimulated cells and cells stimulated with any of the peptides. The time the peptide was left to aggregate also did not change the response observed, there were no differences between the proliferation of unstimulated and peptide stimulated cells. The stimulation index (SI) for each condition was calculated as the antigen response divided by the unstimulated response and a positive response was a value greater than 2.5. Published studies use SI values 2.5 and 3 as the threshold for a positive response, as studies looking at responses to the same peptide used an SI of 2.5 this value was chosen for these experiments [303, 466-468].None of the different conditions in this experiment produced an SI of this value or greater (Fig 5.2).
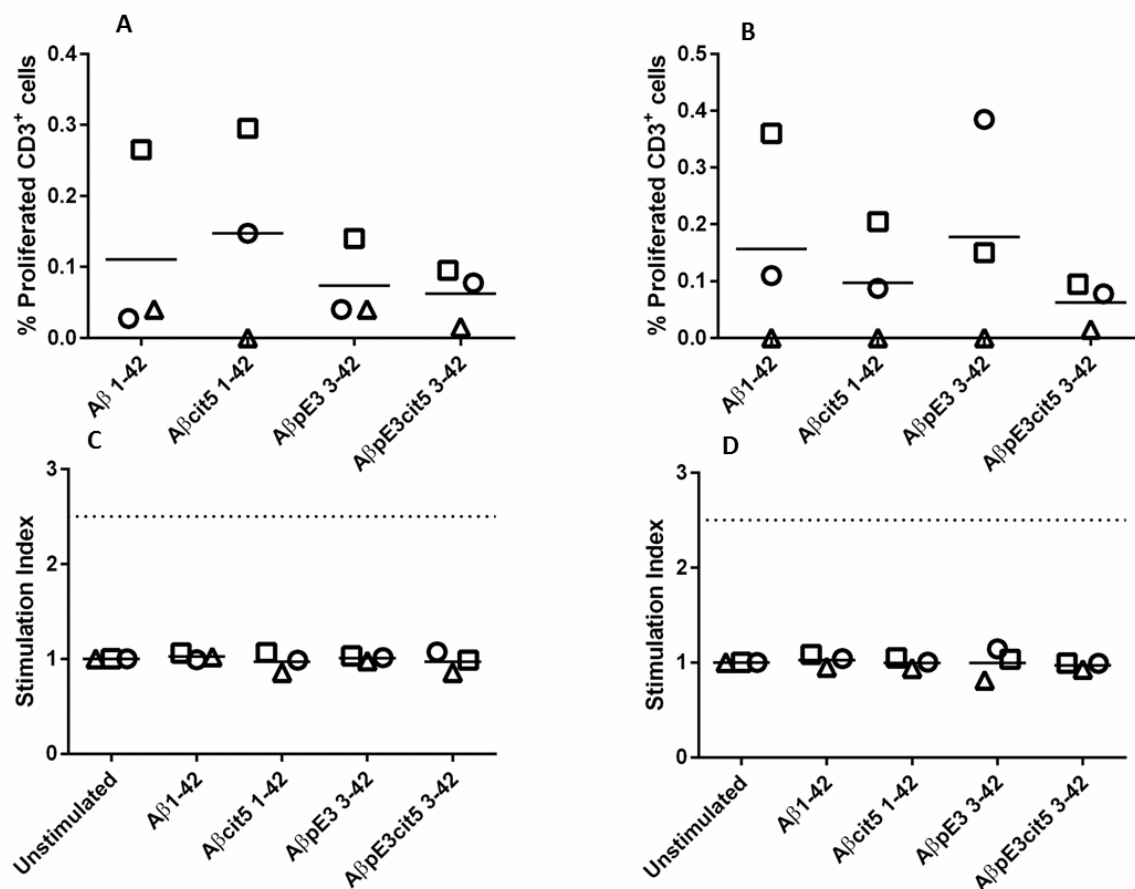
Figure 5 . 2: No T-cell proliferation was observed in response to Aβ or its PTM variants

PBMCs were stained with PKH-lipid dye and stimulated with peptides Aβ 1-42 and its PTM variants. Peptides were disaggregated and dissolved in DMSO, the DMSO was removed and peptide was dissolved in 50mM PB. The peptides were used immediately or left to aggregate for 48h and stimulated the cells which were incubated for five days. Cells were stained with anti-CD3 antibody and acquired using an LSR II flow cytometer. Data displayed as the percentage of proliferated cells minus the proliferation of unstimulated cells: 0h aggregated peptide (A), 48h aggregated peptide (B). Stimulation index was calculated as the percentage of proliferated cells divided by the percentage of unstimulated proliferated cells. 0h peptide aggregation (C), 48h aggregated peptide (D). Dotted line shows positive proliferative response is SI>2.5. Each symbol represents one patient with AD and shows the mean of duplicates. The line shows the overall mean from all the patients. n=3. All samples were obtained and stimulated at the same time.

In order to determine which positive control would provide the best results three stimulants known to activate T-cells were used: SEB, PPD, and PHA. These were all tested on control PBMCs which were initially stimulated for four days to induce the production of antigen-specific T-cells and then restimulated. All three of these stimulants were able to produce a proliferative response from T-cells, however there were some differences. For all T-cell subsets; all $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$, PHA caused over 60% of T-cells to proliferate. For $CD8^+$ cells this was over 80% and for all $CD3^+$ cells was over 68%. In contrast PPD induced the proliferation of T-cells to a lesser extent and was more inconsistent in its activation, for example, in $CD4^+$ T-cells the range of proliferation was from 2.5% to 46.3%. It also had a much smaller effect on $CD8^+$ T-cell proliferation. Furthermore SEB was also able to induce proliferation from all three T-cell subsets, more 60% of cells proliferated in each case.
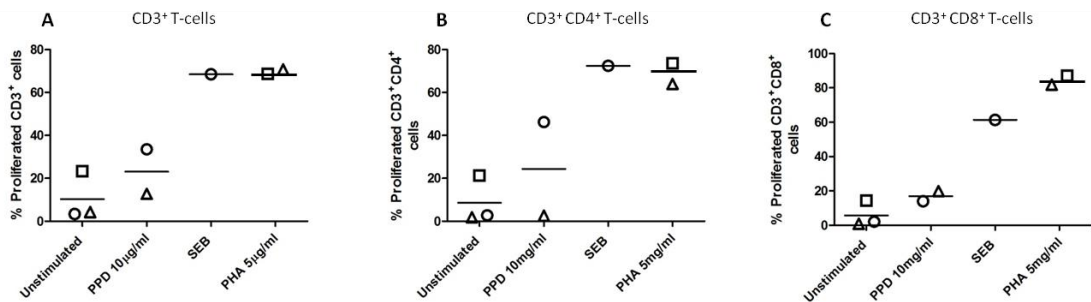
Figure 5 . 3: Test of positive controls on T-cell proliferation

PBMCs were stimulated with 10μg/ml PPD, 5μg/ml PHA or 2μg/ml SEB for 4 days and were then restimulated with the same stimulant for a further 24h. The cells were stained with antibodies for CD3, CD4 and CD8 and the PKH dye. Proliferated cells were calculated as the proportion of cells negative for PKH in each cell subset. A) $CD3^+$ T-cells, B) $CD3^+CD4^+$ and C) $CD3^+CD8^+$. Each symbol represents the mean of duplicates from one participant. The line represents the overall mean. n=3

The proliferation assay had been previously optimised in the lab and the concentration of the Aβ 1-42 peptide used was based on a published study looking at similar responses [303]. The unmodified form of the peptide was used in order to determine the best conditions where T-cell proliferative responses to Aβ occur. Some of the experimental conditions were then altered; firstly the lyophilised peptide was dissolved directly in DMSO as this protocol had been used in similar experiments looking at T-cell responses [303, 308, 309]. As the peptide was initially dissolved to a high concentration, only a small amount of DMSO was added to the cells, at this concentration the DMSO was unlikely induce proliferation of the cells. DMSO treated cells were incubated alongside unstimulated cells and there was no evidence of proliferation in response to DMSO (Fig 5.4). PBMCs were initially stimulated and incubated for 72h, they were then restimulated and incubated for a further 72h. This was to ensure that T-cells had a sufficient amount of time for proliferation.
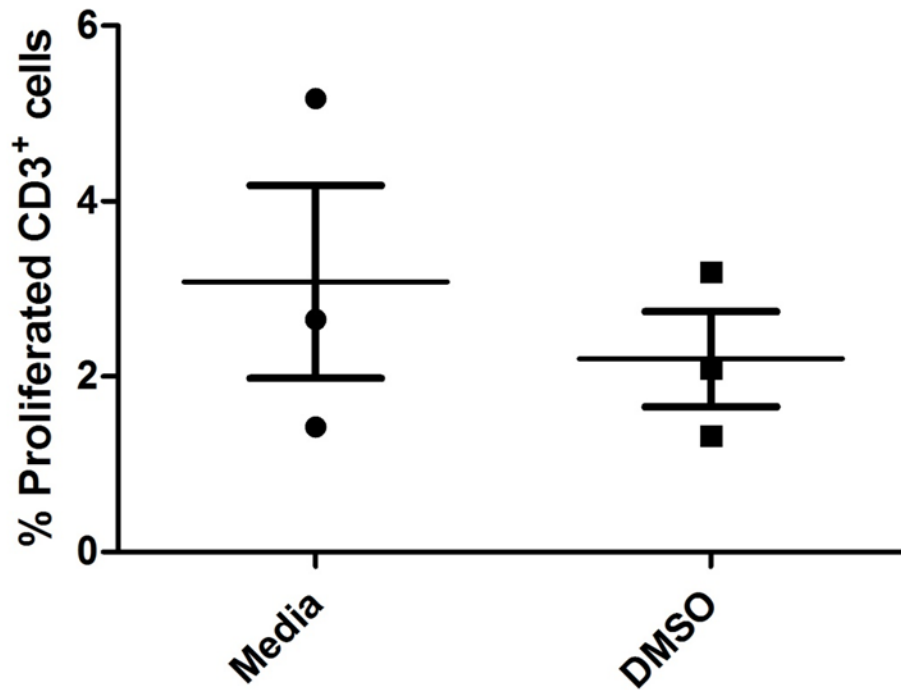
Figure 5 . 4: DMSO did not cause the proliferation of CD3^{+} T-cells

PBMCS were isolated from heparinised blood from healthy controls and were either left unstimulated or were treated with DMSO and allowed to proliferate for 5 days. Each point represents the mean proliferation from duplicates. The lines represent the overall mean ± SD. n=3.

In order to start investigating whether Aβ could induce T-cell proliferation, some pilot experiments were performed to look at whether a response could be observed after changing the experimental conditions. The peptides were required to be unaggregated therefore they were dissolved in DMSO immediately before use. In order to compare these results with the experiments already performed the cells were first gated as described previously to look at the proliferation of CD3$^+$ cells. For the first experiment samples from two healthy controls and one patient were treated with the peptide. PBMCs from all samples responded to the positive control, PHA, for both the patients and healthy controls approximately 70% of T-cells proliferated. However, only one duplicate from one of the healthy controls showed evidence of proliferation after restimulation with Aβ 1-42, this was shown by an SI of over 2.5 (5.5). All the other experimental conditions: Aβ 1-42 no restimulation and Aβ 1-42 restimulated after 72h from all of the other samples showed no evidence of proliferation. In all cases the SI was less than 2.5, and it does not appear that there were any differences between patient and healthy control responses. Furthermore, when the Aβ 1-42 peptide from JPT was used, the results were almost identical, however there were no SI values over 2.5 suggesting there was no proliferation (Fig 5.6).

Figure 5 . 5: Synthetic Aβ 1-42 peptide does not cause a proliferative response in CD3⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A,C) or age-matched controls (B,D) they were stimulated with freshly dissolved 15μg/ml Aβ 1-42, 5 μg/ml PHA or left unstimulated for 96h. Cells were restimulated with the indicated stimulant for 24h and then stained with antibodies for T-cell markers. Proportion of proliferated cells calculated as cells that proliferated in response to stimulant minus negative control. SI was calculated as response divided by negative response. SI>2.5 corresponds to positive proliferative response. A) Proportion of proliferated patient T-cells, B) proportion of proliferated age-matched control T-cells. C) SI calculated from patient T-cells, D) SI calculated from age-matched control T-cells. Each symbol represents one participant, the mean of the duplicates for each participant is plotted, and the line represents the overall mean. n=1 patient, n=2 controls.

Figure 5 . 6: Aβ 1-42 from JPT does not cause a proliferative response in CD3^{+} T-cells

PBMCs were isolated from heparinised blood from patients (A,C) or age-matched controls (B,D) they were stimulated with freshly dissolved 15μg/ml Aβ 1-42 peptide from JPT, 5 μg/ml PHA or left unstimulated for 96h. Cells were restimulated with the indicated stimulant for 24h and then stained with antibodies for T-cell markers. Proportion of proliferated cells calculated as cells that proliferated in response to stimulant minus negative control. SI calculated as response divided by negative response. SI>2.5 corresponds to positive proliferative response. A) Proportion of proliferated patient T-cells, B) proportion of proliferated age-matched control T-cells. C) SI calculated from patient T-cells, D) SI calculated from control T-cells. Each symbol represents one participant, the mean of the duplicates for each participant are plotted. The line represents that overall mean. n=1 patient, n=2 controls

The responses to Aβ may be small and therefore not detectable in the CD3$^+$ subset as a whole due to the background proliferation, so a CD4 antibody was included in the antibody panel to look at different subsets of T-cells and to examine whether a response could be observed. Again the peptide was used immediately after dissolving in DMSO. The initial gating strategy was the same as for CD3$^+$ cells however, after single cells had been gated the cells that were positive for both CD3 and CD4 were selected and then plotted against PKH to look at proliferation (Fig 5.7). Again, all the PBMCs responded to the positive controls regardless of whether they were from a patient or a control, however, there was no response to any of the other experimental conditions apart from one of the duplicates from one of the control samples. No proliferation was observed in response to the Aβ 1-42 peptide from JPT regardless of whether the cells were only stimulated once or were restimulated with the peptide (Fig 5.7). There were no differences observed between the CD3$^+$ (Figs 5.5, 5.6) and the CD4$^+$ (Fig 5.8, 5.9) T-cell subsets for either of the peptides used.
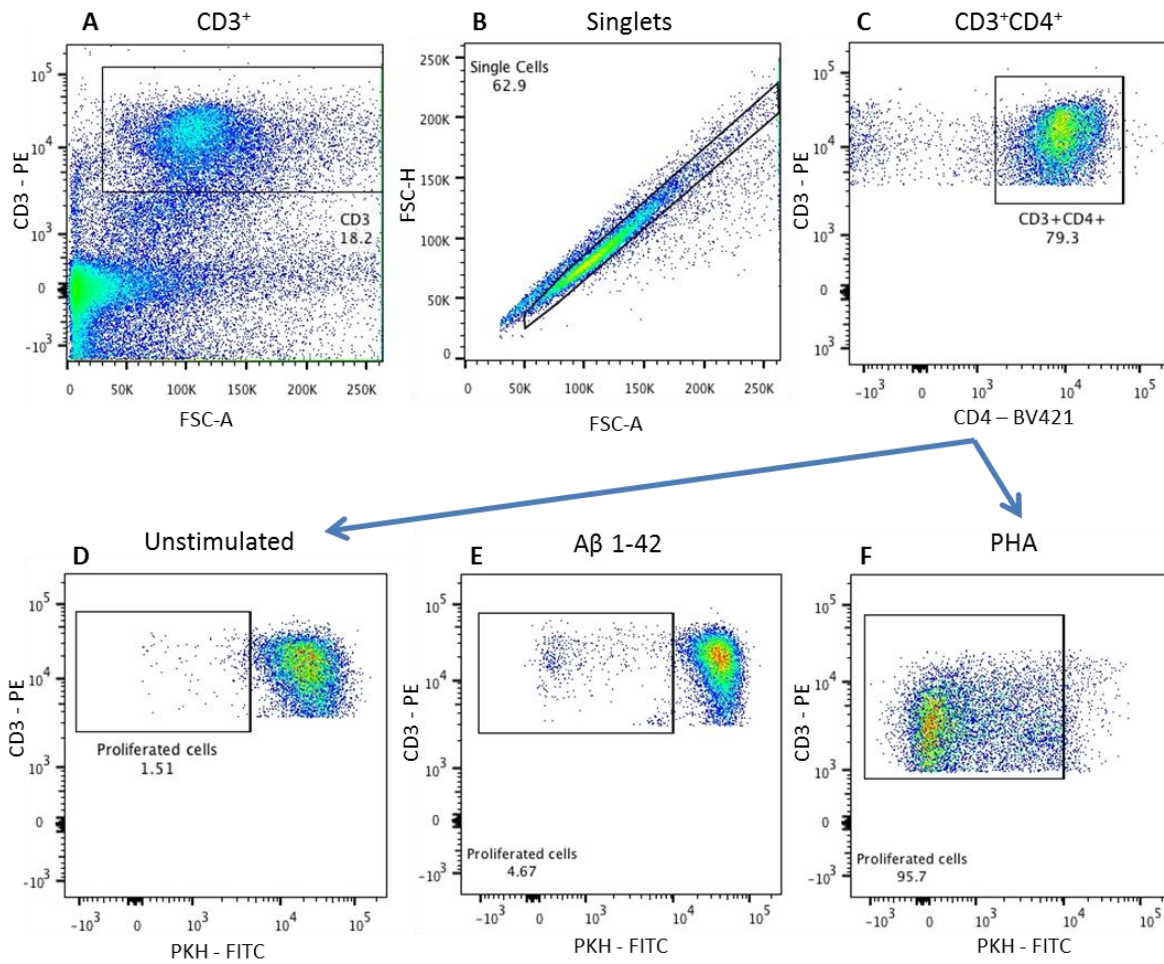
Figure 5 . 7: Gating strategy for CD4+ T-cells

PBMCs were isolated from heparinised blood and stained with antibodies for CD3 and CD4. A) CD3+ cells, B) Single cells, C) CD3+CD4+, D) Proliferated cells, unstimulated control, E) Proliferated cells , Aβ 1-42 stimulated cells and F) Proliferated cells, PHA stimulated.
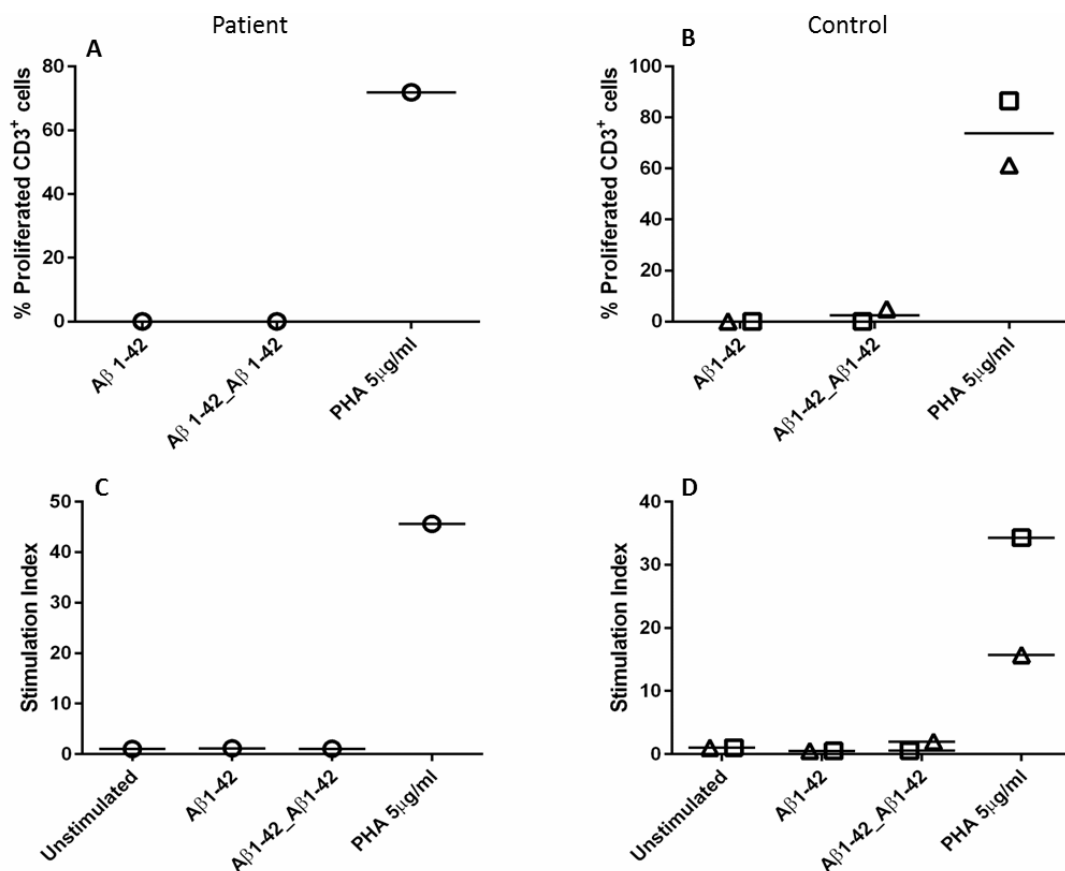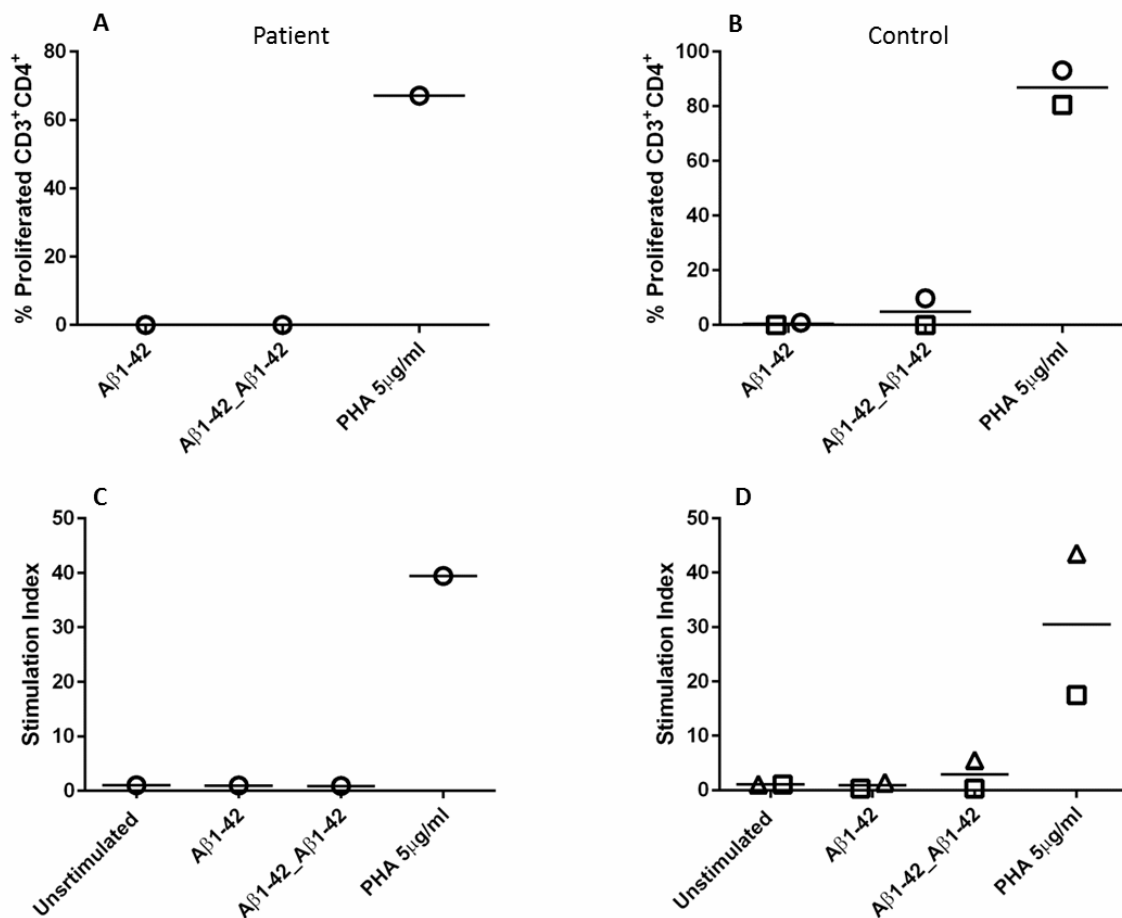
Figure 5 . 8: Aβ 1-42 peptide does not cause a proliferative response in CD3⁺CD4⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A,C) or age-matched controls (B,D) they were stimulated with freshly dissolved 15μg/ml Aβ 1-42, 5 μg/ml PHA or left unstimulated for 96h. Cells were restimulated with the indicated stimulant for 24h and then stained with antibodies for T-cell markers. Proportion of proliferated cells calculated as cells that proliferated in response to stimulant minus negative control. SI calculated as response divided by negative response. SI>2.5 corresponds to positive proliferative response. A) Proportion of proliferated patient T-cells, B) proportion of proliferated age-matched control T-cells. C) SI calculated from patient T-cells, D) SI calculated from control T-cells. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=1 patient, n=2 controls
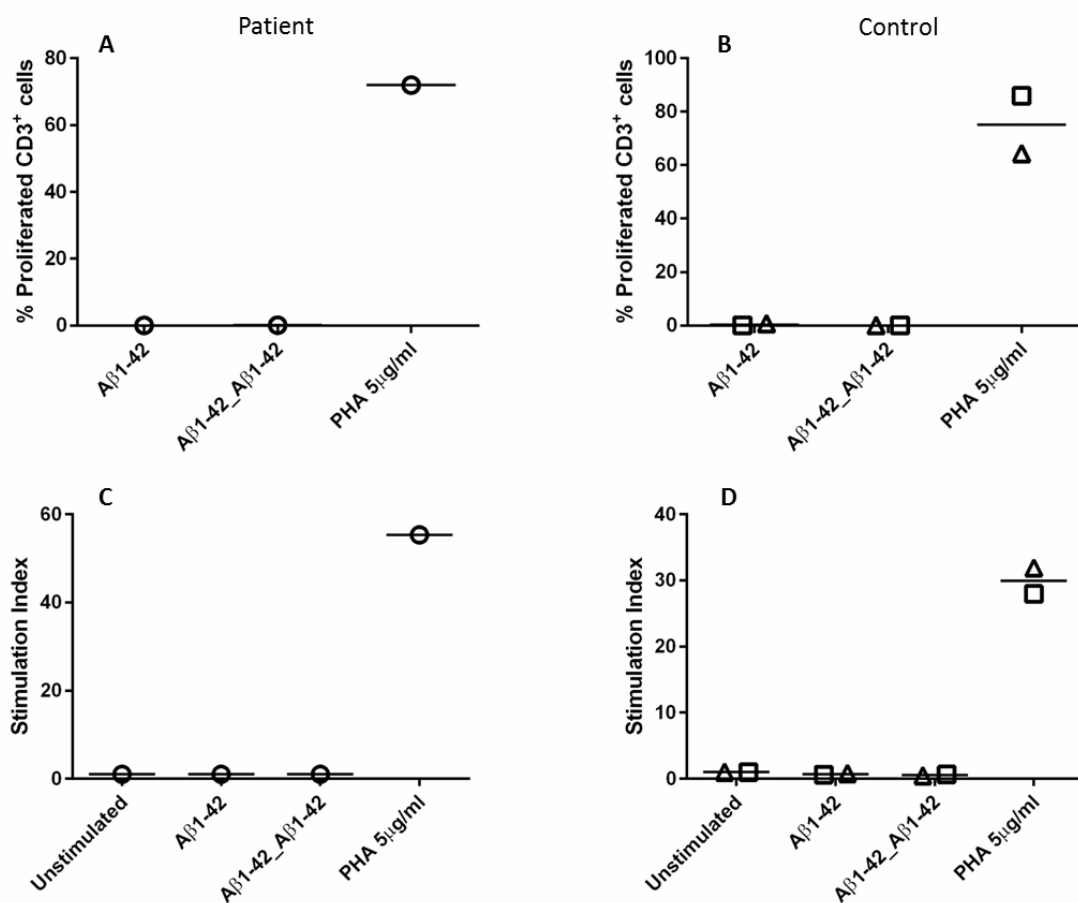
Figure 5 . 9: Aβ 1-42 peptide does not cause a proliferative response in CD3⁺CD4⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A,C) or age-matched controls (B,D) they were stimulated with freshly dissolved 15μg/ml Aβ1-42 from JPT, 5 μg/ml PHA or left unstimulated for 96h. Cells were restimulated with the indicated stimulant for 24h and then stained with antibodies for T-cell markers. Proportion of proliferated cells calculated as cells that proliferated in response to stimulant minus negative control. SI calculated as response divided by negative response. SI>2.5 corresponds to positive proliferative response. A) Proportion of proliferated patient T-cells, B) proportion of proliferated age-matched control T-cells. C) SI calculated from patient T-cells, D) SI calculated from control T-cells. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=1 patient, n=2 controls

