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The role of T cells in the pathogenesis and prevention of the inflammatory and neurodegenerative changes associated with Alzheimer's disease

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A thesis submitted to

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for the degree of

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School of Biochemistry and Immunology
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2009

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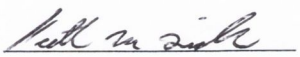

Keith Mc Quillan

15th July 2009

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease, which is characterised by deposition of amyloid-beta (Aβ)-containing plaques and also by neuroinflammatory changes, typified by an increase in proinflammatory cytokine expression in the brain. Activated microglia are considered to be the major source of proinflammatory cytokines in AD, and recent studies have highlighted the importance of adaptive immune responses, in particular T cells, in the pathology of this disease. Clinical trials involving active immunisation with Aβ in adjuvants resulted in development of meningoencephalitis in a subset of patients, which was associated with increased infiltration of T cells into the brain. Furthermore, Aβ-specific T cells have been identified in AD patients.

In this study, *in vivo* and *in vitro* strategies were developed for the generation of Aβ-specific Th1, Th2, and Th17 cells, and these Aβ-specific T cell subtypes were examined for their modulatory effect on Aβ-induced glial activation. It was demonstrated that Aβ promoted glial production of IL-1β, IL-6, and TNF-α, and increased expression of CD80, CD86, and CD40 on microglia. Immunisation with Aβ and CpG followed by a boost with Aβ in incomplete Freund's adjuvant (IFA) induced a predominantly Th1 response to Aβ, while immunisation with Aβ in complete Freund's adjuvant (CFA) followed a boost with Aβ in IFA induced a mixed Th1/Th17 response. Treatment of Aβ-stimulated glia with Aβ-specific T cells enhanced Aβ-induced IL-6 and TNF-α production, as well as microglial expression of MHC class II and CD86. However, there was little difference in glial activation induced by T cells from these different immunisation protocols.

Aβ-specific Th1, Th2, and Th17 cell lines were developed by *in vitro* polarisation of T cells from mice immunised with Aβ and CpG. Co-culture of Aβ-specific Th1 or Th17 cells with glia resulted in increased production of IL-1β, IL-6, and TNF-α, and enhanced expression of MHC class II, CD40, CD80, and CD86 on microglia. Aβ-specific Th2 cells had no effect on Aβ-induced proinflammatory cytokine production from glia, but they enhanced Aβ-induced MHC class II, CD80, CD86, and CD40 expression on microglia. Aβ-specific Th2 cells inhibited IFN-γ and IL-17 production by Th1 and Th17 cells respectively, and they also attenuated IL-1β and IL-6 production induced in co-cultures of glia and Th17 cells. IL-17 enhanced Aβ-induced IL-6 production from mixed glia, while IFN-γ increased Aβ-induced IL-6 and TNF-α production, as well as Aβ-induced CD80, CD86, and CD40 expression on microglia. IL-4 and IL-10 were found to attenuate Aβ and IFN-γ-induced increases in glial proinflammatory cytokine expression, and co-stimulatory molecule expression on microglia.

Activated T cells are capable of crossing the blood-brain barrier (BBB) and entering the CNS, and recent experiments have uncovered links between T cell activation and the development of AD. APPswe/PS1dE9 transgenic mice had increased Aβ burden in the brain and blood, which was not associated with impairments in spatial memory. Transfer of Aβ-specific Th1 cells induced impairments in cognitive function and increased Aβ levels in the brains of transgenic mice. Aβ-specific Th2 cells had no effect on either cognitive function or Aβ levels. Transfer of Aβ-specific Th17 cells reduced the concentration of soluble $A\beta_{40}$ in the hippocampus of APPswe/PS1dE9 mice, but had no effect on spatial memory.

The results presented in this thesis have demonstrated that Aβ-specific T cells differentially modulate Aβ-induced microglial activation. In addition, transfer of Aβ-specific Th1 cells increased Aβ burden and induced cognitive impairment in a transgenic mouse model of AD. Additional research is required to determine whether these T cells play a role in the development of AD.

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Abbreviations

Aβ	Amyloid-beta
AD	Alzheimer's disease
APC	Antigen-presenting cell
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
APPswe	APP gene containing the "Swedish" mutation
BACE	β-APP cleaving enzyme
BBB	Blood-brain barrier
BSA	Bovine serum albumen
CD	Cluster of differentiation
cDNA	Complementary DNA
CIA	Collagen-induced arthritis
CIITA	MHC class II transactivator
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CpG	CpG-containing oligodeoxynucleotide
CSF	Cerebrospinal fluid
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EAE	Experimental autoimmune encephalitis
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FAD	Familial Alzheimer's disease

FITC	Fluorescein isothiocyanate
FcR	Fc receptor
FCS	Foetal calf serum
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HBSS	Hank's Balanced Salt Solution
HRP	Horesradish peroxidase
i.c.v.	Intracerbroventricularly
ICAM-1	Intercellular adhesion molecule-1
IDE	Insulin degrading enzyme
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL-1Ra	IL-1-receptor antagonist
IL	Interleukin
i.p.	Intraperitoneally
IP-10	Interferon-inducible protein-10
i.v.	Intravenously
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MIP-1	Macrophage inflammatory protein-1
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MWM	Morris water maze
NFT	Neurofibrillary tangle
NK	Natural killer
NMDA	N-methyl-D-aspartate

NSE	Neuron-specific enolase
OPD	Orthophenylene diamine
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDAPP	Platelet-derived growth factor promoter expressing APP
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cell
PNS	Peripheral nervous system
PrP	Prion Protein
PRR	Pattern recognition receptor
PS	Presenilin
PS1dE9	Exon 9-deleted presenilin 1
PT	Pertussis toxin
RAWM	Radial-arm water maze
RNA	Ribonucleic acid
RNase	Ribonuclease
RORγT	RAR orphan nuclear receptor
RPMI	Roswell Park Memorial Institute
RT	Room temperature
s.c.	Subcutaneously
sAPPα	Soluble amyloid precursor protein
SEM	Standard error of mean
STAT	Signal transducer and activator o transcription
TCR	T cell receptor
TLR	Toll-like receptor
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
TNFR1	Tumour necrosis factor type 1 death receptor

Th	T helper
Treg	Regulatory T cell
Tr1	T regulatory 1 cell
TLR	Toll-like receptor
TREM	Triggering receptor expressed on myeloid cells
ThT	Thioflavin T
TWB	Tris wash buffer
VLA	Very late antigen
VCAM	Vascular cell adhesion molecule
VLP	Virus-like particle

Chapter 1

Introduction

1.1. The immune system

The immune system is a made up of a network of cells, tissues, and organs that function to protect the host against infection by pathogens. Cells of the immune system recognise foreign organisms and distinguish them from the body's own cells. They then deliver an appropriate effector response to clear the pathogens from the body. The immune system consists of a non-specific innate immune arm and a specific adaptive immune arm. The innate immune system forms the first line of defence against pathogens. It is rapidly activated in response to conserved components of pathogens, and it is not specific to any particular pathogen. If a pathogen manages to circumvent the innate immune response, the adaptive immune response is activated. In contrast to the innate immune response, the adaptive immune response is antigen-specific and reacts only with the organism that induced the response. It is a much slower response, typically taking 5-6 days to develop. In addition, the adaptive immune response has the ability to "remember" past infections and this affords it protection against re-exposure to the same pathogen.

1.1.1. Innate immunity

The first component of the innate immune system is the physical barriers preventing the pathogen from gaining access to the body. The outer layer of the skin consists of several layers of tightly packed dead cells, the surface of which is

maintained between pH 3 and 5 to inhibit the growth of bacteria on these surfaces. In the lungs, coughing and sneezing expel pathogens and other irritants from the respiratory tract, while cilia that line the trachea and bronchi sweep out any bacteria that are inhaled deeper into the respiratory tract. Chemicals such as lysozyme and phospholipase A in saliva and tears are potent antibacterials, protecting these vulnerable surfaces from bacterial colonisation. There are also biological barriers, such as the commensal flora of the gastrointestinal tract, which compete with the pathogenic bacteria for nutrients and space, thus making it harder for them to proliferate in these areas.