As a response was still not observed after these experiments further alterations were made to the experimental conditions, the stimulation conditions were the same as used in the previous experiment in that cells were initially stimulated for 72h and then restimulated for a further 72h. However, since it has been reported that the addition of IFNα reduces background/non-specific proliferation and allows very small proliferative responses to be detected [467], each of the experimental conditions was performed with and without the addition of IFNα.

Further to the use of IFNα, the antibody panel was changed to include CD3, CD4 and CD8 for a more specific measurement of T-cell subsets. The first observation made from the results of these experiments was that there was significant reduction in proliferation of $CD3^+$ and $CD3^+CD4^+$ T-cells, using IFNα, after treatment with the negative control but there was no difference in $CD3^+CD8^+$ T-cells. However, as published data by Von Baehr [467], IFNα has a modest effect on stimulated cells. In these experiments only the $CD3^+CD4^+$ T-cells had a significant reduction in proliferation although there was also a small non-significant reduction in the $CD3^+$ and $CD3^+CD8^+$ T-cells. As IFNα reduced the non-specific, background proliferation it was added to all subsequent experiments (fig 5.10).

Table 5 . 1: Summary of proliferation assay using IFNα

First Stimulation	Restimulation	IFNα	Result
Aβ 1-42	Aβ 1-42	+	Small reduction in background proliferation – no overall proliferation
Aβ 1-42	Aβ 1-42	-	No proliferation
DMSO	DMSO	+	Reduced background proliferation- no overall proliferation
DMSO	DMSO	-	No proliferation
PHA	PHA	+	T-cells proliferated
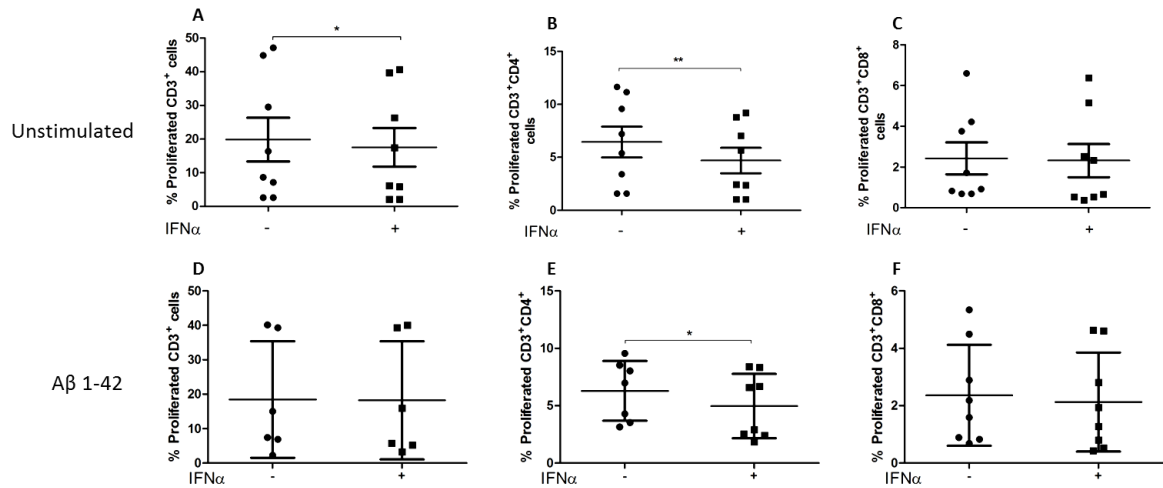
Figure 5 . 10: The effect of IFNα on background proliferation

PBMCs were isolated from heparinised blood and left unstimulated with and without the addition of 125IU/ml IFNα for 72h followed by the addition of fresh media and incubation for a further 72h. Cells were then stained with antibodies for CD3, CD8 and CD4 and the amount of proliferation for each of the cells types was measured as the proportion of cells not expressing PKH dye. Unstimulated A) CD3$^+$, B) CD3$^+$CD4$^+$ and C) CD3$^+$CD8$^+$. Aβ stimulated D) CD3$^+$, E) CD3$^+$CD4$^+$ and F) CD3$^+$CD8$^+$. Paired t-tests performed on cells. P<0.05 *, p<0.01 **, p<0.001 ***. Data is a summary of experiments performed on 8 participants over several different days. n=8.

A timeline of the experiments is shown in figure 5.11 below. Five patients and five controls were tested with the peptides, all of the samples responded to the positive control, PHA, except in one patient where the background proliferation was considerably higher than in any other patient. The results were analysed in several ways, firstly only CD3$^+$ cells were gated to look at overall T-cell responses. There were no observed responses to the peptide under any conditions tested, in other words it made no difference if PBMCs were restimulated or only stimulated once and there were no differences between patients with AD and controls (Fig 5.12). Furthermore, the addition of IFNα to the experiment, although reducing background proliferation, did not reveal any increases in proliferation in response to the peptide.
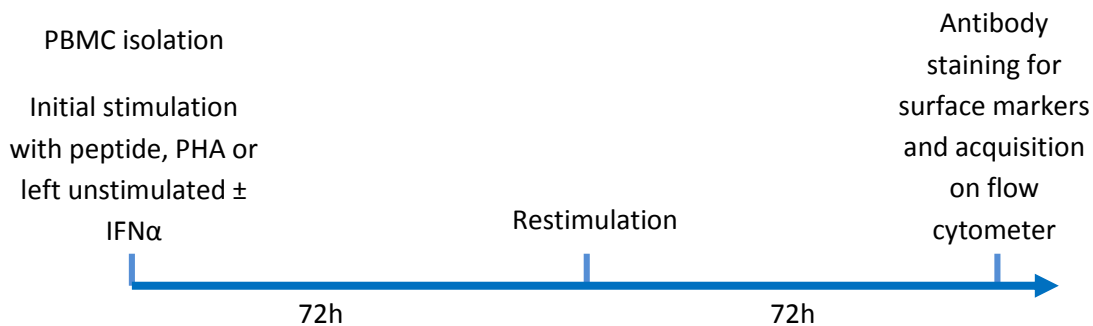
Figure 5 . 11: Timeline of stimulation and restimulation for proliferation assay

A timeline of the steps involved in the proliferation assay, starting from the isolation of PBMCs and initial stimulation with or without IFNα, when restimulation occurs (72h after initial stimulation) and when antibody staining and acquisition occur (72h after restimulation).
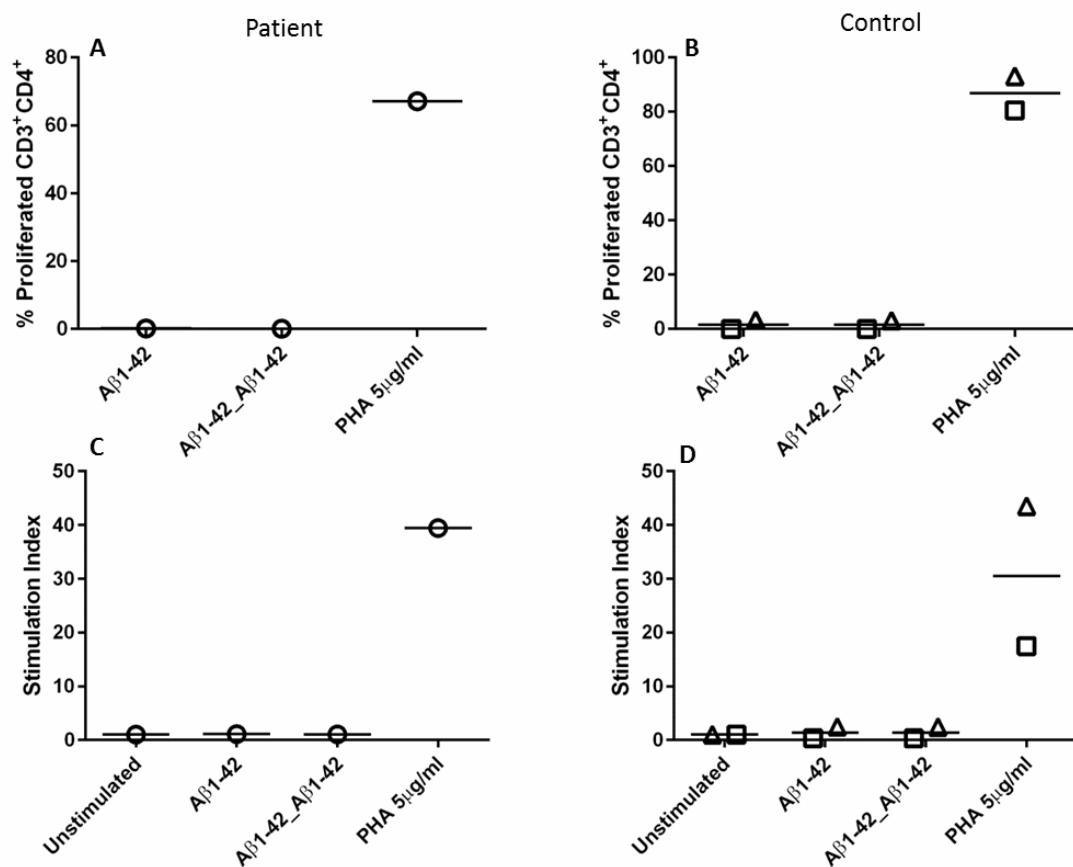
Figure 5 . 12: Synthetic Aβ 1-42 peptide does not cause a proliferative response in CD3⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A,C) or age-matched controls (B,D) they were stimulated with 15μg/ml Aβ 1-42, 5 μg/ml PHA or left unstimulated for 72h. Cells were restimulated with the indicated stimulant and then stained with antibodies for T-cell markers. Proportion of proliferated cells calculated as cells that proliferated in response to stimulant minus negative control. SI calculated as response divided by negative response. SI>2.5 corresponds to positive proliferative response. A) Proportion of proliferated patient T-cells, B) proportion of proliferated age-matched control T-cells. C) SI calculated from patient T-cells, D) SI calculated from control T-cells. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=3 patients, n=3 controls.

It was possible that the response to Aβ would be very small so, because of the amount of background proliferation, it may not be detectable when measuring the proliferation of all CD3$^+$ T-cells, however, by looking at the different subsets individually it may be possible to detect these small proliferative responses. Looking at the different subsets would also determine if proliferation only occurred in one subset of T-cells. Therefore, two subsets of T-cells, CD4$^+$ and CD8$^+$ T-cells, were tested to see if there were any differences in the different types of cell. The gating strategy for the CD4$^+$ and CD8$^+$ T-cells was the same, up to gating for singlets, cells expressing CD3 were gated and then all cells that were not singlets were excluded, CD4 was plotted against CD8 and the population of cells expressing either CD4 or CD8 were selected (Fig 5.13). Again all samples responded significantly to the positive control, except for one patient with elevated background proliferation, but there were no responses to the peptide under any of the conditions regardless of whether the blood samples were from patients or controls. All of the positive controls had a stimulation index above 2.5, in the majority of cases this value was far higher, and all of the peptide stimulations had a value lower than 2.5 suggesting there was no response. (Fig 5.14 5.15)
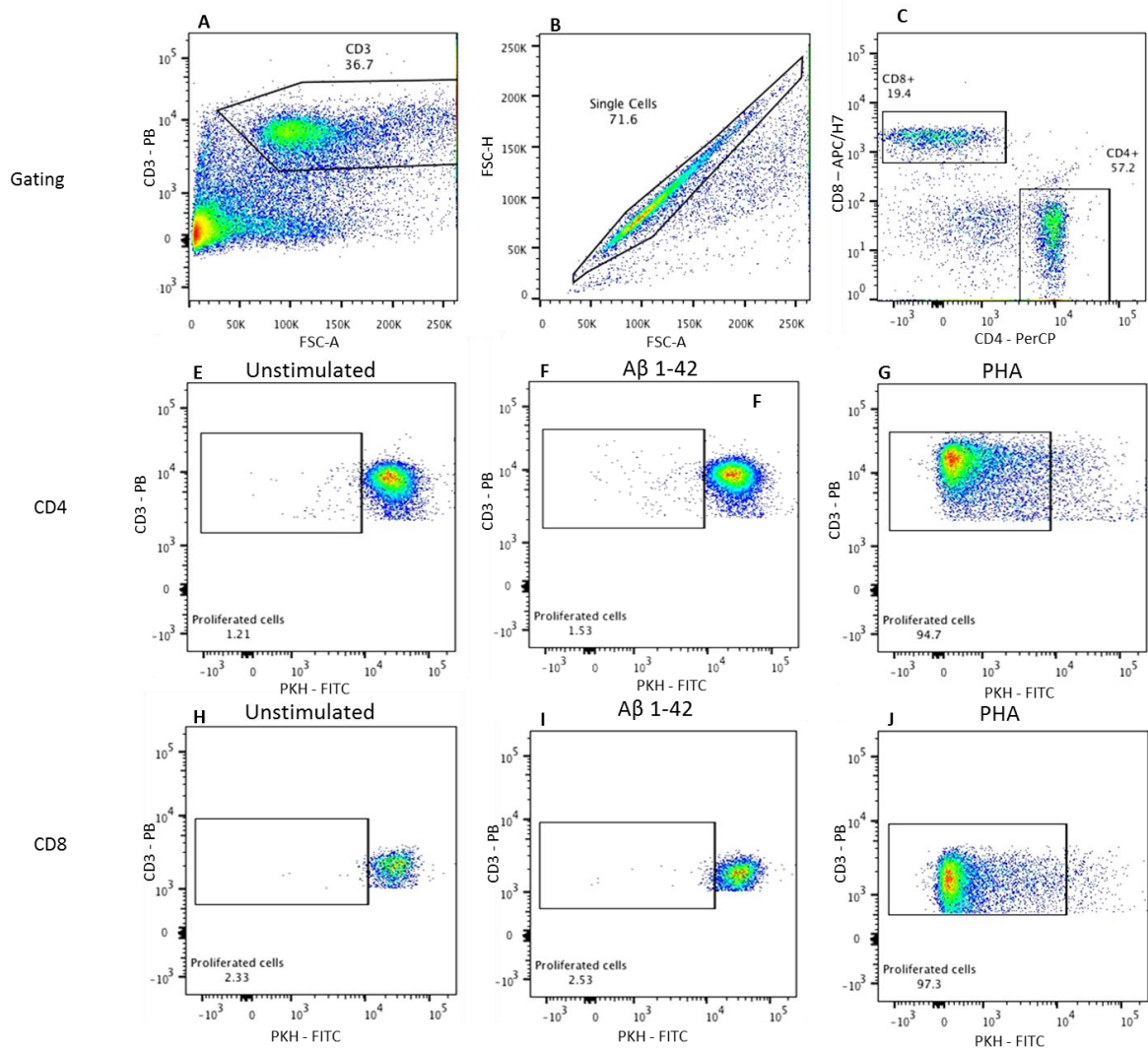
Figure 5 . 13: Gating strategy for CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T-cell proliferation

A) Forward scatter versus CD3, cells expressing CD3 were gated, B) Single cells were selected C) Subsets expressing either CD4 or CD8 (D) CD4, Unstimulated proliferated cells, E) CD4, Aβ 1-42 stimulated proliferated cells, F) CD4, PHA stimulated proliferated cells. G) CD8, Unstimulated proliferated cells, H) CD8, Aβ 1-42 stimulated proliferated cells, I) CD8, PHA stimulated proliferated cells. Representative of one experiment from a healthy control

Figure 5 . 14: Synthetic Aβ 1-42 peptide does not cause a proliferative response in CD3⁺CD4⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A,C) or age-matched controls (B,D) they were stimulated with 15μg/ml Aβ 1-42, 5 μg/ml PHA or left unstimulated for 72h. Cells were restimulated with the indicated stimulant and then stained with antibodies for T-cell markers. Proportion of proliferated cells calculated as cells that proliferated in response to stimulant minus negative control. SI calculated as response divided by negative response. SI>2.5 corresponds to positive proliferative response. A) Proportion of proliferated patient T-cells, B) proportion of proliferated age-matched control T-cells. C) SI calculated from patient T-cells, D) SI calculated from control T-cells. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=3 patients, n=3 controls.
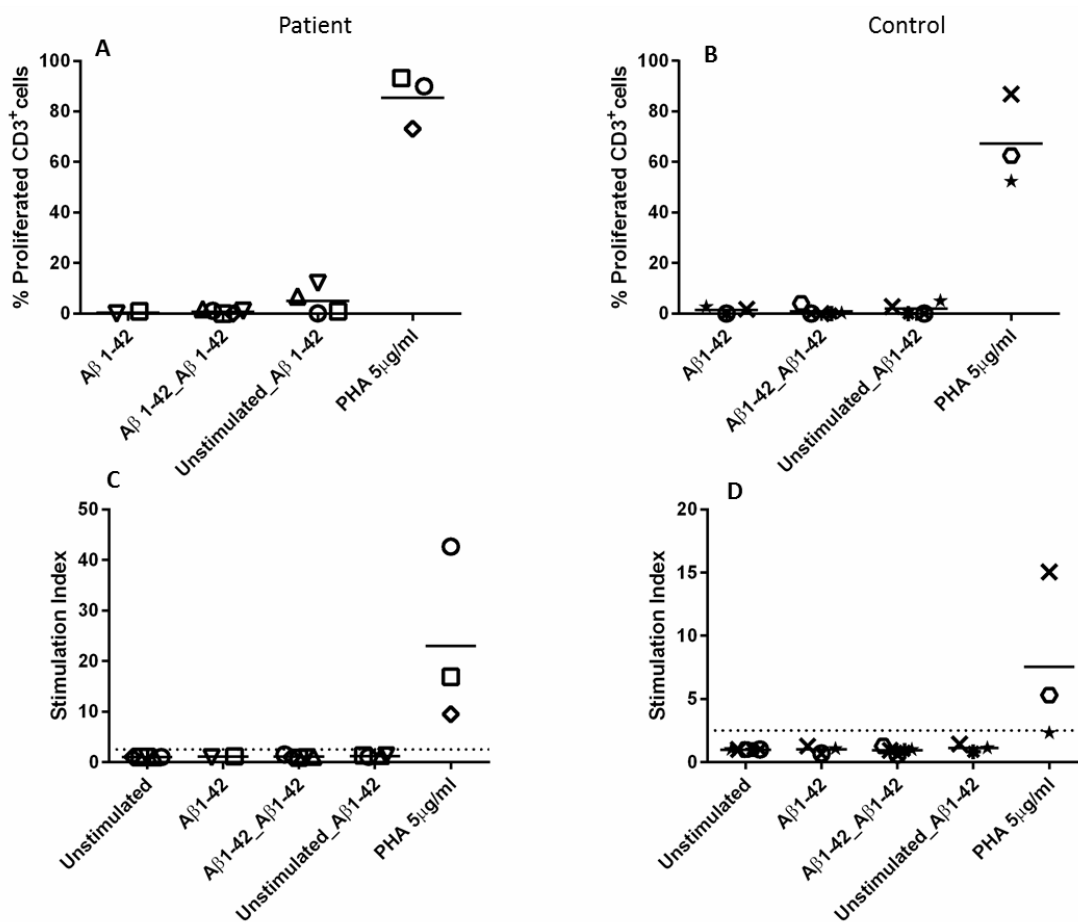
Figure 5 . 15: Synthetic Aβ 1-42 peptide does not cause a proliferative response in CD3⁺CD8⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A,C) or age-matched controls (B,D) they were stimulated with 15μg/ml Aβ 1-42, 5 μg/ml PHA or left unstimulated for 72h. Cells were restimulated with the indicated stimulant and then stained with antibodies for T-cell markers. The proportion of proliferated cells was calculated as the cells that proliferated in response to stimulant minus the response from unstimulated cells. SI was calculated as the response from stimulated cells divided by the response from unstimulated cells. SI>2.5 corresponds to positive proliferative response. A) Proportion of proliferated patient T-cells, B) proportion of proliferated age-matched control T-cells. C) SI calculated from patient T-cells, D) SI calculated from control T-cells. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=3 patients, n=3 controls.

As no positive response had been seen to the peptides in any of the experiments tried, fragments of the Aβ peptide were used. Published studies have suggested that T-cell responses to Aβ are MHC II mediated [469]. The two dominant HLA types associated with Aβ responses are DRB1*1501 and DRB1*0401. We therefore chose 10mer peptides with an overlap of 10 amino acids to cover the whole length of the Aβ 1-42 peptide. Epitope predictions for these alleles show that the fragments chosen for these experiments include the ones with the highest affinity. Table 3.1 below shows five epitopes for each HLA allele with the lowest percentile rank and therefore the highest affinity. If the peptide was not being processed or presented to the T-cells they would not proliferate in response to the peptide, however by using fragments of the peptide processing is bypassed and whether specific fragments of the peptide can induce proliferation of T-cells can be tested.

These experiments were carried out as before, the cells were initially stimulated for 72h and then restimulated for 72h with one of the fragments. They were stained with CD3, CD4 and CD8 antibodies and the gating strategy was as before.

All patient and control samples responded to the positive control when looking at the three different subsets of T-cells, shown by an SI of above 2.5. In addition there was no SI above 2.5 in response to any of the peptide epitopes tested from any of the T-cell subsets. Furthermore there were no differences when comparing the patient responses to the control responses (figures 5.16,5.17,5.18).

Table 5 . 2: Aβ 1-42 epitope predictions for HLA-DRB1 alleles

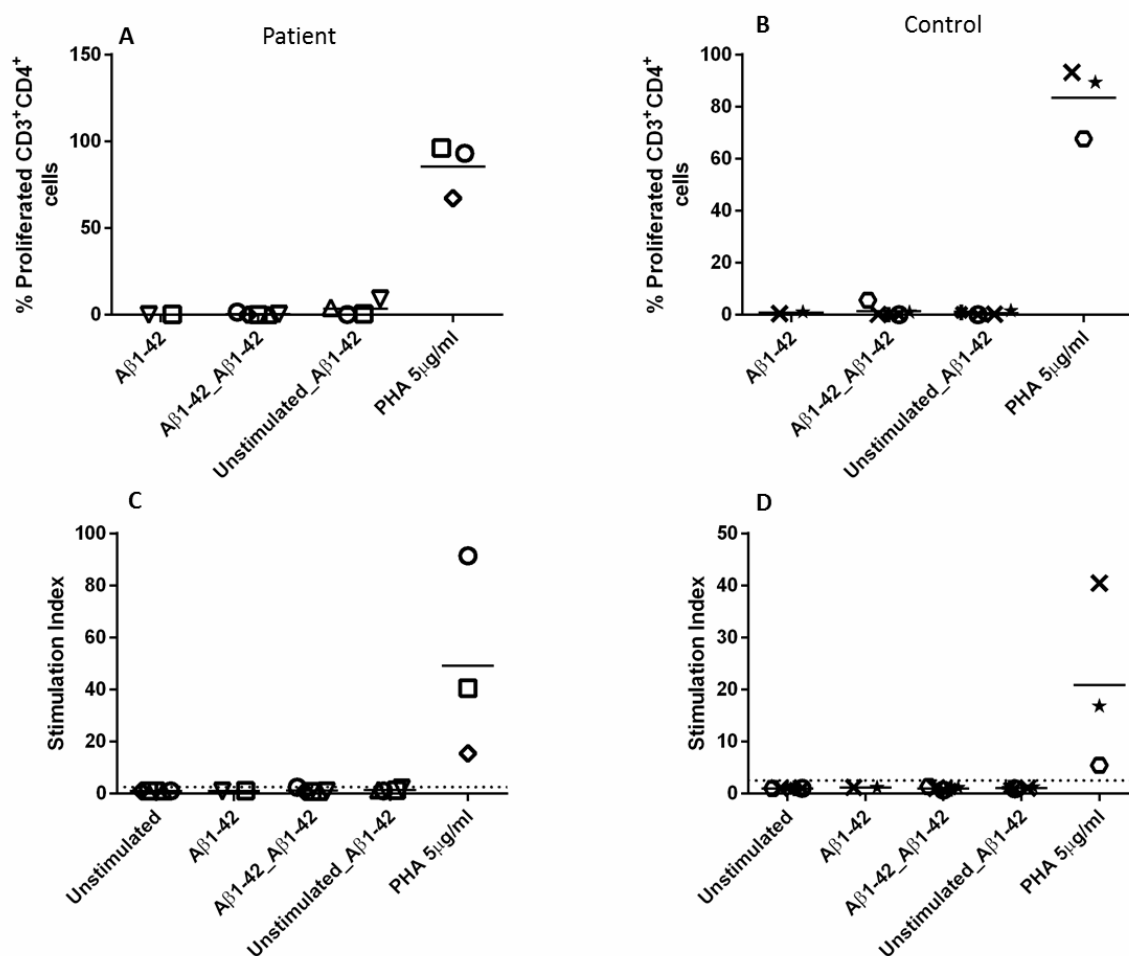| HLA allele	Predicted epitope	Peptide sequence predicted Peptide sequence tested	Peptide fragment	Percentile rank
DRB1*1501	26-40	SNKGAIIGLMVGGVV GSNKGAIIGLMVGGV	Aβ 25-39	2.61
DRB1*1501	27-41	NKGAIIGLMVGGVVI KGAIIGLMVGGVVIA	Aβ 28-42	2.61
DRB1*1501	28-42	KGAIIGLMVGGVVIA KGAIIGLMVGGVVIA	Aβ 28-42	2.61
DRB1*1501	25-39	GSNKGAIIGLMVGGV GSNKGAIIGLMVGGV	Aβ 25-39	8.85
DRB1*1501	11-25	EVHHQKLVFFAEDVG HHQKLVFFAEDVGSN	Aβ 13-27	11.71
DRB1*0401	16-30	KLVFFAEDVGSNKGA LVFFAEDVGSNKGAI	Aβ 17-31	3.44
DRB1*0401	15-29	QKLVFFAEDVGSNKG LVFFAEDVGSNKGAI	Aβ 17-31	3.55
DRB1*0401	14-28	HQKLVFFAEDVGSNK HHQKLVFFAEDVGSN	Aβ 13-27	3.64
DRB1*0401	17-31	LVFFAEDVGSNKGAI LVFFAEDVGSNKGAI	Aβ 17-31	3.64
DRB1*0401	13-27	HHQKLVFFAEDVGSN HHQKLVFFAEDVGSN	Aβ 13-27	7.01
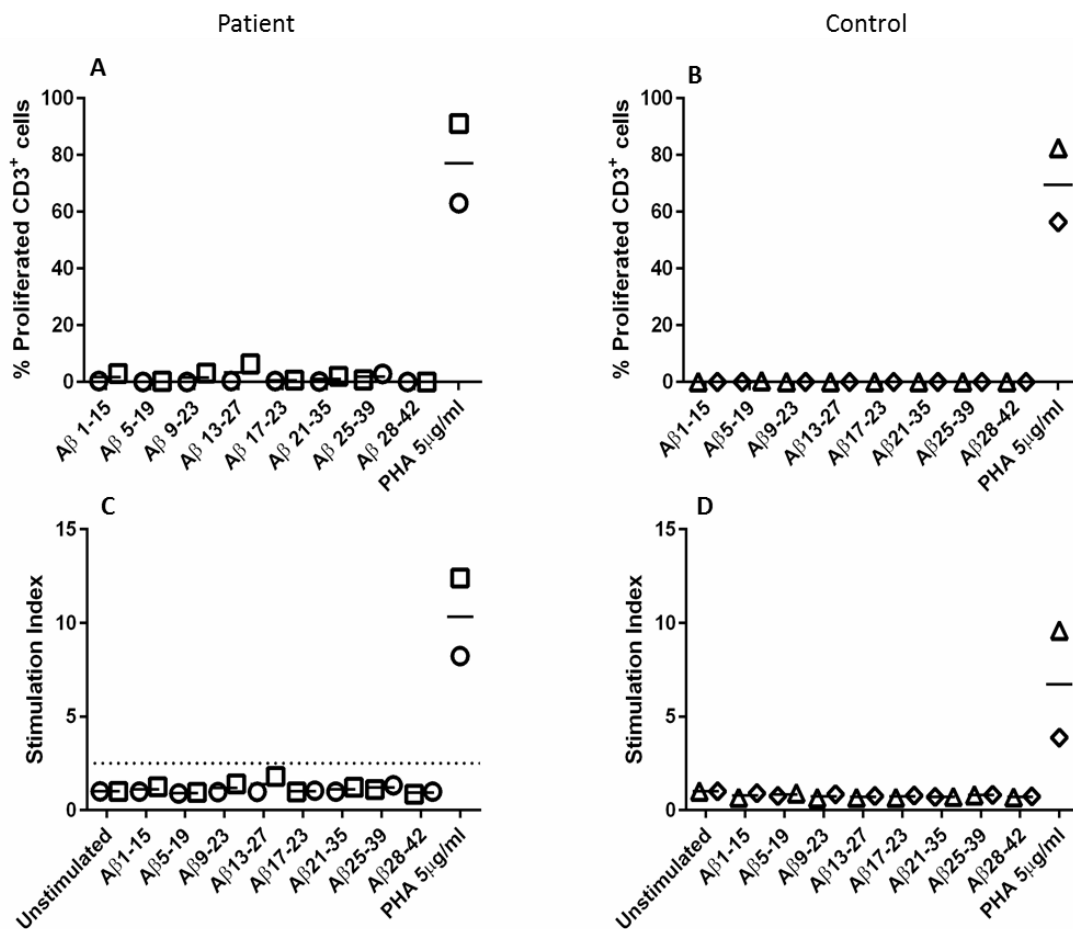
Figure 5 . 16: Fragments of synthetic Aβ peptide do not cause a proliferative response in CD3⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A, C) or age-matched controls (B, D) they were stimulated with 4μg/ml fragments of Aβ for 72h followed by restimulation with the same epitope for a further 72h. The cells were stained with T-cell surface markers and acquired using flow cytometry. The proportion of proliferated cells was calculated as the response to the peptide minus the response in untreated samples. SI was calculated as the response to the peptide divided by the response in untreated samples. A) Proportion of proliferated cells in patient T-cells. B) Proportion of proliferated cells in age-matched controls. C) SI in patient samples. D) SI in control samples. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=2 patients, n=2 controls.
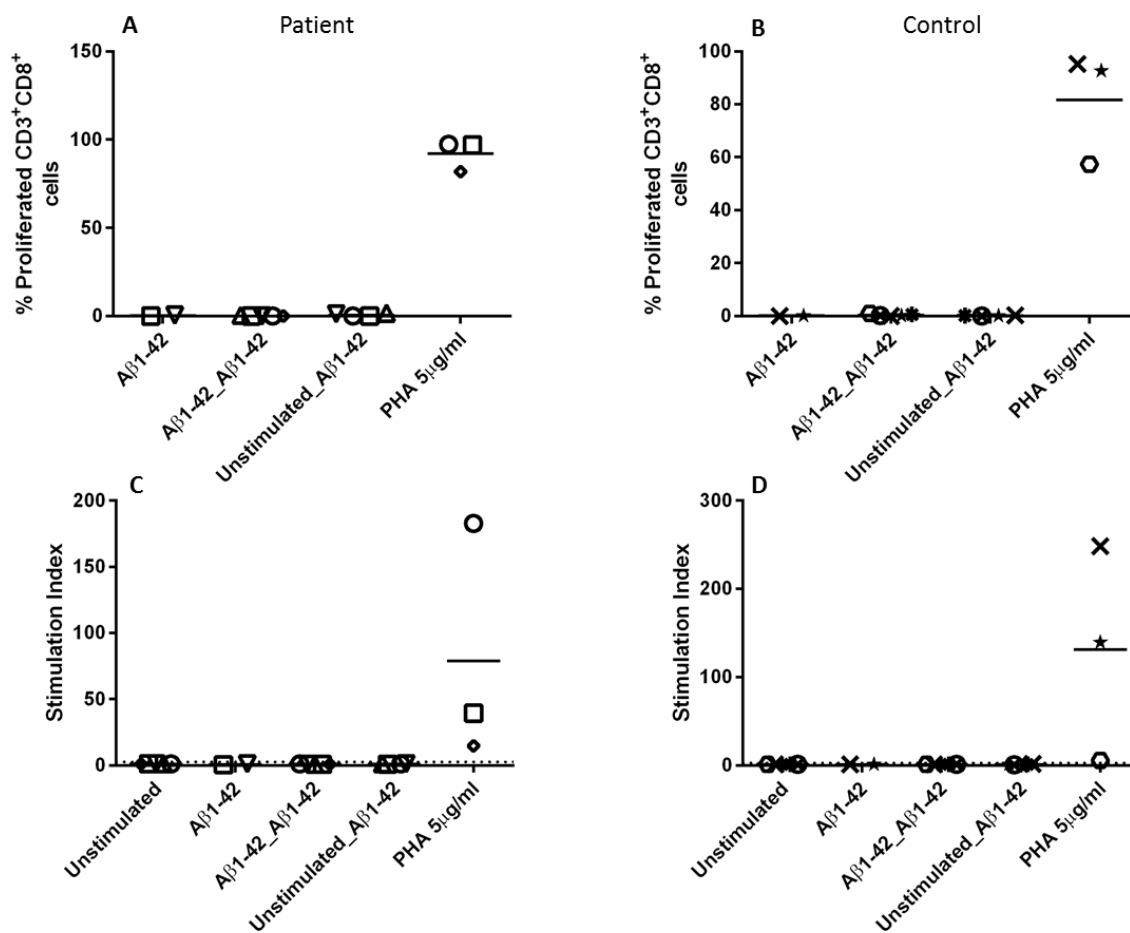
Figure 5 . 17: Fragments of synthetic Aβ peptide do not cause a proliferative response in CD3⁺CD4⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A, C) or age-matched controls (B, D) they were stimulated with 4μg/ml fragments of Aβ for 72h followed by restimulation with the same epitope for a further 72h. The cells were stained with T-cell surface markers and acquired using flow cytometry. The proportion of proliferated cells was calculated as the response to the peptide minus the response in untreated samples. SI was calculated as the response to the peptide divided by the response in untreated samples. A) Proportion of proliferated cells in patient T-cells. B) Proportion of proliferated cells in age-matched controls. C) SI in patient samples. D) SI in control samples. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=2 patients, n=2 controls.

Figure 5 . 18: Fragments of synthetic Aβ peptide do not cause a proliferative response in CD3⁺CD8⁺ T-cells

PBMCs were isolated from heparinised blood from patients (A, C) or age-matched controls (B, D) they were stimulated with 4μg/ml fragments of Aβ for 72h followed by restimulation with the same epitope for a further 72h. The cells were stained with T-cell surface markers and acquired using flow cytometry. The proportion of proliferated cells was calculated as the response to the peptide minus the response in untreated samples. SI was calculated as the response to the peptide divided by the response in untreated samples. A) Proportion of proliferated cells in patient T-cells. B) Proportion of proliferated cells in age-matched controls. C) SI in patient samples. D) SI in control samples. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=2 patients, n=2 controls.

5.2.2 Phosphorylation of PKC-δ and PKC-ζ

Proliferation is not the only way to measure T-cell responses, in addition the
production of cytokines can be measured. In these experiments the production of
CD40L and IL-2 were measure after 72h or initial stimulation followed by 6h of
restimulation. In addition recently published data has shown a specific pattern of
phosphorylation of PKC-δ and PKC-ζ in response to Aβ 1-42 in patients with AD.
The experiments performed also examined these responses to Aβ 1-42. [308].

The experiments looked at Aβ peptides from three different sources, two
commercially available Aβ and one custom made. One of the commercially available
peptides was recombinant and the two other Aβ peptides used were synthetic.

A number of pilot experiments were performed to determine whether Aβ could
induce the production of the cytokine CD40L and IL-2 and whether the peptide was
able to induce the phosphorylation of PKC-δ or PKC-ζ. PBMCs were isolated from
heparinised blood and stimulated for 72h and then restimulated for a further 6h.
After stimulation cells were stained with antibodies for CD3, CD4 and CD8 as well
as CD40L and IL-2, however as the positive controls, PMA and ionomycin affect the
expression of surface receptors, especially CD4, the gating strategy was altered to
account for this. Initially, forward scatter was plotted against side scatter and the
lymphocytes were gated. Any cells that were not single cells were excluded, and
then CD3 was plotted against CD8. The two populations of cells that were then gated
for further examination were the CD3$^+$CD8$^+$ and CD3$^+$CD8$^-$ (Fig 5.19)

As a secondary FITC antibody was used there was a possibility there would be non-
specific fluorescence. Therefore the secondary antibody alone was used to show
where this population of cells would appear in unstimulated cells (fig 5.20) and cells

treated with PMA and ionomycin (Fig 5.21). When these images were compared with samples containing a primary and secondary antibody it was possible to see two populations of cells that did not include the non-specific secondary antibody binding. The gates were set based on these two populations in unstimulated cells and then compared with stimulated samples to see if there was an increase in the proportion with higher P-PKC-δ or ζ staining.
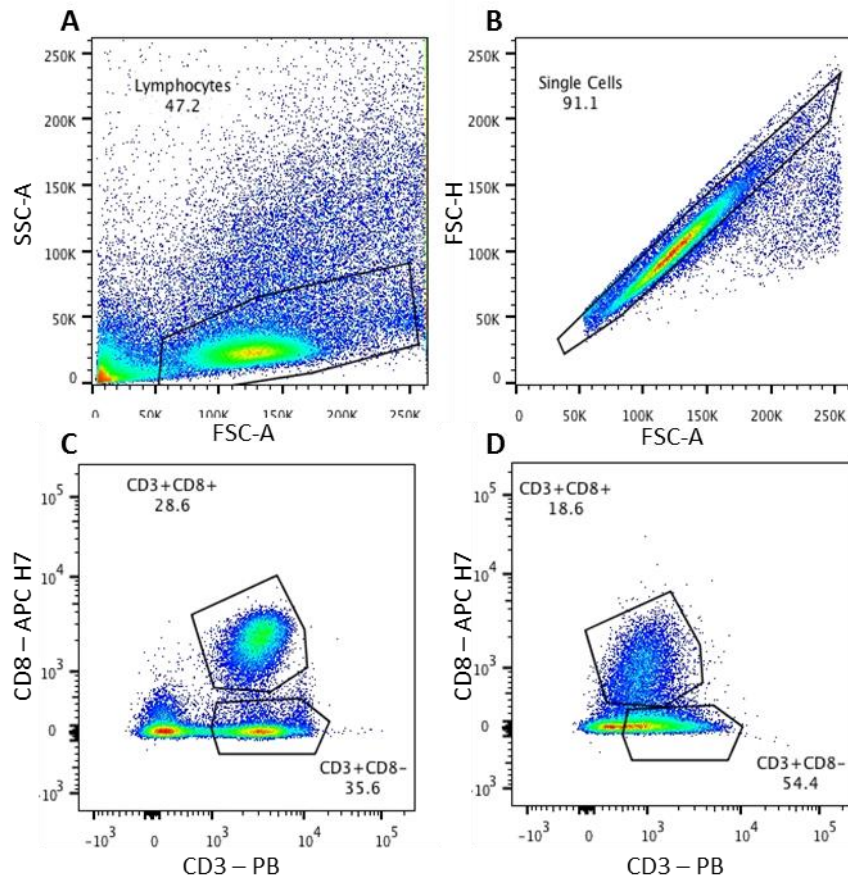
Figure 5 . 19: Initial gating strategy for CD3⁺CD8⁻ and CD3⁺CD8⁺ T-cells

PBMCs were isolated from heparinised blood and either left unstimulated or treated with25ng/ml PMA and 500ng/ml ionomycin for 72h followed by restimulation for 6h. A) Lymphocyte gate, B) Singlet gate, C) CD8 vs CD3 –unstimulated cells, D) CD8 vs CD3 – PMA/ionomycin
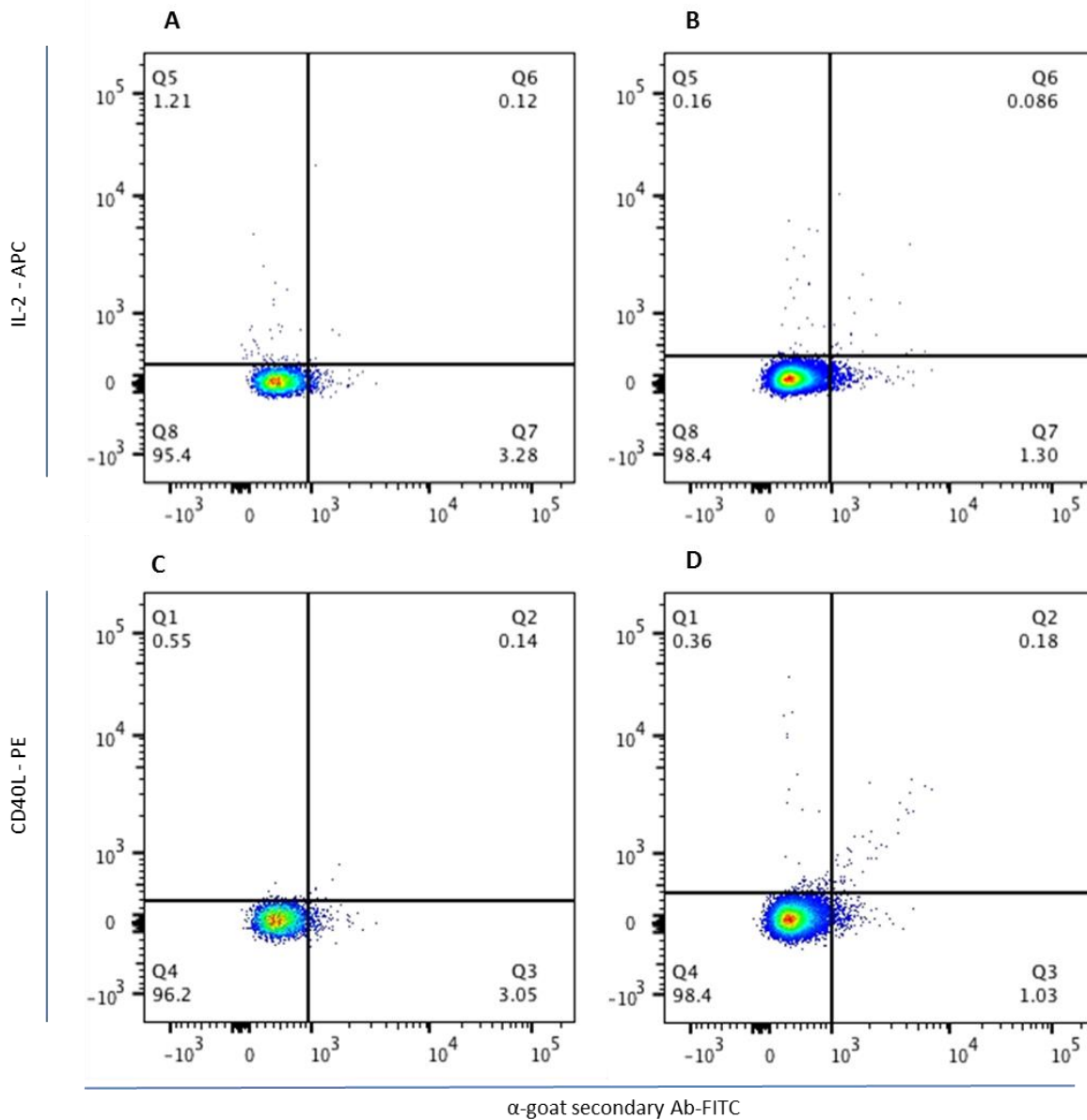
Figure 5 . 20: Background secondary FITC antibody staining in unstimulated cells