Microorganisms that penetrate the physical barriers of the immune system for the first time are met immediately by cells and molecules that mount an innate immune response. One of the body's first responses to invasion by a foreign organism is inflammation. Inflammation is stimulated by chemical factors released from injured cells, and serves to generate a physical barrier against the spread of infection, recruit immune cells to the site of attack, and to promote healing of damaged tissue following the clearance of the pathogen. The chemicals produced during this response, such as histamine and bradykinin, serve to cause vasodilation, thus promoting the attraction of immune cells to the site of infection. Another important component of the innate immune response is the activation of complement. The complement system consists of a number of distinct plasma proteins that react in a cascade to generate proteins that bind covalently to pathogens, targeting them for destruction by phagocytes with complement receptors. It can also directly kill pathogens by forming a membrane attack complex, which punches holes in bacterial cell membranes.

The cells of the innate immune system rely on the detection of conserved molecular structures, known as pathogen-associated molecular patterns (PAMPs) that are produced by pathogens but not by host cells (Janeway & Medzhitov 2002). These PAMPs are recognised by receptors on the surface of innate immune cells known as pattern recognition receptors (PRRs). One of the principal

functions of PRRs is in triggering phagocytosis by innate immune cells. Macrophage mannose receptor is a C-type lectin that is expressed primarily on the surface of macrophages. It binds to specific types of sugar molecules found on the surfaces of bacteria and some viruses, rendering them susceptible to phagocytosis (Stahl & Ezekowitz 1998). In addition to inducing phagocytosis, recognition of PAMPs by PRRs can also result in the production of proinflammatory cytokines such as interleukin-1 beta (IL-1β), IL-6, and Tumour necrosis factor-alpha (TNF-α), and the expression of the co-stimulatory molecules CD80 and CD86 (Janeway 2005). The best characterised PRRs that activate the immune response in this manner are the Toll-like receptors (TLRs). To date 10 TLRs have been identified in humans, each of which recognise different PAMPs (Kawai & Akira 2006). TLR4 was the first mammalian TLR to be identified (Medzhitov *et al.* 1997), and it was found to be required for the induction of an inflammatory response to lipopolysaccharide (LPS), a component of the cell wall of Gram negative bacteria (Hoshino *et al.* 1999). Other TLRs include TLR2, which recognises zymosan from yeast cell walls and lipoproteins, and TLR9, which recognises the CpG-containing oligodeoxynucleotide (CpG) motif in bacterial DNA. Because PRRs bind to features of microorganisms that are conserved across multiple species, a limited number of receptors enable the innate immune system to react to a very wide range of pathogens.

1.1.1.2. Polymorphonuclear cells (PMNs)

Neutrophils are the most abundant white blood cells, normally accounting for between 50 and 60% of the total circulating leukocytes. They are normally found in the bloodstream, but in response to infection they migrate into the tissues. Neutrophils are phagocytic cells, and can internalise and destroy many pathogens. They attack these internalised pathogens by means of a "respiratory burst" of nitric oxide, the superoxide anion, and hydrogen peroxide. Their main mechanism

of destruction of pathogens is to release lytic and bactericidal substances from their granules, thereby killing the invading pathogen.

Eosinophils are also phagocytic and secrete a range of highly toxic proteins and free radicals upon activation. These substances are highly destructive to pathogens, but are also responsible for much of the "bystander" damage to healthy cells that occurs during allergic reactions. Their activation is thus tightly regulated. They also play a major role in fighting viral infections, as their granules contain ribonucleases (RNAses) and deoxyribonucleases (DNAses) (Domachowske *et al.* 1998).