PBMCs were isolated from heparinised blood and left unstimulated for 72h. A) CD3$^+$CD8$^+$ secondary Ab vs IL-2. B) CD3$^+$CD8$^-$ secondary Ab vs IL-2, C) CD3$^+$CD8$^+$ secondary Ab vs CD40L, D) CD3$^+$CD8$^-$ secondary Ab vs CD40L.
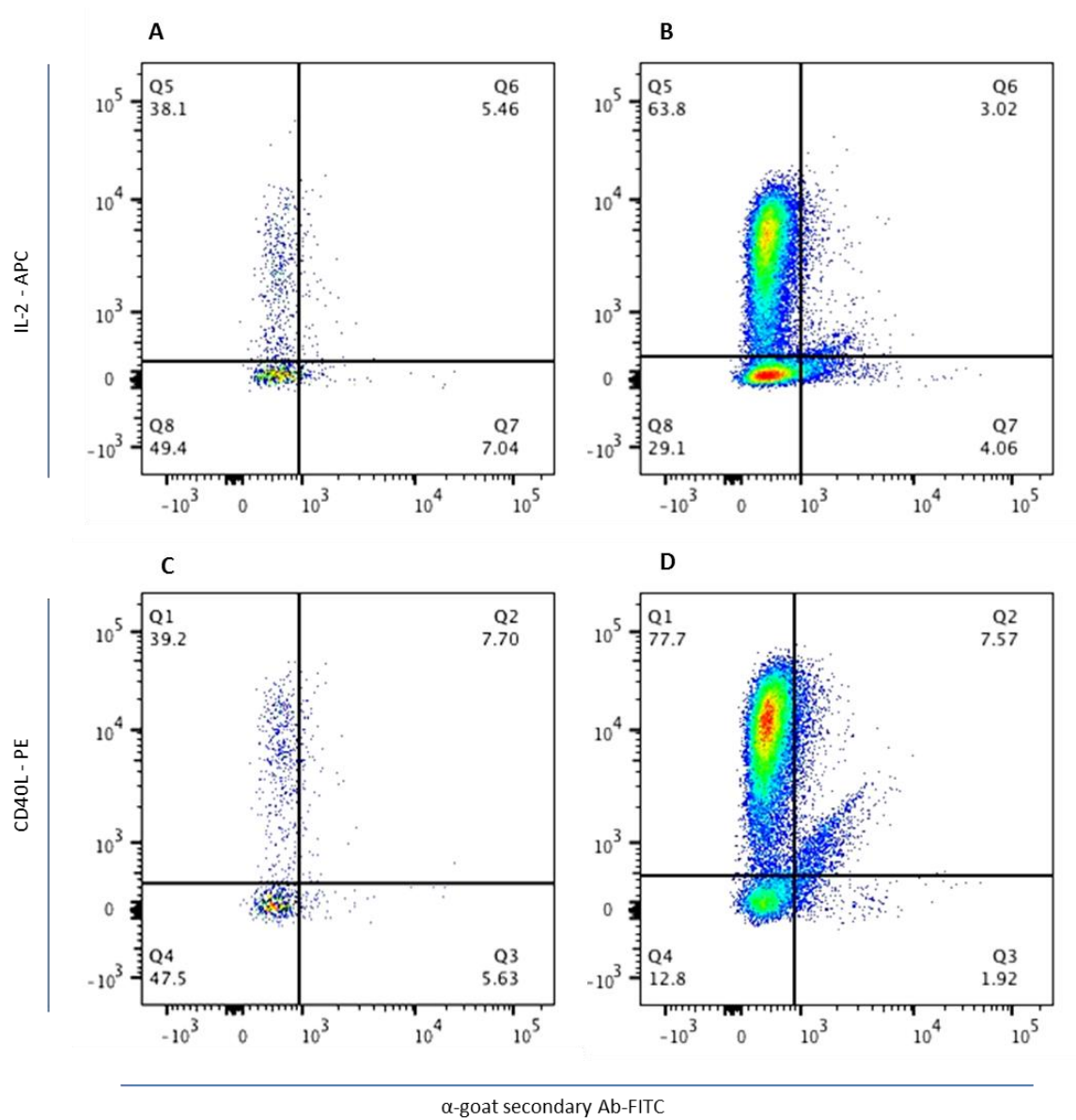
Figure 5 . 21: Background secondary FITC antibody staining in PMA/ionomycin stimulated cells

PBMCs were isolated from heparinised blood and stimulated with 25ng/ml PMA and 500ng/ml Ionomycin for 72h. They were then restimulated for a further 6h. A) CD3$^+$CD8$^+$ secondary Ab vs IL-2. B) CD3$^+$CD8$^-$ secondary Ab vs IL-2, C) CD3$^+$CD8$^+$ secondary Ab vs CD40L, D) CD3$^+$CD8$^-$ secondary Ab vs CD40L.

To measure the production of the two cytokines, PKC was plotted against IL-2 and CD40L and the proportion of cells expressing those cytokines were measured. On the same plots the cells positive for either P-PKC-δ or P-PKC-ζ were also measured (Fig 5.22. Fig 5.23).

Initially the experiment was performed with PMA and ionomycin on control blood only to ensure the experimental procedure would result in phosphorylation. The stimulation with PMA and ionomycin resulted in the production of CD40L and IL-2 as well as causing the phosphorylation of PKC-ζ and PKC-δ (CD40L) (Fig 5.22, Fig 5.23).
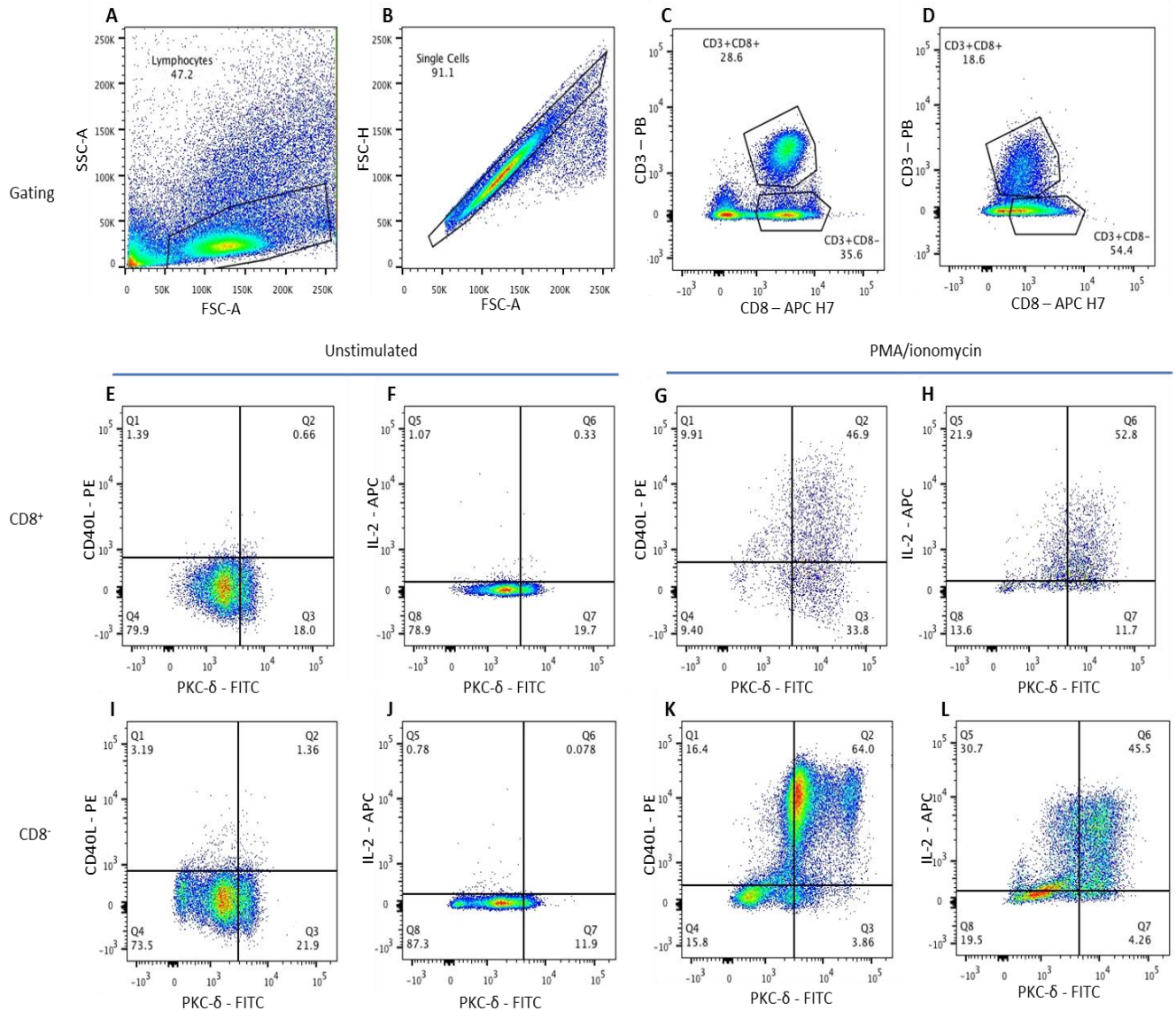
Figure 5 . 22: Gating strategy for P-PKC-δ

PBMCs were isolated from heparinised blood and either left unstimulated or treated with25ng/ml PMA and 500ng/ml ionomycin for 72h followed by restimulation for 6h. A) Lymphocyte gate, B) Singlet gate, C) CD8 vs CD3 –unstimulated cells, D) CD8 vs CD3 – PMA/ionomycin, E) Unstimulated CD3+CD8+ , PKC-δ vs CD40L F) Unstimulated CD3+CD8+ , PKC-δ vs IL-2., PKC-δ vs IL-2. G) PMA/ionomycin CD3+CD8+, PKC-δ vs CD40L. H) PMA/ionomycin CD3+CD8+, PKC-δ vs IL-2. I) Unstimulated CD3+CD8-, PKC-δ vs CD40L. J) Unstimulated CD3+CD8- K) PMA/ionomycin CD3+CD8-, PKC-δ vs CD40L. L) PMA/ionomycin CD3+CD8-, PKC-δ vs IL-2.
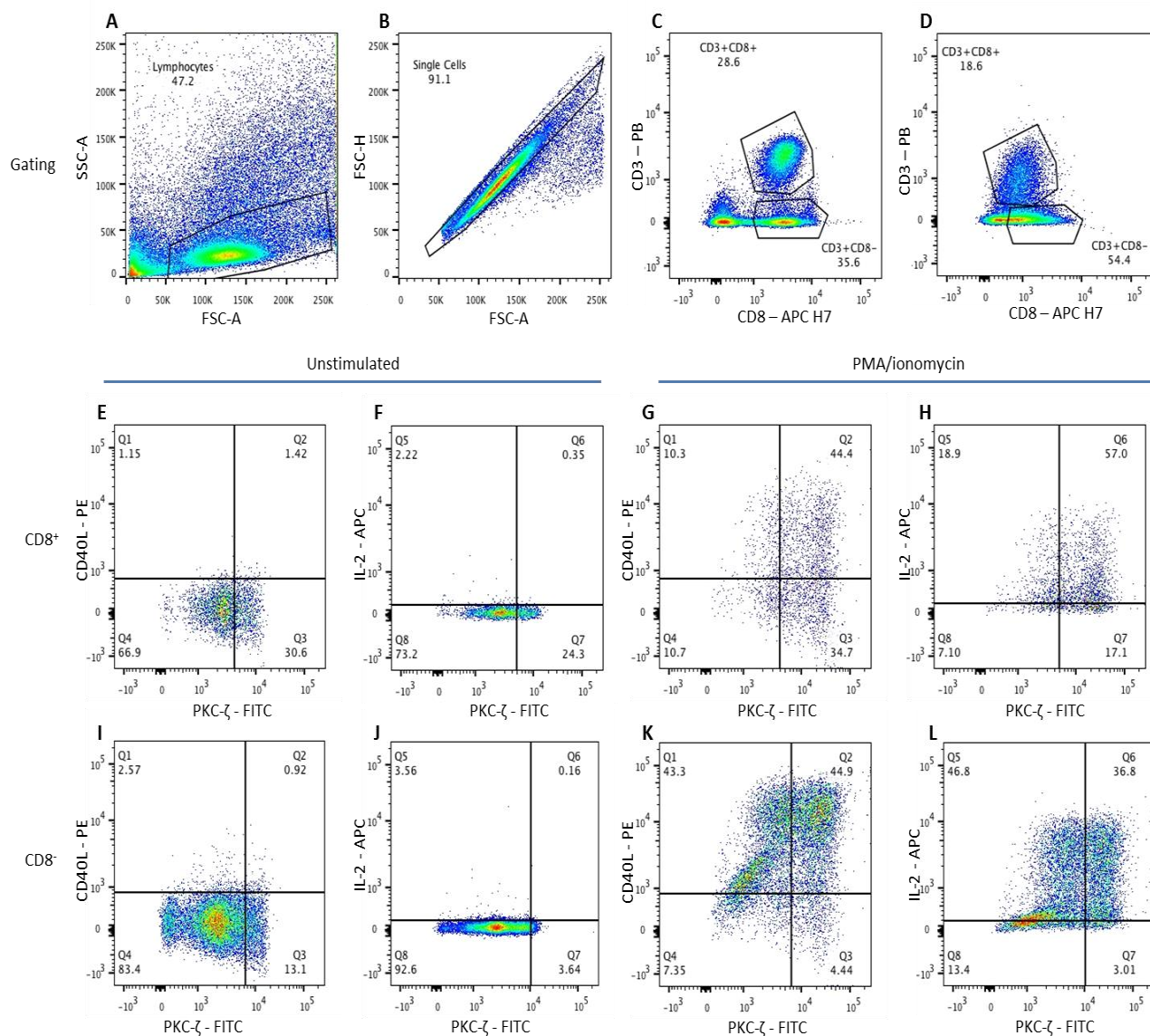
Figure 5 . 23: Gating strategy for P-PKC-ζ

PBMCs were isolated from heparinised blood and either left unstimulated or treated with25ng/ml PMA and 500ng/ml ionomycin for 72h followed by restimulation for 6h. A) Lymphocyte gate, B) Singlet gate, C) CD8 vs CD3 –unstimulated cells, D) CD8 vs CD3 – PMA/ionomycin, E) Unstimulated CD3$^+$CD8$^+$, PKC-ζ vs CD40L. F) Unstimulated CD3$^+$CD8$^+$, PKC-ζ vs IL-2. G) PMA/ionomycin CD3$^+$CD8$^+$, PKC-ζ vs CD40L. H) PMA/ionomycin CD3$^+$CD8$^+$, PKC-ζ vs IL-2. I) Unstimulated CD3$^+$CD8$^-$, PKC-ζ vs CD40L. J) Unstimulated CD3$^+$CD8$^-$, PKC-ζ vs IL-2. K) PMA/ionomycin CD3$^+$CD8$^-$, PKC-ζ vs CD40L. L) PMA/ionomycin CD3$^+$CD8$^-$, PKC-ζ vs IL-2.

5.2.3 Activation marker up-regulation

The Aβ peptides were then used to stimulate the PBMCs, the gating strategy used was identical as that used with PMA/ionomycin stimulated cells so that conditions could be compared. After stimulation with the Aβ 1-42 peptide there was no evidence of either CD40L or IL-2 production from either of the T-cell subtypes examined despite a response being observed to the positive control. The expression of cytokines was calculated as the percentage of cells positive for each of the activation markers. The mean of the cells expressing either CD40L or IL-2 was determined and plotted for both CD8$^+$ (Fig 5.24) and CD8$^-$ (Fig 5.25) cells. In addition, there were no differences between patient and control responses for either of the cytokines.

Figure 5 . 24: Aβ 1-42 peptide does not cause the production of CD40L or IL-2 from CD3$^+$CD8$^+$ T-cells

PBMCS were isolated from heparinised blood from patients with AD or age-matched controls. Cells were stimulated as indicated for 72h followed by restimulated for 6h. Cells were stained with antibody for T-cell surface markers and cytokines, CD40L and IL-2. They were acquired using flow cytometry and the proportion of cells expressing either IL-2 or CD40L was calculated. A) Patient samples producing CD40L. B) Control samples producing CD40L. C) Patient samples producing IL-2. D) Control samples producing IL-2. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=3 patients, n=3 controls.

Figure 5 . 25: Aβ 1-42 peptide does not cause the production of CD40L or IL-2 from CD3$^+$CD8$^-$ T-cells

PBMCS were isolated from heparinised blood from patients with AD or age-matched controls. Cells were stimulated as indicated for 72h followed by restimulated for 6h. Cells were stained with antibody for T-cell surface markers and cytokines, CD40L and IL-2. They were acquired using flow cytometry and the proportion of cells expressing either IL-2 or CD40L was calculated. A) Patient samples producing CD40L. B) Control samples producing CD40L. C) Patient samples producing IL-2. D) Control samples producing IL-2. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=3 patients, n=3 controls

As the Aβ 1-42 peptide showed no evidence of cytokine production, despite publications using this protocol seeing a response, different Aβ peptides were used. One recombinant Aβ 1-42 rPEP and one synthetic Aβ 1-42 from JPT were used in addition to ensure that the origin of the peptide does not affect response.

The Aβ 1-42 peptide from JPT produced the same response as Aβ 1-42 peptide, again there was no evidence of either CD3$^+$CD8$^+$ (Fig 5.26) or CD3$^+$CD8$^-$ (5.27) T-cells expressing CD40L or IL-2 despite all the samples responding to PMA/ionomycin. There was no difference between patient and control responses and the mean was calculated for both cytokines.
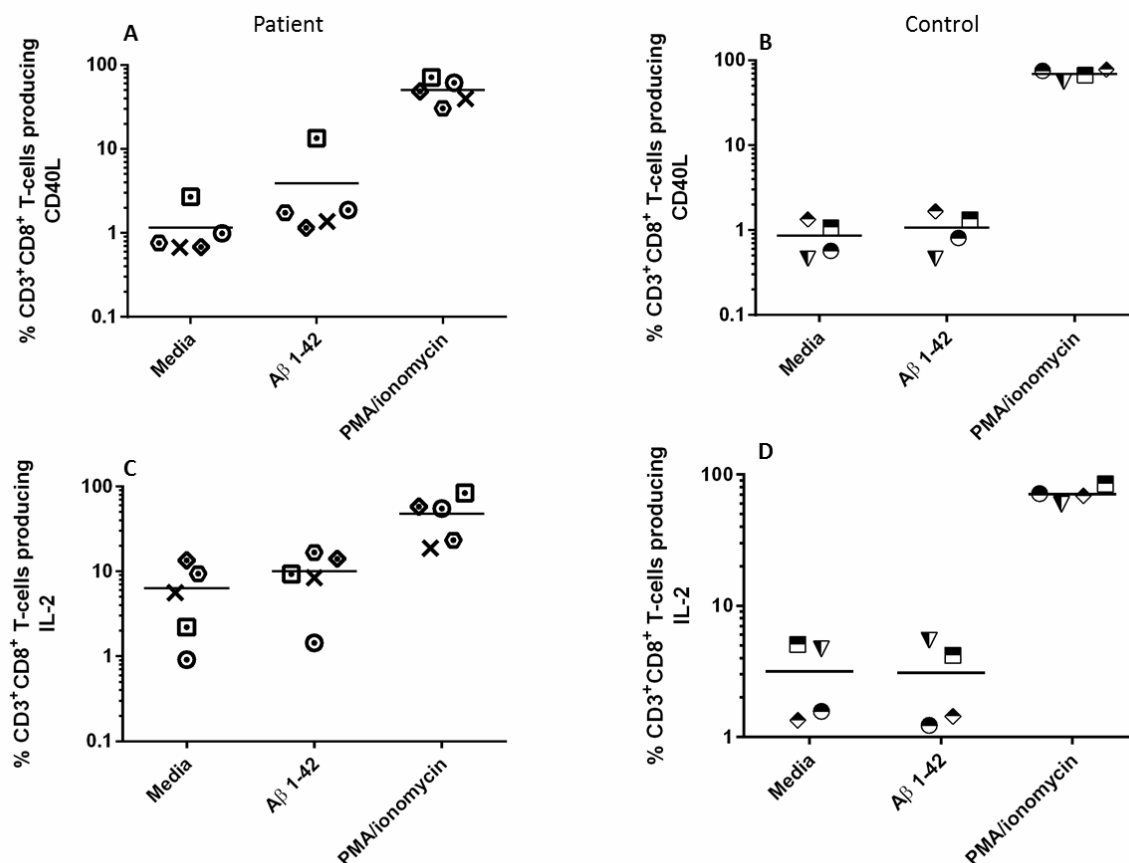
Figure 5 . 26: Aβ 1-42 peptide from JPT does not cause the production of CD40L or IL-2 from CD3$^+$CD8$^+$ T-cells

PBMCS were isolated from heparinised blood from patients with AD or age-matched controls. Cells were stimulated as indicated for 72h followed by restimulated for 6h. Cells were stained with antibody for T-cell surface markers and cytokines, CD40L and IL-2. They were acquired using flow cytometry and the proportion of cells expressing either IL-2 or CD40L was calculated. A) Patient samples producing CD40L. B) Control samples producing CD40L. C) Patient samples producing IL-2. D) Control samples producing IL-2. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=5 patients, n=5 controls.

Figure 5 . 27: Aβ 1-42 peptide from JPT does not cause the production of CD40L or IL-2 from CD3⁺CD8⁻ T-cells

PBMCS were isolated from heparinised blood from patients with AD or age-matched controls. Cells were stimulated as indicated for 72h followed by restimulated for 6h. Cells were stained with antibody for T-cell surface markers and cytokines, CD40L and IL-2. They were acquired using flow cytometry and the proportion of cells expressing either IL-2 or CD40L was calculated. A) Patient samples producing CD40L. B) Control samples producing CD40L. C) Patient samples producing IL-2. D) Control samples producing IL-2. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=5 patients, n=5 controls

The same was also true for the recombinant Aβ peptide from rPEP, there was no increase in IL-2 or CD40L after stimulation from either of the two subsets investigated, although a response was seen after PMA stimulation. The patient status did not affect whether a response was observed nor did the type of T-cell subset examined, suggesting that none of the Aβ peptides were able to induce the response of CD40L or IL-2 from T-cells. (Fig 5.28, 5.29)
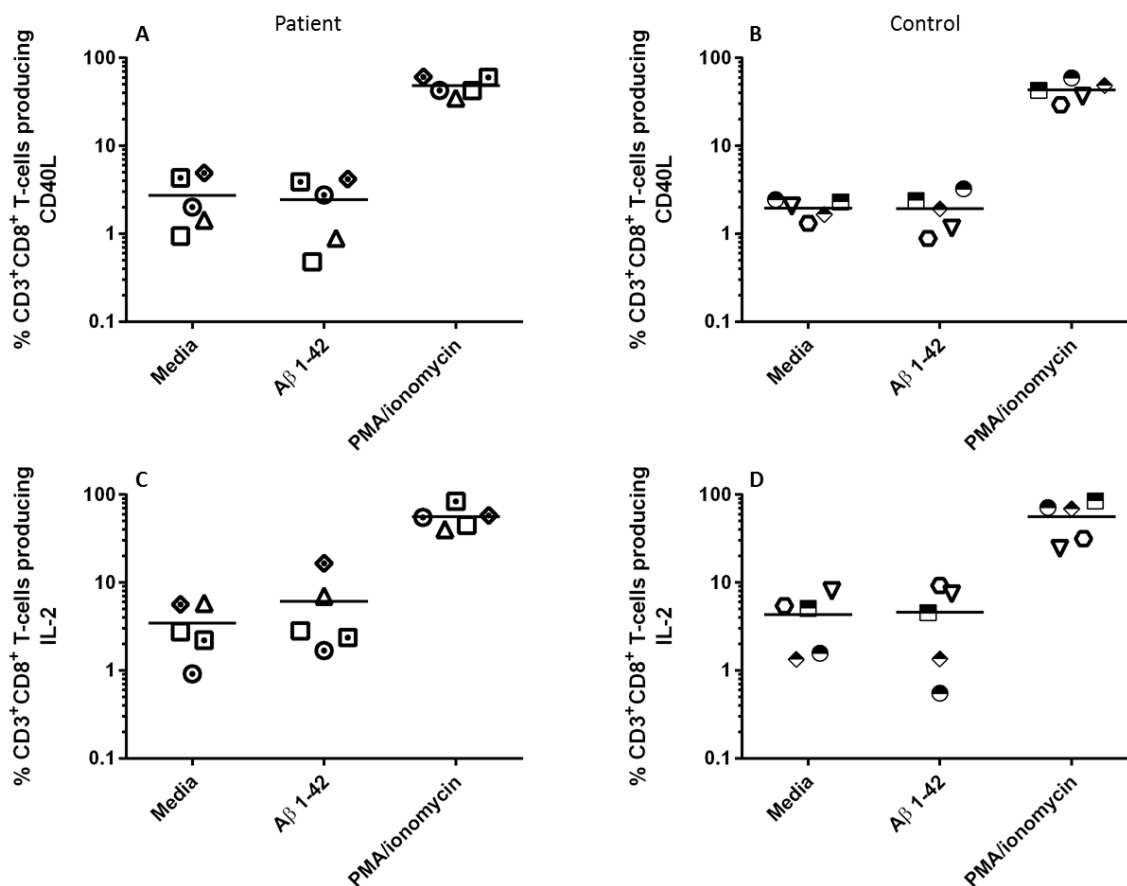
Figure 5 . 28: Recombinant Aβ 1-42 from rPEP does not cause the production of CD40L or IL-2 from CD3⁺CD8⁺ T-cells

PBMCS were isolated from heparinised blood from patients with AD or age-matched controls. Cells were stimulated as indicated for 72h followed by restimulated for 6h. Cells were stained with antibody for T-cell surface markers and cytokines, CD40L and IL-2. They were acquired using flow cytometry and the proportion of cells expressing either IL-2 or CD40L was calculated. A) Patient samples producing CD40L. B) Control samples producing CD40L. C) Patient samples producing IL-2. D) Control samples producing IL-2. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=5 patients, n=5 controls

Figure 5 . 29: Recombinant Aβ 1-42 peptide from rPEP does not cause the production of CD40L or IL-2 from CD3⁺CD8⁻ T-cells

PBMCS were isolated from heparinised blood from patients with AD or age-matched controls. Cells were stimulated as indicated for 72h followed by restimulated for 6h. Cells were stained with antibody for T-cell surface markers and cytokines, CD40L and IL-2. They were acquired using flow cytometry and the proportion of cells expressing either IL-2 or CD40L was calculated. A) Patient samples producing CD40L. B) Control samples producing CD40L. C) Patient samples producing IL-2. D) Control samples producing IL-2. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=5 patients, n=5 controls

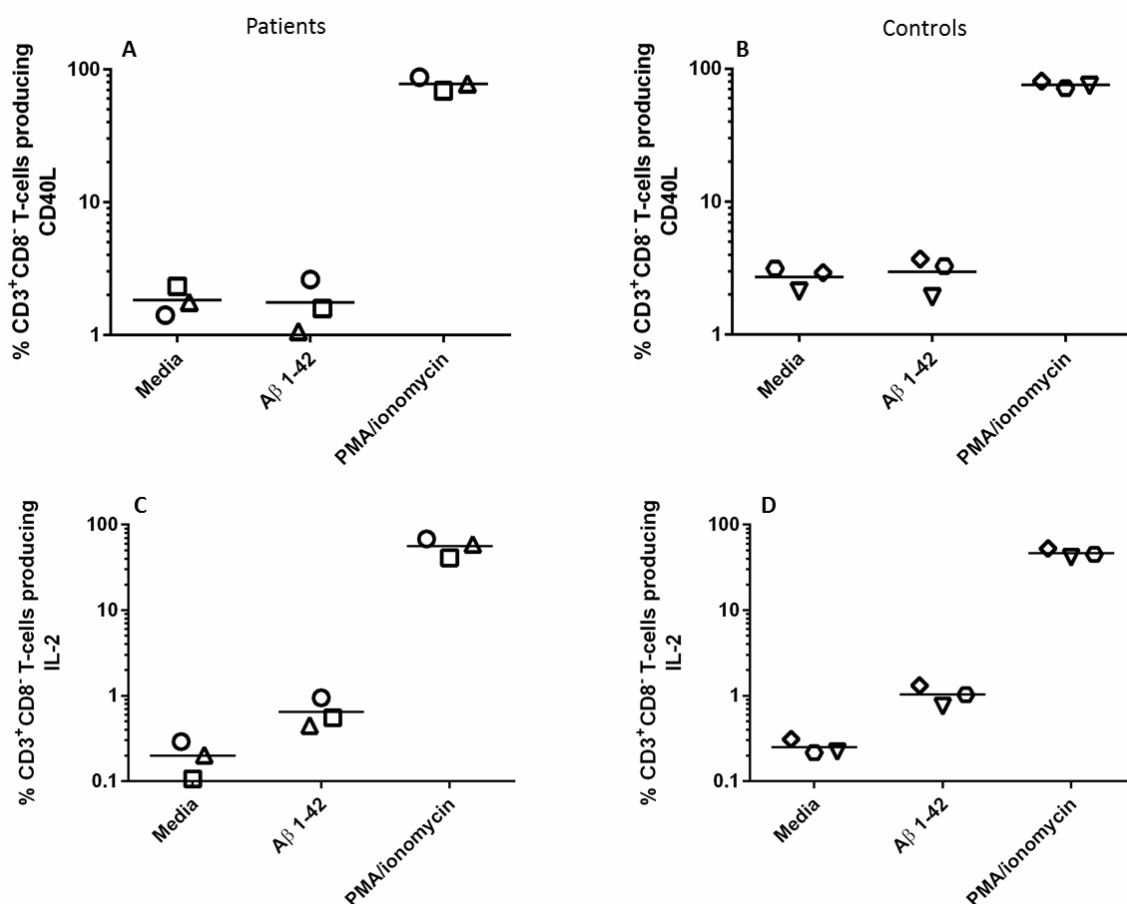The data displayed in the previous experiments were performed on a number of different patients and healthy controls over several months. This was due to sporadic patient and control recruitment which limited how many experiments were performed at once. In addition, it limited the peptides that were available for each experiment. As the peptide begins to aggregate as soon as they are dissolved they could only be used immediately after preparation, this meant that all of the peptides could not be used for every patient and control experiment. Furthermore because of the unknown number of participants being recruited each time there was not always enough material available to stimulate all the samples with the same peptides. For these reasons the experiments were not all run in parallel, however, the conditions of all the experiments remained the same and at least three patients and controls were stimulated with each of the peptides. The symbols used in the figures for these experiments shows one specific patient or control and is consistent throughout all the activation marker and PKC-phosphorylation experiments.

The same three peptides were used to look at the phosphorylation of PKC-ζ and PKC-δ. One initial observation was that there was an elevated level of background phosphorylation of PKC-ζ in almost all samples. The mean amount of P-PKC-δ that was measured in unstimulated cells was 11% in CD8$^+$ and 10% in CD8$^-$ cells whereas the mean P-PKC-ζ was 26% in CD8$^+$ cells and 21% in CD8$^-$. However, there were no differences in background phosphorylation between patients and controls.

PKC-δ and PKC-ζ phosphorylation was looked at in the two T-cell subsets, CD3$^+$CD8$^+$ and CD3$^+$CD8$^-$. In CD3$^+$CD8$^+$ cells, PMA and ionomycin caused an increase in cells expressing P-PKC-δ in all the age-matched controls although there was no evidence of a response to any of the peptides when compared with the

unstimulated cells. In patients with AD there was a smaller increase in the proportion of cells positive for P-PKC-δ after PMA/ionomycin stimulation than in the healthy controls, however this difference was not significant. There was also no response to the three peptides tested.

An increase in cells positive for P-PKC-ζ was observed after PMA/ionomycin stimulation of all healthy controls but none of them showed any response to the peptides tested. There was a significantly smaller increase in the proportion of $CD3^+CD8^+$ cells with positive P-PKC-ζ expression after PMA/ionomycin stimulation from patients, when compared with controls. As there were no differences in expression in the unstimulated cells between the two groups it suggests that there is less of a response to PMA/ionomycin from patients compared with healthy controls. The increase in cells expressing P-PKC-ζ and P-PKC-δ after PMA/ionomycin stimulation is shown in figure 5.34 and the proportion of cells expressing P-PKC-δ in response to the three peptides tested is shown in figure 5.30 and to P-PKC-ζ is shown in figure 5.31.

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Figure 5 . 30: Aβ 1-42 peptide does not cause the phosphorylation of PKC-δ in CD3⁺CD8⁺ T-cells

PBMCs were isolated from patient or age-matched control blood and stimulated with 10μg/ml of the indicated Aβ peptide for 72h followed by restimulation with the same peptide. Cells were stained with CD3 and CD8 and acquired using flow cytometry. Proportion of phosphorylated cells were calculated as the CD3⁺CD8⁺ cells positive for P-PKC-δ. Each symbol represents one sample. A) Aβ 1-42 peptide in patient samples, B) Aβ 1-42 response in control samples, C) response to Aβ 1-42 from JPT in patient sample, D) response control samples to Aβ 1-42 from JPT, E) response in patient samples to Aβ 1-42 from rPEP, F) response in control samples to Aβ 1-42 from rPEP. Each symbol represents one participant; the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=4 patients, n=4 controls
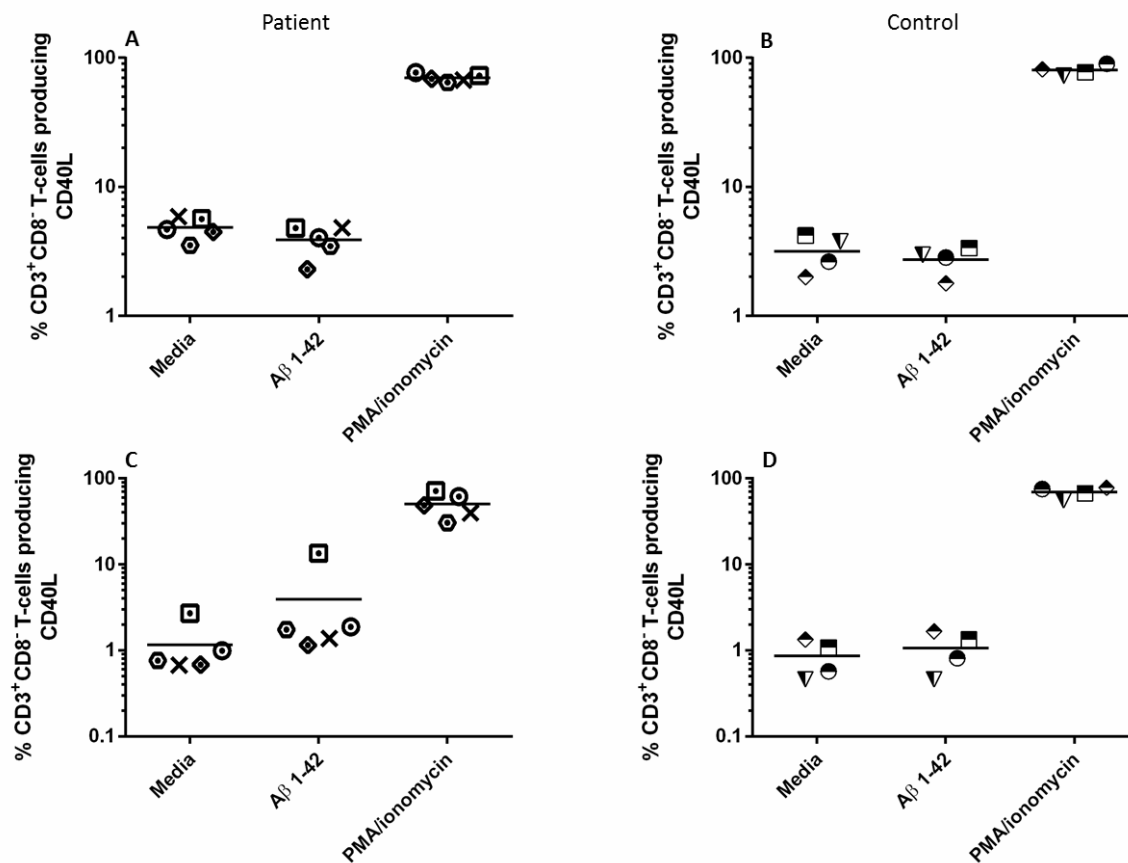
Figure 5 . 31: Aβ 1-42 peptide does not cause the phosphorylation of PKC-ζ in CD3⁺CD8⁺ T-cells

PBMCs were isolated from patient or age-matched control blood and stimulated with 10μg/ml of the indicated Aβ peptide for 72h followed by restimulation with the same peptide. Cells were stained with CD3 and CD8 and acquired using flow cytometry. Proportion of phosphorylated cells were calculated as the CD3⁺CD8⁺ cells positive for P-PKC-ζ. Each symbol represents one donor. A) Aβ 1-42 peptide in patient samples, B) Aβ 1-42 response in control samples, C) response from patient sample to Aβ1-42 from JPT, D) response from control samples to Aβ 1-42 from JPT, E) response in patient samples to Aβ1-42 from rPEP, F) response in control samples to Aβ1-42 from, rPEP. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=4 patients, n=4 controls
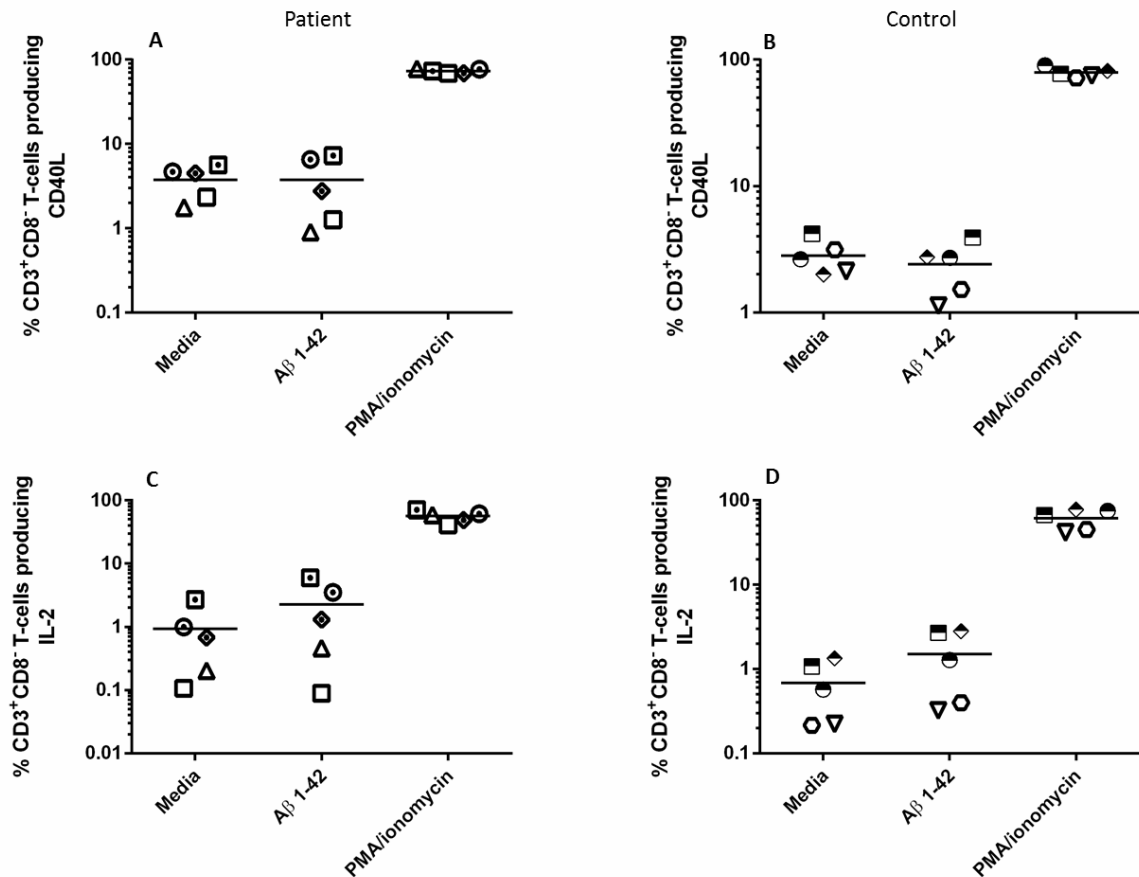
When looking at the second population of cells, $CD3^+CD8^-$ T-cells, there were a number of similarities but also a number of differences. Firstly, the age-matched controls all showed PKC-δ phosphorylation after stimulation with PMA and ionomycin but none of the peptides caused any increase in the proportion of cells expressing P-PKC-δ. It was also true that none of the patients with AD responded to any of the peptides (Fig 5.32), there was also a smaller increase in the cells expressing P-PKC-δ after PMA/ionomycin stimulation when compared with the controls (Fig 5.34).

There was a small increase, for example from 17% to 28%, in the proportion of cells expressing P-PKC-ζ, in some patients after stimulation with the Aβ peptides (Fig 5.33) and there were similar responses observed from some of the healthy controls, 11% to 18% (Fig 5.33). The increases observed occurred in a minority of participants, two patients and two controls, and these were the same two patients or controls in almost all cases. Furthermore, the experiments were designed to show a population of cells that was specific for patients with AD [308, 309]. The fact that there were both patients and controls and that the response was not observed in all patients suggests that this is not that specific population of phosphorylated $CD8^-$ cells.

There were also significantly different responses observed to PMA/ionomycin between controls and patients, the controls showed a larger increase in the proportion of cells expressing P-PKC-ζ after PMA/ionomycin stimulation, this is shown in figure 5.34. Unstimulated cells from patients and controls did not show a different proportion of P-PKC-$ζ^+$ cells, suggesting that patient cells responded less to PMA/ionomycin.

Figure 5 . 32: Aβ peptide does not cause the phosphorylation of PKC-δ in CD3⁺CD8⁻ T-cells

PBMCs were isolated from patient or age-matched control blood and stimulated with 10μg/ml of the indicated Aβ peptide for 72h followed by restimulation with the same peptide. Cells were stained with CD3 and CD8 and acquired using flow cytometry. Proportion of phosphorylated cells were calculated as the CD3$^+$CD8$^+$ cells positive for P-PKC-δ. Each symbol represents one sample. A) Aβ 1-42 peptide in patient samples, B) Aβ 1-42 response in control samples, C) response from patient sample to Aβ1-42 from JPT, D) response from control samples to Aβ 1-42 from JPT peptide, E) response in patient samples to Aβ 1-42 from rPEP, F) response in control samples to Aβ1-42 from rPEP. Each symbol represents one participant; the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=4 patients, n=4 controls
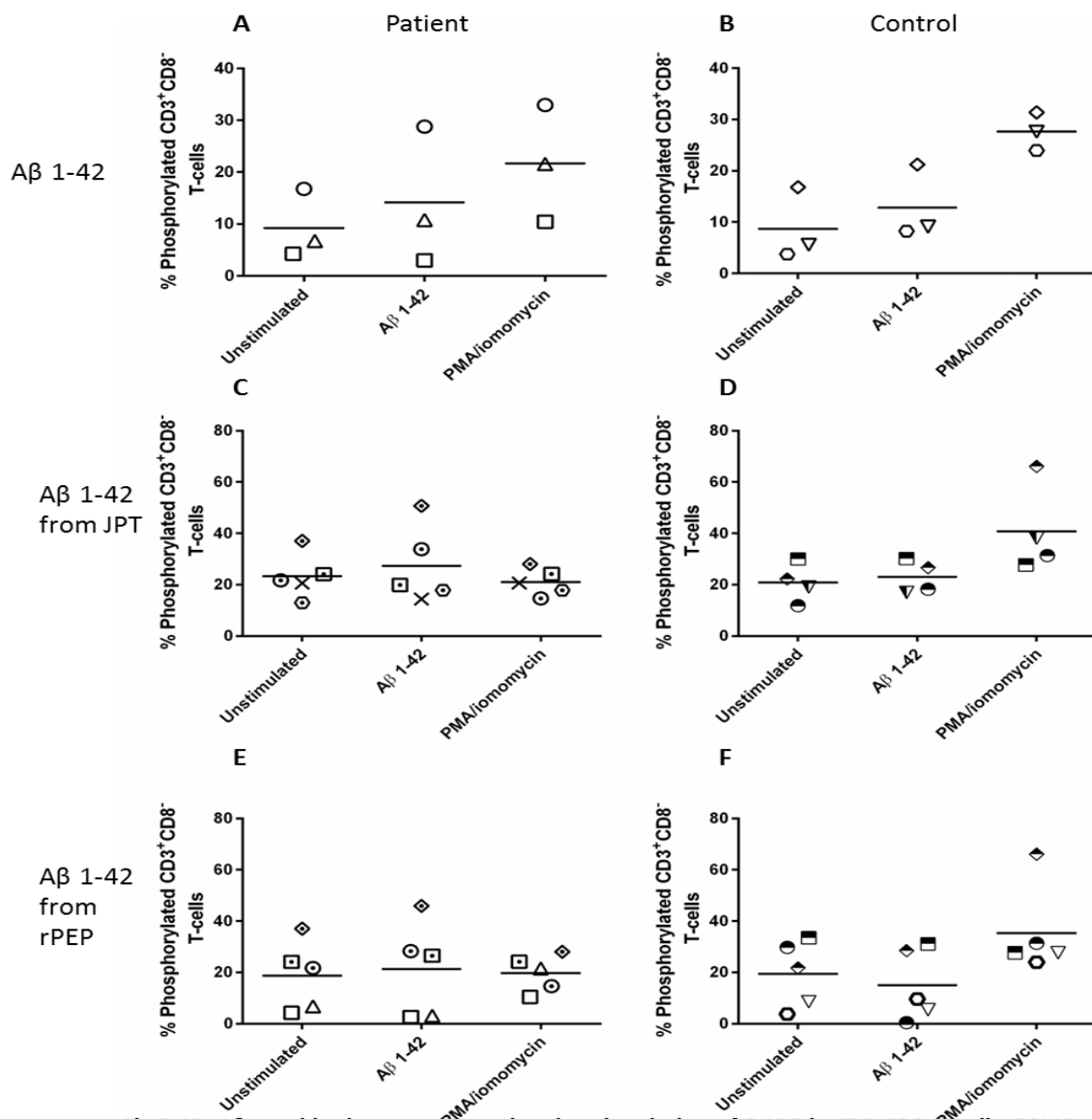
Figure 5 . 33: Aβ peptide does not cause the phosphorylation of PKC-ζ in CD3$^+$CD8$^-$ T-cells

PBMCs were isolated from patient or age-matched control blood and stimulated with 10μg/ml of the indicated Aβ peptide for 72h followed by restimulation with the same peptide. Cells were stained with CD3 and CD8 and acquired using flow cytometry. Proportion of phosphorylated cells were calculated as the CD3$^+$CD8$^+$ cells positive for P-PKC-ζ. Each symbol represents one donor. A) Aβ 1-42 peptide in patient samples, B) Aβ 1-42 response in control samples, C) response from patient sample to Aβ 1-42 from JPT, D) response in from control samples to Aβ 1-42 from JPT, E) response in patient samples to Aβ -142 from rPEP, F) response in control samples to Aβ 1-42 from rPEP. Each symbol represents one participant, the mean of the duplicates is shown by the symbols. The line represents the overall mean. n=3 patients, n=4 controls.