Basophils release histamine upon activation, which causes vasodilation and increased permeability. This allows recruitment of other immune cells to the site of infection. They also secrete heparin, preventing the blood from clotting too quickly. Basophils are important in host defence against parasites. For example, production of cytokine, interleukin-4 (IL-4), by basophils plays an important role in clearance of the gastrointestinal nematode parasite, *Nippostrongylus brasiliensis* (Min *et al.* 2004), and in driving Th2 responses to parasites and allergens (Sokol *et al.* 2008).

1.1.1.2. Natural Killer (NK) cells

NK cells are distinctive from other cells of the innate immune system in that they attack host cells that are infected with pathogens, but do not attack the pathogens directly. NK cells kill virally infected cells and tumour cells, which express lower levels of major histocompatibility complex (MHC) class I than host cells. They are activated in response to interferons or macrophage-derived cytokines. Upon activation and detection of an infected or cancerous cell, NK cells release cytotoxic granules containing perforin, which punches holes in the cell membrane

of the target cell, and granzymes, which enter the cell through these holes causing apoptosis.

1.1.1.3. Macrophages

Macrophages are derived from monocytes that leave the circulation to migrate into the tissues in pursuit of pathogens. They are highly versatile cells that play a number of different roles in the immune response. As scavenger cells, they engulf and destroy dead cells and debris. They are the most efficient phagocytic cells in the body, and can phagocytose large numbers of bacteria. Macrophages in the sub mucosal tissues are the first cells to encounter pathogens. Upon encountering a pathogen, as well as engulfing and destroying it using a process known as the respiratory burst, the macrophage also produces chemokines, which summon other immune cells to the site of infection, and cytokines, which stimulate the activation of these immune cells. Ingestion of microbes by macrophages can also induce the expression of MHC class II molecules, which bind pathogen-derived peptides and display them on the surface of the macrophage, enabling them to present antigen to T cells. Macrophages can also phagocytose pathogens that are opsonised by either antibody or complement, which greatly increases the antigen recognising abilities of the phagocyte (Stuart & Ezekowitz 2005).

1.1.1.3. Dendritic cells (DCs)

DCs are phagocytes in tissues that are in contact with the external environment. They are efficient stimulators of B cells and by far the most potent stimulators of naïve T cells. They are produced in the bone marrow and migrate via the blood to the peripheral tissues where they exist in an immature state. Here, they routinely sample antigen from the surrounding tissue, but are not yet equipped to stimulate naïve T cells. Recognition of PAMPs by PRRs on the DC stimulates recruitment

of the DC to the local lymph node where they lose the ability to take up antigen, but have enhanced expression of MHC class I and II, enabling them to present antigen to and stimulate cytotoxic T lymphocytes (CTLs) and helper T (Th) cells respectively. Like macrophages, DCs serve as a link between the innate and adaptive immune systems, as they present antigen to T cells of the adaptive immune system (Guermonprez *et al.* 2002). They are much more efficient as antigen-presenting cells (APCs) than macrophages, and unlike macrophages they can sample large amounts of soluble antigen from extracellular fluid by macropinocytosis (Norbury 2006).

1.1.2. Adaptive immunity

Unlike innate immunity, which mediates non-specific responses to infection, adaptive immunity involves an antigen-specific immune response to the pathogen. The adaptive immune response is more complex than the innate response as the antigen must first be processed and presented to T cells before a response can be mounted. Once an antigen has been recognised, the T and B cells of the adaptive immune system proliferate, giving rise to effector cells that can attack and eliminate any cell displaying that antigen. Adaptive immunity also has immunological memory for pathogens, and responses against subsequent attacks by the same pathogen are stronger and faster.

Adaptive immunity is mediated by T cells, which mature in the thymus, and B cells, which mature in the bone marrow. Both T and B cells express receptors on their surface that are specific for a particular peptide. Upon recognition of their specific peptide:MHC complex on the surface of an APC, they are induced to proliferate and differentiate. The adaptive immune response is linked to the innate by virtue of the fact that its activation depends initially upon the presentation of antigen by APCs, such as dendritic cells and macrophages, to a naïve T cell. The adaptive immune system can also be activated independently of

the innate by direct recognition of certain antigens presented by B cells (Janeway 2005).