CD3⁺CD8⁺

CD3⁺CD8⁻
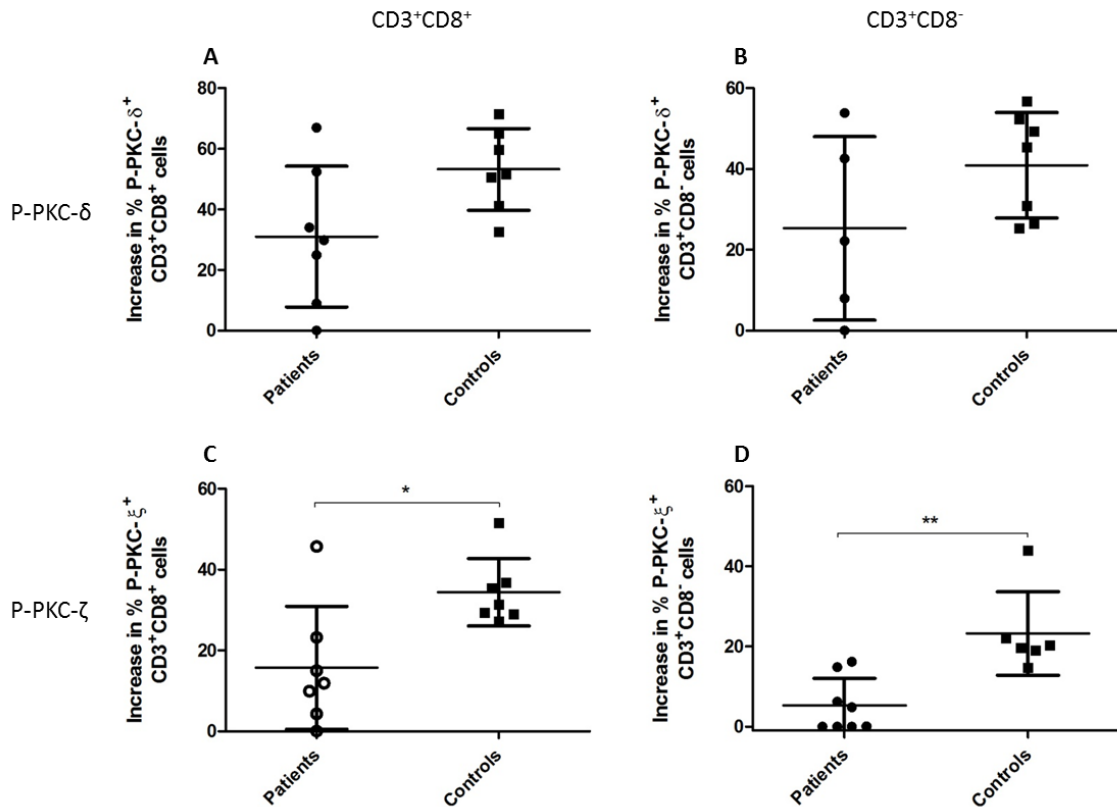
Figure 5 . 34: The increase in the proportion of P-PKC-ζ T-cells is greater in healthy controls than in patients with AD

PBMCs were isolated from patient or healthy control blood and stimulated with 25ng/ml PMA/500ng/ml ionomycin or were left unstimulated. The difference between the proportion of cells expressing P-PKC-δ or P-PKC-ζ detected in the unstimulated cells and after PMA/ionomycin stimulation were calculated and the mean of the duplicates was plotted. The line shows the overall from all patients or all controls ± SD. A) P-PKC-δ in CD3⁺CD8⁺ T-cells, B) P-PKC-δ in CD3⁺CD8⁻ T-cells, C) P-PKC-ζ in CD3⁺CD8⁺ T-cells, D) P-PKC-ζ in CD3⁺CD8⁻ T-cells. n=7

5.3 Discussion

Although there are a number of studies that have suggested the presence of Aβ-specific T-cells in people with and without AD, there is some debate over the role they play. For example, they may be involved in the characteristic inflammation observed in the disease, in the clearance of Aβ from the brain and in the recruitment/influencing the activation of other immune cells. Despite, the evidence that there are T-cells in the brain, relatively few studies have looked at the activation of Aβ-specific T-cells and the studies that have been published do not all agree on the action of Aβ on T-cells.

Despite the published data suggesting that there are Aβ-specific T-cells, in this study there was no evidence that Aβ activated T-cells in either patients with AD or in age-matched controls. Although, both patients and control T-cells were able to respond to the positive control, PHA, suggesting that this lack of Aβ-specific proliferation was not due to an inability of the T-cells to proliferate or a technical issue with the protocols used.

When looking at the proliferative studies of T-cells in response to Aβ there are several inconsistencies in the published data. For example, early studies suggested that there was a decrease in the reactivity of T-cells to Aβ in patients with AD [305]. The investigators postulated that increased exposure to products from APP processing that have an increased concentration in AD may result in T-cell anergy leading to a decrease in responsiveness of T-cells, particularly to antigens that cause a small response [305, 470].The authors also showed that it was not only patients with AD that were unable to respond to the peptide but that a proportion of the young and healthy old people also failed to respond, suggesting it was not a disease

specific response [305] and that there was wide variation between people. Furthermore, the responses that were observed in the young participants were small, the majority of which had a stimulation index above 2 but below 2.5.

In contrast Monsonego et al. performed a similar study and found that there was increased reactivity in patients with AD and the elderly compared with younger adults, although all of the groups investigated showed some proliferation. The explanation hypothesised by the group for the differences in results was: the use of two different isotypes of the Aβ peptide, Aβ1-40 and Aβ1-42, as the Aβ1-42 peptides used by Monsonego et al is known to be more immunogenic [303], and the difference in the protocols used to stimulate the cells. Since this paper was published some other studies have also suggested increased reactivity of T-cells in AD, although this is not just observed in response to Aβ but to a number of different antigens including PHA, PMA and ionomycin [471].

A third study looking at the proliferation of T-cells in response to Aβ showed yet another outcome of Aβ stimulation, in this study no proliferation was observed in either healthy controls or in patients with AD [306]. The study used the same [^{3}H]thymidine incorporation to measure proliferation of T-cells as had been used by both Trieb and Monsonego although there are no more specific details given about the protocols used [303, 305]. Although the Aβ1-40 peptide was used which is likely to produce a smaller response than the Aβ1-42 peptide, a number of overlapping epitopes of the peptide were also used, none of which produced a response. In addition to the lack of proliferation in response to the peptides, there was also no evidence of cytokine production after ELISPOT was performed further suggesting the peptide had not activated T-cells.

One further study that looking into T-cell activation showed that there were no responses from healthy adults and only small responses in patients with AD [304], moreover the study also observed that Aβ had an enhancing effect on the proliferation of T-cells in response to other antigens. For example anti-CD3 stimulation with the addition of Aβ peptide produced more T-cell proliferation than anti-CD3 alone.

Combined all the data from these papers shows the inconsistencies in generating a Aβ specific T-cell response from both patients with AD and healthy controls, the differences in protocols used or the peptides used could explain these differences and it is therefore not altogether surprising that a different response was also observed in this study, particularly as one of the study also reported that no response was seen.

In addition to the differences observed in different investigations, a study by Zoto et al highlighted the importance of HLA type in determining whether a response was observed to the Aβ peptides. It was shown that there were specific HLA-DR alleles that were required for the proliferation of Aβ-specific T-cells and although several alleles were tested there were two dominant alleles that were involved in Aβ-specific T-cell responses, these were: HLA-DRB1*1501 and 0401. The investigation showed that T-cells bearing the HLA-DRB1*1101, 0801 and 0102 but not 1501 or 0401 all failed to proliferate in response to the peptide. The conclusions from the study emphasised the importance of these dominant alleles were in generating specific Aβ responses [469].

Although the patients in this study were not HLA-typed it is unlikely that none of the patients or controls expressed either of these alleles. Therefore, while the HLA-DR

type may influence the results there may have been other factors affecting T-cell activation that would result in the lack of Aβ-specific responses.

One observation about the literature was that in the majority of cases proliferative T-cell responses to the Aβ peptides were fairly low, for example Jozwik et al reported less than 2 % of T-cells proliferated in response to Aβ, although this was said to be a significant number it is still far lower than proliferation in response to other antigens [304]. Further to this, as mentioned before the Trieb et al paper showed that most of the responses observed had a stimulation index less than 2.5 suggesting that T-cells react weakly to the Aβ peptides [305]. It could therefore be possible that with the protocols used in this study the signal was too small to be detected. This was believed to be a potential problem in measuring Aβ-specific T-cell proliferation, therefore IFNα was included in the experiments as it has been shown to reduce non-specific background proliferation and is believed to make it possible to measure the smallest proliferative responses. However, even with the addition of IFNα to the experiment and the subsequent reduction in background proliferation it was still not possible to measure a response to the Aβ peptides. This suggests that the Aβ peptides were not causing a T-cell response.

Further evidence for the role of T-cells in AD has been seen in experiments examining immunisation with Aβ. In the brain there was evidence of a T-cell response to the peptides, however in some patients this was excessive and lead to the development of meningoencephalitis and the stopping of clinical trials. It is believed that Aβ immunisation leads to the clearance of Aβ in the brains and the infiltration and activation of T-cells [296]. The effect of immunisation on the periphery in transgenic mouse models of AD have also been performed. Immunisation elicited an

Aβ antibody response and peripheral T-cell response. Due to the high IgG titres seen it suggested a Th2 response, further supported by the detection of IL-4 and IL-10 and a decrease in the secretion of INFγ [285, 472, 473]. In contrast to this further mouse model investigations suggested that a Th1 response is elicited by Aβ immunisation [474]. Although this adds to the evidence that there are T-cell specific responses to Aβ there is still more research that needs to be performed to determine more specifically what occurs in humans. In addition, it has been suggested that the immunisation with Aβ induces the activation of microglia, which can have a phagocytic role but also an antigen presentation role and therefore contribute to inflammation [475]. It has been suggested that activation of microglia can lead to presentation of Aβ and B7-dependent proliferation. It is possible that this would also be true for monocytes, if these cells were not activated by the non-fibrillar forms of Aβ used in these experiments that it was not possible to induce the proliferation of T-cells [285, 303].

It is well known that Aβ is a difficult peptide to synthesise and that there can be batch to batch differences in the peptide. The published data looking at the differences have focused mainly on the aggregative properties of the peptides however it is also believed that different batches can lead to differences in the biological activity of the peptide [405, 406, 476, 477]. This was observed in this study where two different batches of peptide from the same source caused different responses i.e. one peptide caused a response from PBMCs whereas the second batch did not. There are very few publications that have addressed the issue of batch-to-batch differences in responses to Aβ, however, as shown in this study using different batches of peptide can result in different biological responses. It is therefore possible that one of the reasons for such variance in T-cell responses to Aβ could be due to

differences in the peptides used and may explain why no T-cell responses were observed in this study.

Although one of original aims was to compare the proliferation of T-cells in response to unmodified Aβ as well as its PTM variants, it was decided to use only the unmodified one to determine what conditions were needed to detect the proliferation or the PKC-δ and ζ phosphorylation. If a response had been seen the variants would have been tested to determine if there were any differences. However, as these responses were not observed under any of the conditions used it was decided not to proceed with the PTM peptides.

6. Exploring differences between batches of synthetic peptide exhibiting different biological effects.

6.1 Introduction

The use of synthetic peptides is a well-established practice in biological experimentation, different methods can be used to synthesise a huge variety of peptides including insulin and Aβ but one of the most commonly used is solid-phase peptide synthesis (SPPS). Although there have been many advances in the techniques used to produce these peptides, making pure peptides or proteins with a high yield can be a problem and some peptides cause more problems than others. SPPS is a technique that uses a resin to form the peptide from the C-terminal to the N-terminal of the peptide. However there are a number of steps involved in the process that can lead to formation of truncated peptides that contaminate the overall yield of the peptide [361].

One part of the process that is crucial to synthesise the correct peptide is known as deprotection. In order to prevent the build-up of side chains in the peptide, each amino acid that is added to the peptide chain is protected, however for the peptide to be 'pure' the protective groups need to be removed. Trifluoroacetic acid (TFA) is one of the most popular reagents for deprotection. The combinations of reagents may differ depending on the peptide being synthesised and complications that arise in the deprotection step can cause problems with the generation of the peptide [361] .

The common problems that occur in the synthesis of peptides has led to the term 'difficult sequence phenomenon' being used to describe the peptides [379]. There are a number of peptide properties that have been observed to cause difficulties in peptide synthesis for example, long amino acid chains, which is a particular problem

with proteins. Another property that has been observed to cause problems is hydrophobicity. The hydrophobic nature of such peptides prevents them from being efficiently solvated whilst attached to the solid resin and causes incomplete N-amino acid acylation and deprotection. This leads to the build-up of side reactions leading to reduced length and branching of the peptide [379, 440, 469].

In addition to hydrophobicity a property of a number of peptides that is thought to underlie poor synthesis is the ability to aggregate. The aggregation can occur whilst the peptide is bound to the resin support, and is thought to be due to the secondary β-sheet structure of peptides. This makes amyloid peptides particularly difficult to effectively synthesise and there are a number of reports of batch to batch discrepancies when purchasing synthetic Aβ [379, 440, 469, 478].

The problems that occur during the synthesis could potentially cause differences in the actions of the peptides. This part of the study aimed to understand the absence of effects seen with the use of the second batch of Aβ and its PTM variants. This includes the aggregation of the peptides, the immune responses observed in patients with AD and age matched controls and mass spectrometry of the two batches.

6.2 Results

Several experiments were performed using a second batch of peptides, the first of which was to check that they were able to aggregate to form fibrils like those seen with the previous batch. The peptides were all prepared using preparation protocol 3. They were all diluted to 100μM and allowed to aggregate for 216h at 4°C. After this time, the Aβ peptides had all formed fibrils with no evidence of any intermediate species of the peptides in any of the images. This was the same for the original batch of peptides which, as described previously, all had a fibrillar morphology after 216h of incubation. Figure 6.1 shows a representative image of the morphology of each of the peptides from each of the batches. The images shown for the original batch are the same as the images showed earlier in the chapter 3 of this thesis (Fig 3.6).
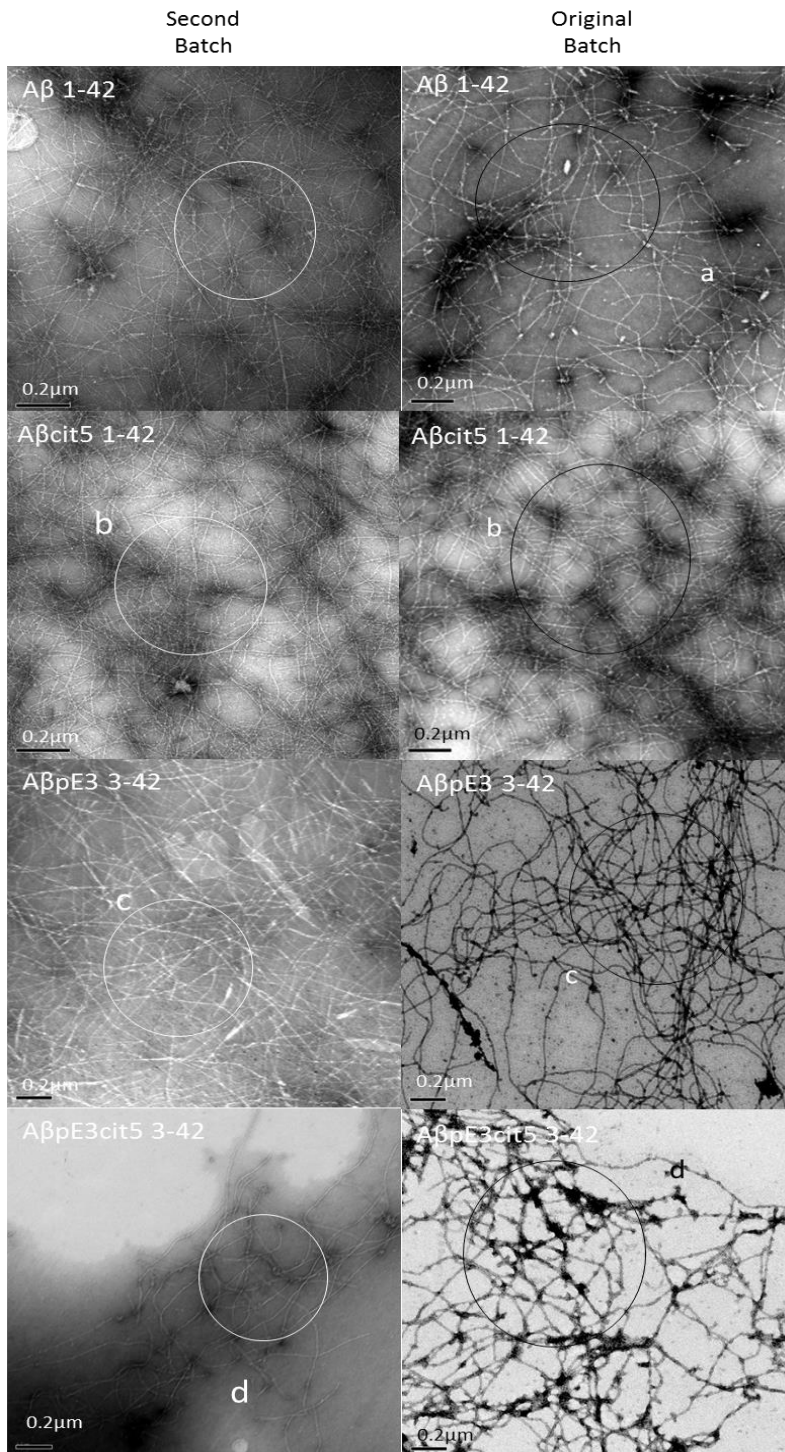
Figure 6 . 1: Aβ peptides from the second and original batch aggregate to form fibrils

Peptides from both batches were disaggregated and dissolved in tissue culture grade water and allowed to aggregate for 216h at 4°C. TEM grids were prepared for each of the peptides and images were generated for each of them. Displayed are representative images of each of the peptides.

As the peptides appeared to aggregate normally they were used to stimulate PBMCs from patients with AD and age-matched controls. The PBMCs were isolated from heparinised blood samples, and plated in 96-well plates. They were then stimulated with 30μM fibrillar Aβ peptide for 18h and the supernatants were collected for IL-1β and TNFα ELISAs. These were identical experiments to the ones performed with the original batch of peptide. However, unlike with the original batch of peptide, there was no significant release of IL-1β (Fig 6.2) or TNFα (Fig 6.3) from either patients or controls, although a response can be seen to the positive control. In contrast, as described previously all the peptides from the original batch were able to induce the release of IL-1β but not TNFα from patients and controls. The figures displaying the original batch data can be seen in chapter 4 (Fig 4.20 and 4.21).

In addition to the ELISAs performed on the supernatants an MTT assay was also carried out to determine if there was any reduction in cell viability. If the peptide was toxic to the cells it would provide an explanation for why there was no cytokine production. The activity of the cells was measured and presented as a comparison to the unstimulated cells. In all cases, whether stimulated with peptide or positive control the cellular activity was at least 90% of the unstimulated control (Fig 6.4).
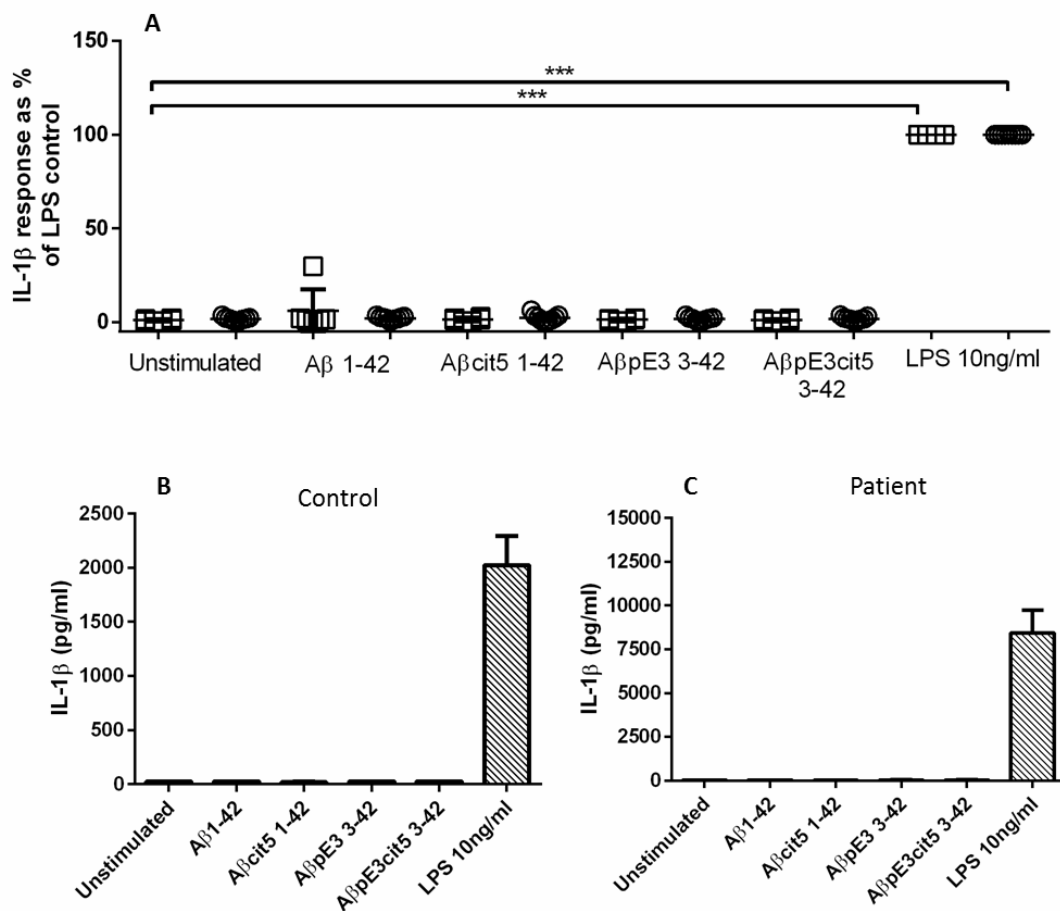
Figure 6 . 2: PBMCs from patients with AD and age-matched controls do not release IL-1β in response to Aβ peptides from the second batch

PBMCs were isolated from patients with AD and age-matched controls. Peptides were disaggregated and diluted in TC grade water, they were allowed to aggregate for 216h at 4°C. PBMCs were stimulated with 30μM peptide for 18h, cell supernatants were collected and an IL-1β ELISA was performed. A) IL-1β produced by patient PBMCs as a percentage of LPS response, B) IL-1β produced by control PBMCs as a percentage of LPS response. C) Representative IL-1β production from patients, D) representative IL-1β production from controls. Unstimulated cells were used as the negative control and 10ng/ml LPS was used as the positive control. Each point represents the mean of triplicates from one participant. The lines show the overall mean ± SD. n=5. $p<0.05$*, $p<0.01$** and $p<0.001$***.

Figure 6 . 3: PBMCs from patients with AD and age-matched controls do not release TNFα in response to Aβ peptides from the second batch

PBMCs were isolated from patients with AD and age-matched controls. Peptides were disaggregated and diluted in TC grade water, they were allowed to aggregate for 216h at 4°C. PBMCs were stimulated with 30μM peptide for 18h, cell supernatants were collected and an IL-1β ELISA was performed. A) IL-1β produced by patient PBMCs as a percentage of LPS response, B) IL-1β produced by control PBMCs as a percentage of LPS response. C) Representative IL-1β production from patients, D) representative IL-1β production from controls. Unstimulated cells were used as the negative control and 10ng/ml LPS was used as the positive control. Each point represents the mean of triplicates from one participant. The lines show the overall mean ± SD. n=5. p<0.05*, p<0.01** and p<0.001***.

Figure 6 . 4: Aβ peptides from the second batch do not reduce cell viability

PBMCs were isolated from patients with AD or from age-matched controls. Peptides were disaggregated and diluted in water for 216h at 4°C to form fibrils. PBMCs were stimulated with 30μM Aβ peptides for 18h, supernatants were removed and an MTT assay was performed on cells. A) Age-matched controls, B) patients with AD. Unstimulated cells were used as the negative control and 10ng/ml LPS was used as the positive control. Cell viability was calculated as the cellular activity of stimulated cells as a percentage of cellular activity of the untreated controls. The means of triplicates from each participant was calculated and plotted is the overall mean ± SD. n=5.

Alongside the ELISA experiments, flow cytometry was also used to measure whether there was any production of intracellular cytokines. This could show whether there were any cytokines produced but not released. The antibody would also detect the inactive pro-IL-1β and therefore could indicate whether TLR was being activated but the cells were not releasing the active form of the cytokine. As monocytes express the greatest number of TLRs these were the main focus of the analysis and the surface markers used reflect this. The cells were first plotted as HLA-DR against FSC-A and the population positive for HLA-DR were gated. The cells that were positive for CD14 but negative for CD3 were then gated as the monocyte population. A quadrant was then added to this monocytic population of cells to show the expression of TLR2 and TLR4. The vast majority, 85%, of the monocytes expressed both TLR2 and TLR4, the proportion of cells expressing intracellular IL-1β and TNFα were then measured from this population of cells. The gating strategy is shown in Fig 6.5.
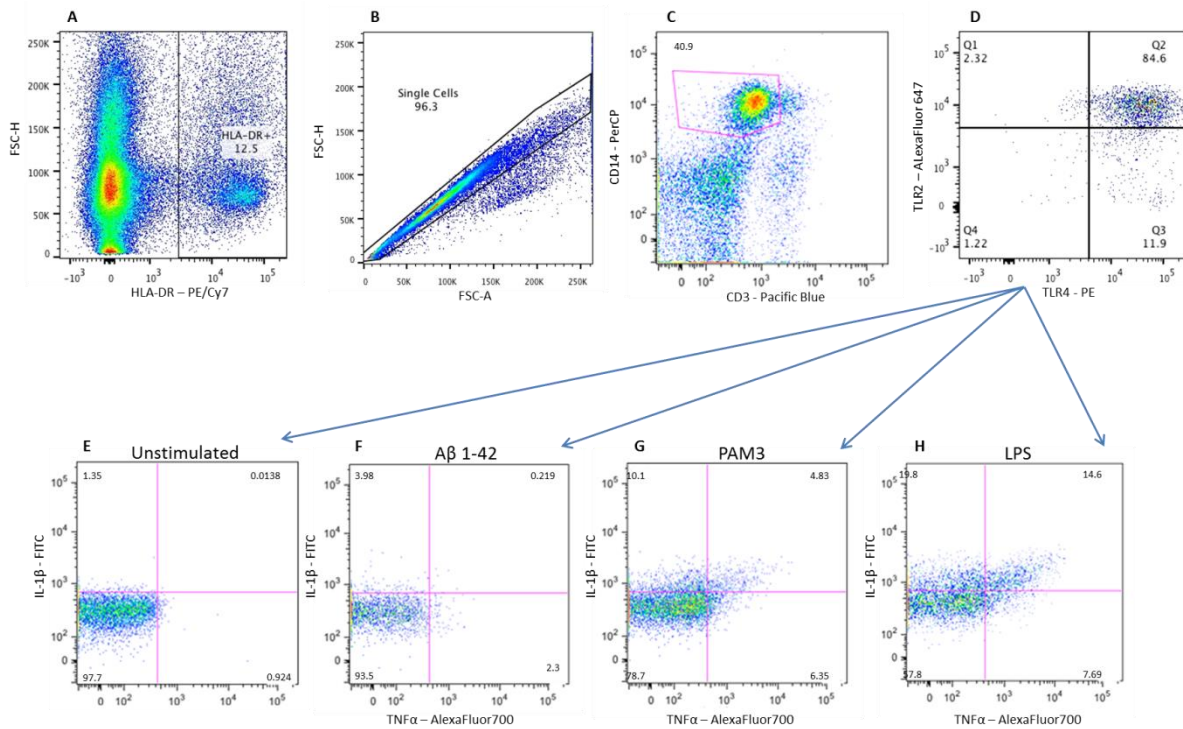
Figure 6 . 5: Gating strategy for CD14$^+$ monocytes

PBMCs were isolated and acquired using LSR-II. A) HLA-DR$^+$ population, B) Single cells, C) CD14$^+$CD3$^-$ population D) TLR4 versus TLR2 – TLR2$^+$TLR4$^+$ population. E) Unstimulated cells, F) peptide stimulated cells, G) PAM3 100ng/ml stimulated cells, and H) LPS 10ng/ml stimulated cells. Black boxes represent the population of interest: IL-1β producing cells.

After peptide stimulation with the second batch of peptide the proportion of cells expressing IL-1β or TNFα were measured. In addition the proportion of cells that expressed IL-1β and TNFα were also measured. Although there were 2 patients that appeared to have a small increase in IL-1β expression the rest of them showed no response to any of the peptides. Furthermore, the mean response from all patients or all controls after peptide stimulation was not significantly different to the unstimulated cells. In age-matched controls, one sample had a very high response to the AβpE3cit5 3-42 peptide, but again there was no significant difference when comparing the means from each peptide stimulation (Fig 6.6A). There was, however, a significant increase in IL-1β in response to PAM3 and LPS stimulation. Again there were two patients that expressed a small increase in the proportion of cells expressing TNFα, this goes from 3% to 15% of cells. All other patients and age-matched controls showed no increase in the proportion of cells expressing TNFα. There was also no increase in response to PAM3 or LPS (Fig 6.6B). One patient and one age matched control also showed an increase in the proportion of cells expression TNFα and IL-1β in response to Aβcit5 1-42, however, the increase was from 2% to 7% for the patient and 1.5% to 8% from the control. There was an increase in the proportion of cells expressing both TNFα and IL-1β after LPS stimulation but not after PAM3 stimulation (Fig 6.6C).
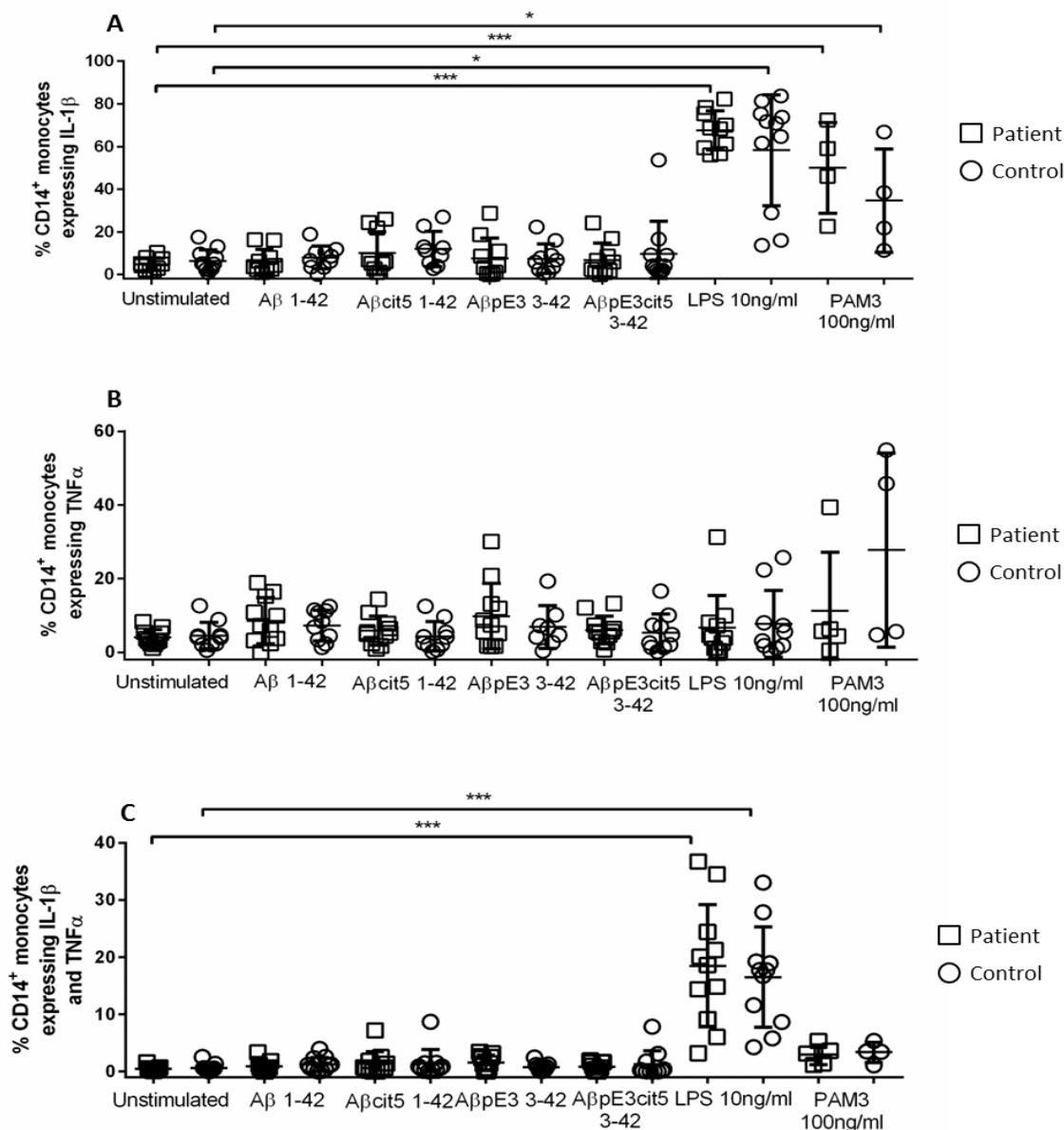
Figure 6 . 6: The second batch of Aβ peptides does not cause an increase in intracellular IL-1β or TNFα

PBMCs from patients with AD and age-matched controls were isolated and stimulated with 30μM Aβ peptides. Peptides were disaggregated and dissolved in TC grade water and allowed to aggregate for 216h before stimulating cells. PBMCs were then stained with surface and intracellular antibodies and acquired using LSR-II flow cytometer. Cells were gated as HLA-DR$^+$CD14$^+$CD3$^-$ monocytes. A) Intracellular IL-1β B) intracellular TNFα, patients C) Double positive IL-1β and TNFα. Patients shown by squares, controls by circles. Each mark represents the mean of duplicates from one participant, the line represents the overall mean ±SD. 10ng/ml LPS and 100ng/ml PAM3 were used as positive controls and unstimulated cells were used as the negative control. n=8.

As a response had been observed to the original batch of peptides but not the second a direct comparison of the intracellular staining was performed to confirm that the two batches had a different effect. The same experiments were performed as described above. As there was not a sufficient amount of the AβpE3cit5 3-42 peptide from the original batch, only Aβ 1-42, Aβcit5 1-42 and AβpE3 3-42 were used.

The gating strategy was as described above (Fig 6.5) and the proportion of cells expressing IL-1β, TNFα or both were measured. As seen in the previous experiments there was no increase in cells expressing IL-1β, TNFα or double positive for both after stimulation with the peptides from the second batch, however when stimulation was performed with the original batch of peptide there was an increase in the proportion of cells expressing IL-1β but there was no increase in either TNFα or double positive cells (Fig 6.7).
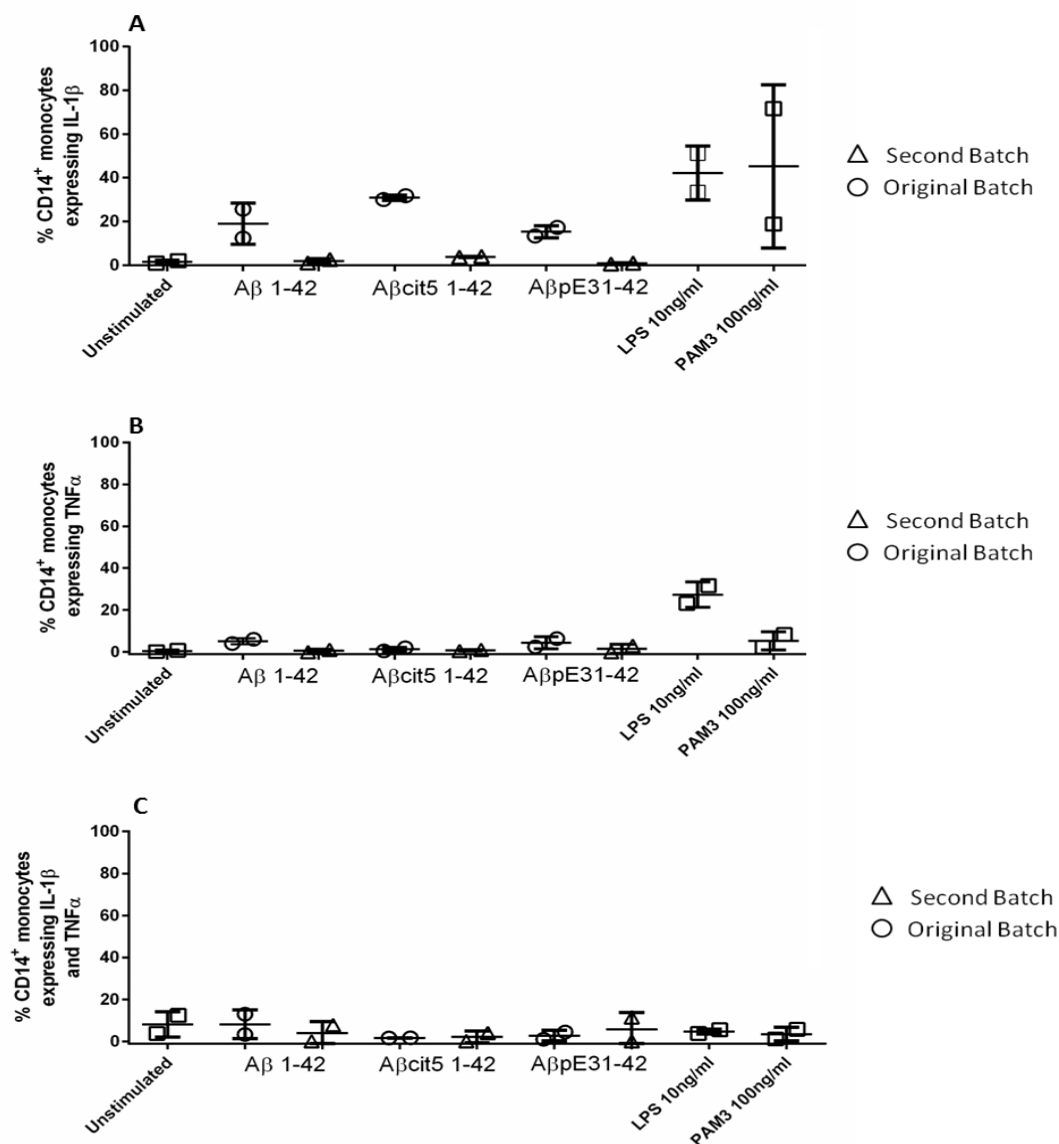
Figure 6 . 7: Comparison of intracellular IL-1β and TNFα from original batch and second batch of peptide in healthy controls

PBMCs were isolated from healthy controls and stimulated with 30μM Aβ 1-42, Aβcit5 1-42 or AβpE3 3-42 peptide. Peptides were disaggregated and dissolved in tissue culture grade water, they were left to aggregate for 216h at 4°C and then used to stimulate PBMCs. Cells were stained with intracellular and surface antibodies and gated as HLA-DR⁺CD14⁺CD3⁻ monocytes. A) Intracellular IL-1β, B) intracellular TNFα, C) double positive. 10ng/ml LPS and 100ng/ml PAM3 were used as positive controls. Unstimulated cells were used as the negative control. Each point represents the mean of duplicates from one participant. The line represents the overall mean ± SD. Circles show the original batch of peptide, triangles show the second batch of peptide and squares show shared controls. n=2 patients, n=2 controls.

To investigate what may be causing the difference in the results between the two batches, mass spectrometry was performed on Aβ 1-42 and Aβcit5 1-42 from each of the batches.

Electrospray ionisation mass spectrometry was used to investigate whether the original batch of peptides were the unmodified Aβ 1-42 and citrulline modified Aβ 1-42. For each of the peptides peaks were observed with masses of 903.5, 1129.1 and 1505.1 for Aβ 1-42 and 903.7, 1129.3 and 1505.4 for Aβcit5 1-42. Analysis of the second batch of peptide was performed using matrix-assisted laser desorption/ionization (MALDI) and liquid chromatography mass spectrometry (LCMS) and kindly performed by JPT peptide technologies in Berlin (Germany). This analysis showed that the main component had a molecular weight of 4519.317 for Aβ 1-42 and 4525.823 for Aβcit5 1-42. The analysis also showed that there were a number of other peaks present with masses of 1010.294m/z, 1397.443m/z, 2778.884m/z and 2935.216m/z. The mass spectrometry of the original batch are shown in Fig.6.8 and 6.9 and from the second batch in 6.10 and 6.11.
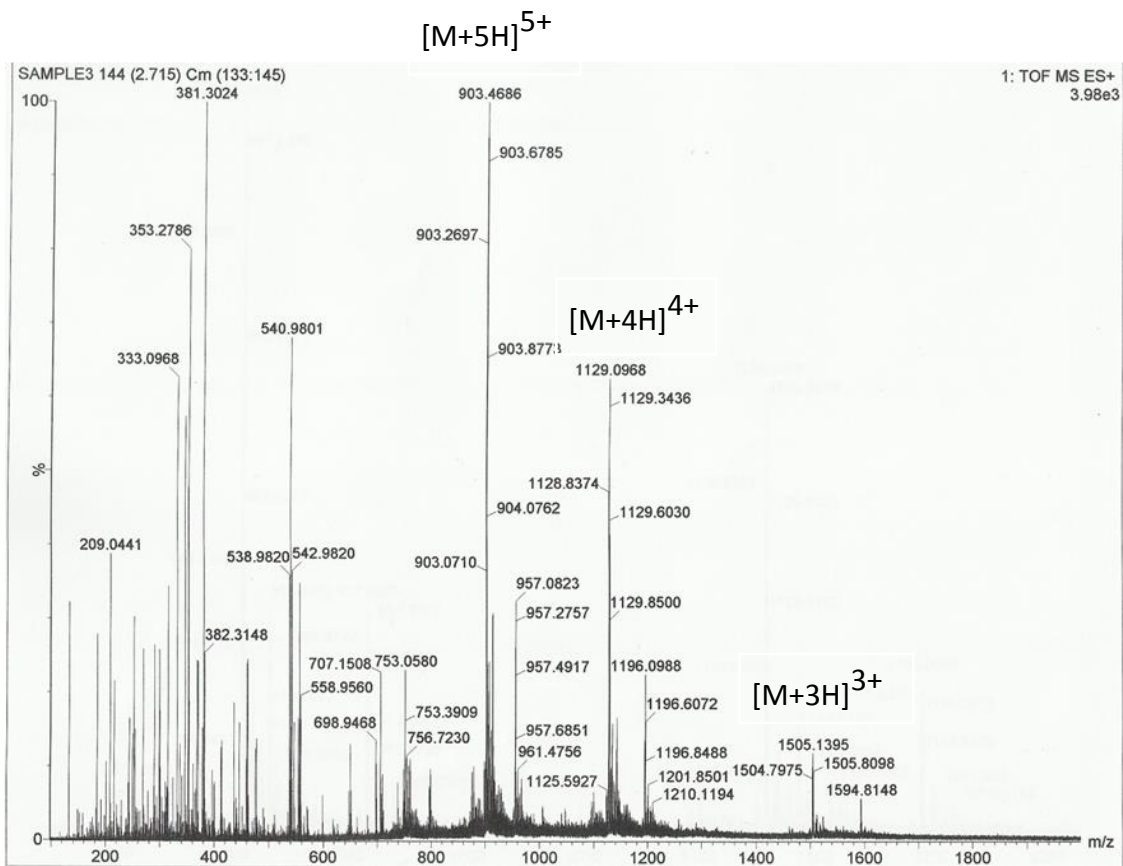
Figure 6 . 8: Electron spray ionisation mass spectrometry spectra of Aβ 1-42 from the original batch of peptides

Mass spectrometry was performed on lyophilised Aβ 1-42 peptide from the original batch of peptide. The relative abundance of each peak is shown as a percentage of the most abundant peak (y-axis). The m/z ratio is shown by the x-axis.
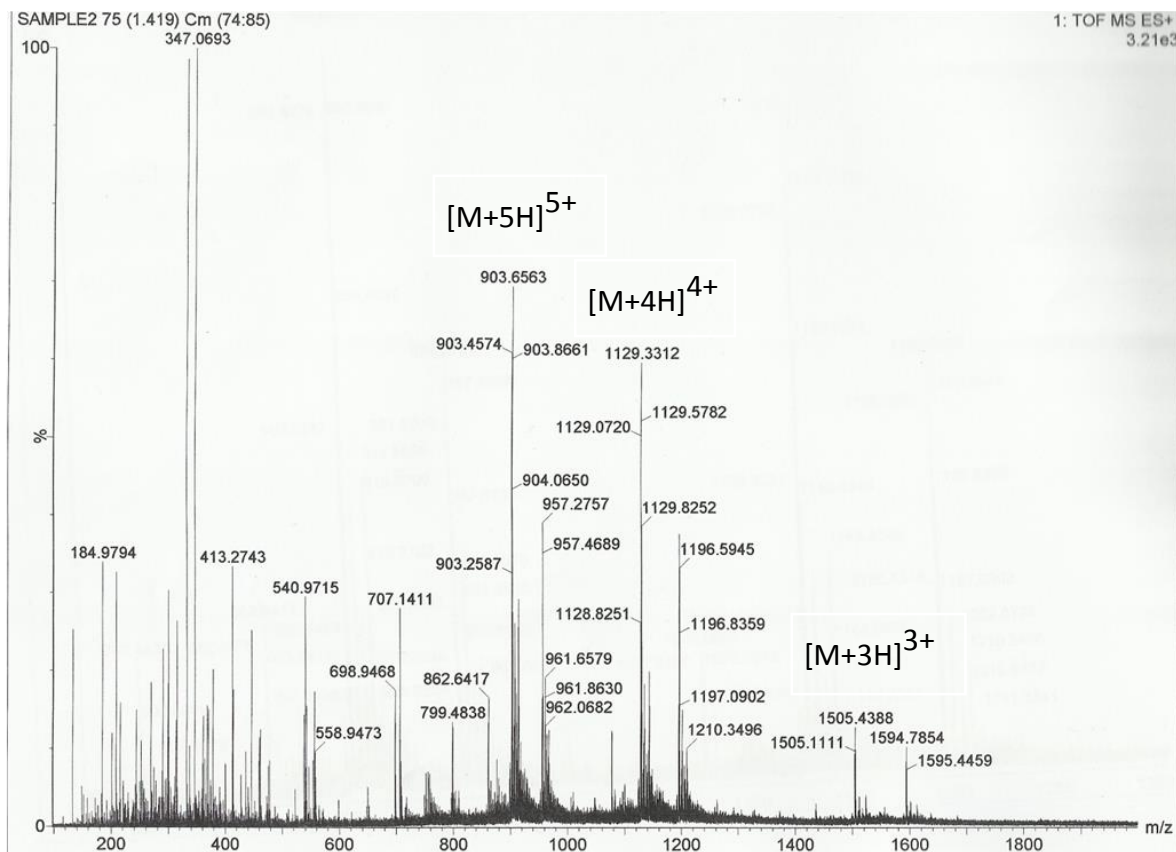
Figure 6 . 9: Electron spray ionisation mass spectrometry spectra of Aβcit5 1-42 from the original batch of peptides

Mass spectrometry was performed on lyophilised Aβcit5 1-42 peptide from the original batch of peptide. The relative abundance of each peak is shown as a percentage of the most abundant peak (y-axis). The m/z ratio is shown by the x-axis.
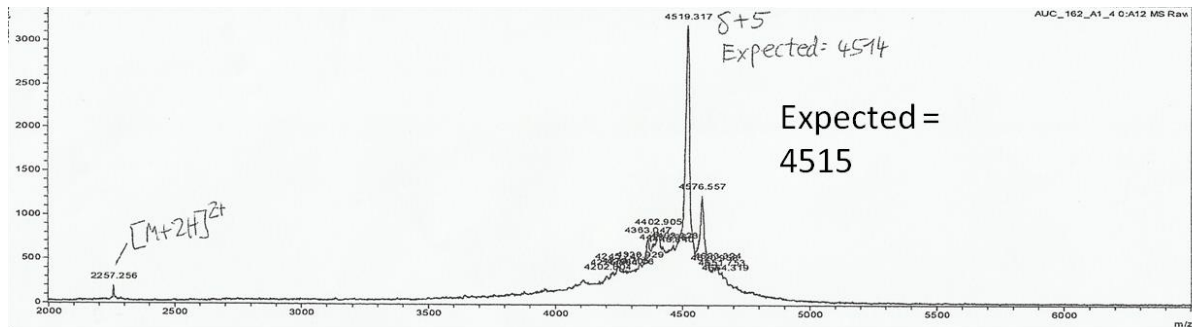
Figure 6 . 10: MALDI analysis of Aβ 1-42 peptide from the second batch

The image shows the molecular weight of the main component found in the sample of Aβ 1-42 peptide from the second batch. The y-axis shows the relative abundance of the different peaks and the x-axis shows the m/z ratio
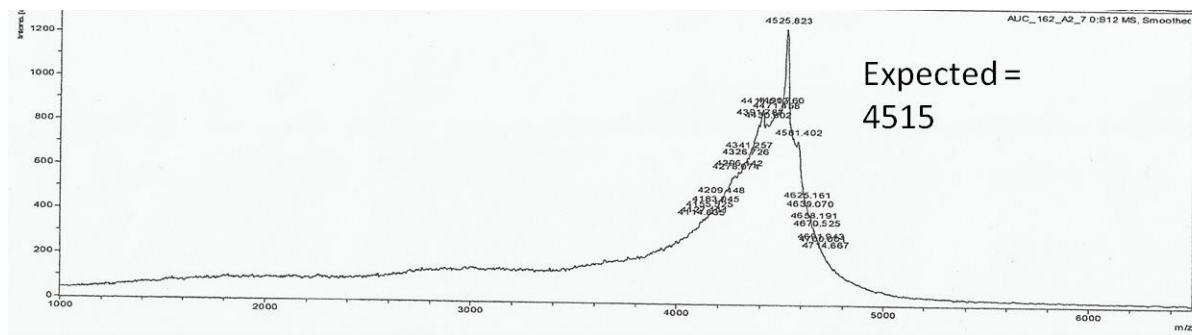
Figure 6 . 11: MALDI analysis of Aβcit5 1-42 peptide from the second batch

The image shows the molecular weight of the main component found in the sample of Aβcit5 1-42 peptide from the second batch. The y-axis shows the relative abundance of the different peaks and the x-axis shows the m/z ratio

6.3 Discussion

It has been reported that there can be differences between batches of Aβ peptide, although published data has focused on the effect that these differences have on aggregation and less so on the effect they may have on other processes, such as their ability to activate the immune system, [404-406, 476, 477]. The results from this study highlight the possibility that different batches of peptide may lead to different biological responses.

The TEM analysis performed on all the peptides from both batches provided evidence for the morphology of the peptides and showed that all of them were able to form fibrils. The images of the fibrils obtained were all very similar which would suggest that there were no significant differences in the morphology of the peptides. As it had been found that the peptide had to have a fibrillar morphology in order to induce a response this was the property of the peptide that was the most important. As the morphology of the peptides appear to be the same, it is possible that this is not the only property of the peptide that allows it to induce an immune responses. TEM does not provide specific information about the molecular structure of the peptide fibrils. It is possible that there may have been differences in the molecular structure of the two batches leading to the differences in response.

However, despite the similarities in the aggregative properties of the peptide there were a number of differences observed between the two batches. Firstly all the original batch of peptide caused the release of IL-1β from PBMCs whereas the second batch did not, this result suggests that there is a difference in the way the peptides were able to act on immune cells. Using flow cytometry it was determined that the IL-1β was being produced by monocytes, as expected, however, when the

same experiments were performed with the second batch of peptide this was not the case.

An MTT assay was performed to ensure that the peptides were not reducing the cell viability of the PBMCs and therefore preventing the production of any cytokines, however the results showed that the viability was not reduced suggesting that the second batch of peptide was simply unable to activate PBMCs.

One explanation for the difference between the batches could have been a contamination in the original batch of peptide, as this would cause a signal even if the peptide was not able to. Although contamination is more likely to occur in recombinant peptides, the techniques in peptide synthesis are non-sterile so there is a possibility that contaminations could occur and despite every effort being made to keep peptide preparations sterile it is not possible to entirely rule out that endotoxin contamination could occur [417, 479, 480]. However, if there was a contamination present that was able to activate either TLR2 or TLR4 a response would have been observed after stimulation of isolated primary human monocytes. In addition to no response from isolated monocytes there was also no response from HEK293 expressing TLR4. This would suggest that the most common endotoxin contamination, from LPS, was not present in the peptide. Furthermore, as all the peptides in the original batch caused a response but none of the peptides in the second batch were able to it would be more likely that another factor, such as the synthesis process, was responsible. All together it appears unlikely that it was a contamination in the original batch of peptide that was responsible for the immune responses observed.

Mass spectrometry was performed on the Aβ 1-42 and Aβcit5 1-42 peptide from both of the batches, the analysis showed that the main component in each of the samples was the expected peptide. Different techniques were used to look at the two batches, however, both techniques are able to provide evidence about each of the peptides. The expected molecular weight of the unmodified Aβ 1-42 peptide was 4515.04 [64]. The analysis of the second batch by JPT showed that 4515 was the molecular weight of the main component of the peptide. The same was true for the Aβcit5 1-42 peptide, the JPT analysis showed that the main peptide was Aβ 1-42 with a citrulline 5 modification.

The analysis performed on the original batch of Aβ 1-42 and Aβcit5 1-42 showed three specific peaks that represent three different charged ions from the peptide. These three peaks correspond to the ions: $[M+3H]^{3+}$, $[M+4H]^{4+}$ and $[M+5H]^{5+}$ which are associated with peaks at 1505m/z, 1129m/z and 903m/z. From these peaks it is possible to determine the molecular weight of the peptides, which were calculated to be the expected mass, 4514.04 for Aβ 1-42 and 4515.02 for Aβcit5 1-42 [64, 481-483]. In addition to the peaks that correspond to peaks associated with Aβ 1-42, there were also some peaks with values of 353.3 and 381.3 in the Aβ 1-42 peptides and 347.1 and 413.3 in the Aβcit5 1-42 peptide. These are not peaks associated with Aβ. It is possible that the two peaks in the Aβ 1-42 peptide are due to the presence of the detergent Triton [484]. This is most likely left over from a step in the preparation of the peptide, as it is often used to promote coupling and to disrupt β-sheets in peptide synthesis, however it is unlikely to be responsible for the immune responses observed as they would have been present in all the experiments not just the one that involved fibrillar Aβ 1-42 peptides [485].

In the Aβcit5 1-42 the two peaks that are present are the peaks for polypropolene glycol ubiquitous polyether, a common solution for the tuning of mass spectrometry equipment [486]. Furthermore, the second peak is caused by Diisoocytyl phthalate plasticiser which is a common unwanted peak in mass spectrometry analysis and is cause by vaccum O-rings used in electrospray mass spectrometry [484]. Neither of these peaks would have affected the immune response results as they were not contaminants in the peptide batch but in the machine.

The mass spectrometry analysis was performed to determine whether there were any differences within the actual peptides that could explain why different results were obtained. However, the analysis of the results by experts, in peptides Dr Karsten Schnatbaum (JPT) and Alaa Abdul Sada (University of Sussex), did not highlight any major differences between the two batches, suggesting that it was full length Aβ1-42 peptide that was present in the both batches.

Despite the fact that the main component within the peptides is the expected peptide, the analysis of the peptide also shows broad peaks, suggesting the peptide is not homogenous [487]. Broad peaks such as these have been seen when Aβ was purified from cerebral cortices [487]. This could suggest that there are a number of different peptides present with molecular weight between 4050 and 4700. Another analysis of Aβ peptides found in different regions of brain suggest there are a variety of peptides that are found within this range including Aβ1-40, Aβ3-42, Aβ4-42, Aβ5-52 and Aβ2-42 [64, 488]. It is therefore possible that in addition to the full length Aβ 1-42/AβCit5 1-42 there are a variety of shorter fragments of the peptide that could influence the immune responses between batches. Input from Karsten Schnatbaum suggested that the peptides Aβ1-25, Aβ1-23, Aβ24-42 and Aβ26-42 were also present in the peptide batch. The truncation of these peptides is potentially due to the

synthesis method used, SPPS. As the peptides are synthesised from the C-terminal to the N-terminal, it is possible that the last few amino acids at the N-terminal end are not always added to the full peptide, one part of the process of this type of peptide synthesis is known as deprotection and is necessary to ensure that peptides form the full length peptide without any side chains, however this process is more difficult to perform with hydrophobic peptides, such as Aβ, increasing the number of short peptide fragments. In addition, amyloid peptides that aggregate also cause problems with peptide synthesis contributing to an increase in truncated peptides. It is likely that there were a number of these short peptides in both of the batches, however there may have been more peptide fragments in the second batch that could have contributed to the differences observed [379, 440, 469, 478].

While it remains unclear why the two batches of peptides caused such different responses, there were very obvious differences between them. The results support the idea that batch-batch differences can arise in Aβ peptides and can have quite dramatic effects on the responses observed, in this case in immune responses.

7. General Conclusions

Overall, the aims of this study were to look at immune responses to Aβ peptide and variants containing PTMs. It was designed to look at whether there were differences between unmodified Aβ 1-42 and altered versions of the peptide in their ability to activate both the adaptive and innate immune system. This was done by investigating the activation of T-cells through TCRs and their engagement with TLR2 and TLR4. Furthermore, interactions between the adaptive and innate immune system in response to these peptides were also investigated.

The presence of post-translationally modified Aβ peptides in the brains of people with AD is now well known and it is believed there are a wide range of modifications that can occur, including oxidation, phosphorylation and glycosylation [59]. The most predominant Aβ modification observed in AD is a pyroglutamate modification, AβpE3/11-42, this is one of the most researched Aβ modifications and the studies that have been performed, in the large part, have been investigations into the aggregation and deposition of the peptide [59, 64, 65, 72, 74, 225, 361, 385, 489, 490]. However, there are also studies looking at the toxicity of the peptide as well as its effects on cognition, [73, 75, 109, 217, 491-496] in addition to a study showing the effect that AβpE3 has on phagocytosis [66]. Further to the modifications mentioned, it has recently been observed that there is a citrullinated form of Aβ found in the brains of people with AD [60, 61, 69]. However, to date there are no published studies that have looked at the effect of this modification on the properties of the peptide.

It has been reported that immune responses are observed to Aβ peptide, however almost all of these have used unmodified forms of the peptide, usually Aβ 1-40 or 1-

42 [274-276, 279, 280, 283, 284, 376, 497]. However, as there is such an abundance of modified Aβ peptides in AD brains it is important that differences between the 'wild-type' and the PTM variants also be explored. In other diseases, such as rheumatoid arthritis, PTMs, specifically a citrulline modification, has been shown to alter immune responses to peptides [498, 499]. This would suggest that the same modification in Aβ could also alter immune responses in AD.

In this study TEM was used to look at the four peptides being investigated and mainly provided information about the morphology of the peptide. The results obtained suggested that the final structure of the mature peptide fibrils and the aggregation process was the same. Although there were also some differences observed between the peptides. The main difference observed was the time at which the more aggregated species of Aβ appeared, all of the modifications appeared to reduce the time needed for aggregated peptide to be present but this was most significant for AβpE3 1-42. These results were supported by unpublished data, using the same batch of peptide, that utilised a variety of techniques to investigate differences in aggregation kinetics and propensity and showed that although the structure of the peptides was the same the propensity of the modified peptides, particularly AβpE3 1-42, to aggregate was increased (manuscript in preparation).

The morphology of the peptide has been proposed to be important in inducing immune responses, specifically in innate immune responses [435]. A large body of research has postulated that in order for Aβ 1-42 to activate receptors such as TLRs [274-276], the peptide requires a fibrillar morphology, however there are also studies that have observed that Aβ oligomers, the most toxic form of the peptide, cause inflammation through PRRs [284]. In this study, all the responses that were observed were after stimulation with fibrillar forms of the peptides. Initially the timecourse

experiment showed activation of TLR2 expressed on HEK293 cells when fibrillar peptide was used but not when the peptide had an oligomeric or protofibrillar morphology. Furthermore, PBMC stimulation by the peptide only resulted in IL-1β production when fibrillar peptides were used. An Aβ peptide unable to form fibrils, confirmed by TEM, was unable to produce the same response.

The experiments carried out in this study have supported published data showing that Aβ can activate TLR2 and has provided evidence that the PTM variants of the peptides used are able to act in the same way. It has been proposed that the quaternary β-sheet structure of fibrillar Aβ peptide is how it is able to activate TLRs. This has been observed in a number of amyloid proteins, in addition to Aβ, that all contain a β-sheet structure, supporting the importance of peptide morphology in TLR activation [416, 500-502]. Considering that the final structure of fibrils of the unmodified Aβ is the same as the three PTM variants, they will all share the same β-sheet structure suggesting that they would all activate TLR2 in the same way. Even though the data from this study suggests that there may be another mechanism contributing to the actions of Aβ, the similarities in the fibrillar structure of the peptide could still explain why there were no differences in the responses to the modified forms of the peptide, in regards to TLR2. This would also suggest that modified peptides found in the brains of people with AD may contribute to the inflammation observed through the same mechanisms as the unmodified peptide. However, as it has been suggested that AβpE3 1-42 has an increased propensity to aggregate, it stands to reason that this form of the peptide would form aggregates earlier. In fact it has been reported that it starts accumulating earlier in AD than the 'wild-type' form and has been proposed to be the seeding species leading to an increase in the deposition of pathological aggregates [65, 503]. The increase in

deposition of Aβ aggregates and the increased propensity of the peptide to aggregate would suggest that aggregated AβpE3 1-42 would be 'available' in the brain for longer and may therefore have an increased contribution to the inflammation that is characteristic of AD [115, 390].

Some of the data obtained in this study points towards the involvement of TLR2 in the recognition of Aβ, however, it also suggests that it is not mediated entirely through this receptor. HEK293 expressing TLR2 but not TLR4 were activated by fibrillar Aβ as well as the fibrillar forms of all the PTM variants tested, however when the peptide was tested on isolated monocytes no response was observed. This suggested that in primary human cells there was no response through TLRs. Despite published data suggesting that the concentration used in the experiment was sufficient to induce a response [278, 504, 505], it is still possible that the concentration of the peptide was not enough to produce detectable levels of either IL-1β or TNFα from isolated monocytes. The role of TLR2 in Aβ recognition was supported in part by the use of the neutralising antibody for TLR2, on PBMCs; the effect of the antibody was, however, dependent on the donor tested. The fact that some donors showed a reduction in the proportion of cells expressing IL-1β after the addition of the antibody does suggest that TLR2 is involved but on the other hand that not all donors showed this reduction suggests the involvement other mechanisms too.

One such involvement could be the interaction of T-cells with monocytes. As no response was seen in isolated monocyte cultures but was observed in monocytes as part of PBMCs, it is possible that the presence of T-cells in the culture influences the activation of monocytes. It has already been shown that monocytes in PBMCs are able to produce more inflammatory cytokines, like IL-1β, in response to LPS

stimulation than purified monocytes [422]. As LPS induction of IL-1β occurs predominantly through TLR4 on monocytes, the paper suggested that T-cell interaction can amplify the effect LPS has on monocytic production of cytokines [422]. The effect was particularly relevant to IL-1β and had a greater effect in aging donors. Based on this research it is possible that the action of Aβ through TLR2 is amplified by the presence of T-cells and could also explain why a signal was seen in monocytes in PBMCs but not in purified monocytes (if the signal was too small to detect with the concentration used).

When looking at Aβ activation of T-cells through TCRs, the majority of the work was performed with unaggregated forms of the peptide, this was because we were working under the assumption that the peptide needed to be processed and presented by APCs to the T-cells [303, 308, 309]. Groups such as Monsonego et al, specifically tested the peptide to ensure that it would not form fibrils for the duration of experiment highlighting the importance of using non-aggregated forms of the peptide [303]. Although there is evidence that Aβ can be internalised by APCs, it has also been proposed that large fibrillar structures of the peptide undergo processes such as frustrated phagocytosis that results in the ineffective internalisation and digestion of the peptide [438]. In addition, the insoluble, stable structure associated with fibrillar amyloid proteins has also been proposed to be resistant to proteolysis [506], suggesting that fibrils of Aβ would not be effectively processed and therefore are not likely to be presented by MHC molecules and are therefore not able to activate T-cells via TCRs. PTM variants of the Aβ peptide are thought to have different properties, for example the AβpE3 1-42 modified peptide, which is strongly associated with AD, has been observed to be more stable and are therefore more resistant to proteolysis [75, 361], suggesting that it would not just be fibrils of the

unmodified forms of the peptide that are unable to be processed and presented to T-cells. However, in this study there was no evidence of the activation of T-cells by Aβ peptide even when it was used in its non-aggregated form.

Despite no activation of T-cells being observed with unaggregated forms of the peptide, it is possible that fibrillar forms of the peptide may be able to produce a response, although this is unlikely to be through TCRs. As it has been documented that T-cells are able to induce the production of cytokine from monocytes [262, 263, 428, 429, 507, 508] , it is a possible reason that T-cells may be involved. This may suggest that fibrillar Aβ is able to activate T-cells causing the release of cytokines from monocytes, either through amplification of Aβ induced cytokine production or by direct activation. However, we were unable to shown this effect.

It appears that PKCs are involved in a variety of immune responses, including those mediated through TLRs. It is possible that Aβ causes the phosphorylation of PKC through a mechanism that is not necessarily TCR mediated. A fairly large body of evidence suggests a strong link between TLRs and PKC, in particular PKC-α, δ and ζ, these are the three isoforms of PKC that were initially tested by Miscia et al. A number of studies have looked at these three different isoforms in regards to the effect they have on TLR signalling [509-512]. For example, it was shown that activation of p38 MAPK and NF-κB after stimulation with TLR2 and TLR4 ligands was depressed when PKC-δ was downregulated. In addition, studies have shown that LPS stimulation causes the activation of PKC-ζ in macrophages, and that PKC-ζ is directly involved in TLR2 and TLR4 activation of NF-κB.

It is also possible that PKCs may be involved in other areas of innate immunity that do not involve TLRs [511]. PKC-δ is thought to be involved in TNFR1 signalling and neutrophil activation and PKC-ζ in signalling through IL-1R and TNFR [511].

All these reports supported the idea that the phosphorylation of PKC-δ and -ζ observed in previous reports may not be the result of TCR activation but a less specific action on cells, like TLRs that are expressed in low numbers on T-cells.

Overall, this study has confirmed previous reports about the aggregation of unmodified Aβ peptide as well as providing new evidence about the aggregation of Aβ peptides containing specific PTMs. The increased aggregation propensity of the modified peptides could mean that they play an important role in inflammation in AD. The study has also confirmed how important the morphology of the peptides is in inducing innate immune responses and that the similarities in the final structure of all of the peptides investigated may be responsible for the similarities observed in response to them. In addition to data suggesting that TLR2 does play a role in Aβ recognition it also appears that it is not the only mechanism involved in triggering inflammation in AD and all four of the peptides are able to cause activation of the same pathways.

8. References

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