1.1.2.1. The humoral immune response

The extracellular spaces in the body are protected by the humoral immune response, whereby specific antibodies produced by B cells cause the destruction of extracellular pathogens. B cells are produced in the bone marrow of most animals. Each B cell expresses a unique B cell receptor that recognises a specific antigen. Any B cells that recognise self-antigen during the maturation process undergo apoptosis before they are released into the body. Once a B cell encounters its specific antigen and receives an additional signal from a helper T cell, it differentiates into a plasma cell. Plasma cells secrete large amounts of antibodies, which bind to their antigen in a process known as opsonisation, making it easier for phagocytosis and activation of the complement system. Antibodies can also neutralise bacterial toxins by binding to the receptor-binding site on the toxic molecule, and can block viral infection of cells by binding to virus receptors. Until recently, plasma cells were thought to survive only for a few days before dying due to the stress caused by massive production of antibody, but it is now known that these cells can survive for a long period in the bone marrow or at the site of inflammation (Radbruch *et al.* 2006). As well as differentiating into plasma cells, some B cells differentiate into memory B cells. These memory cells are long-lived and have the ability to proliferate rapidly to produce antibody-producing plasma cells upon subsequent exposure to the same antigen.

There are five different antibody isotypes: immunoglobulin M (IgM), IgD, IgG, IgA, and IgE, each of which is adapted to operate in different compartments of the body. IgM is the first class of antibody to be produced in a humoral response. IgM normally exists as a pentamer, and as a consequence of this it is very good at activating complement and at agglutinating antigens. It tends to have

a relatively low affinity for antigen but a high avidity due to its pentameric structure. Due to its size, it is usually found in the blood. IgA is smaller, usually a monomer or dimer, and is the major antibody isotype found in mucosal secretions. It is thus an important isotype in mucosal immunity, where it functions mainly in the neutralisation of viruses and extracellular bacteria. IgD is primarily found on the surfaces of B cells, and is thought to be involved in B cell development and fine-tuning of the humoral immune response (Geisberger *et al.* 2006). IgE is the least common antibody isotype found in the blood, and is tightly bound to mast cells and basophils before ever encountering antigen. Binding of antigen to IgE triggers release of chemicals from mast cells that induce allergic reactions such as sneezing. IgG is the most common isotype and four subtypes of IgG have been identified to date (IgG1-4), named in order of their relative abundance in the serum. They have a number of functions: for example IgG3 is very good at activating complement, while IgG4 cannot fix complement at all. The cytokines produced by T cells during B cell activation can direct the subtype of antibody produced by the resulting plasma cell. For example, interferon-gamma (IFN-γ), a Th1-type cytokine, promotes IgG2a production and inhibits IgG1, IgG2b, and IgG2e (Snapper & Paul 1987, Finkelman *et al.* 1988). Conversely, production of IgG1 is induced by the Th2-type cytokine, interleukin-4 (IL-4), which inhibits IgG2a, IgG2b, and IgM (Coffman *et al.* 1988, Snapper & Paul 1987). In this way, the antibody subtype produced reflects the subtype of the accompanying T cell response.

1.1.2.2. T cell-mediated immunity

In contrast to B cells, T cells respond to both exogenous and endogenous antigens in a cell-mediated manner. T cells are produced in the bone marrow and then migrate to the thymus where they mature. They then enter the bloodstream and circulate between the blood and the peripheral lymphoid organs. At this point, they have not yet encountered antigen and are known as naïve T cells. Like B

cells, T cells each express a unique surface T cell receptor (TCR) that is specific for a particular antigen. Naïve T cells are kept from being destroyed by receiving periodic survival signals from their encounters with self-peptide:self-MHC complexes, similar to those that originally positively selected them, and also through their IL-7 receptors (Schluns & Lefrancois 2003, Jaleco *et al.* 2003). In order to become activated, a T cell must recognise its cognate antigen in the context of an MHC molecule. This interaction normally occurs in the draining lymph node, but may also occur in the spleen. There are two types of MHC molecule, and each type presents antigen to a corresponding type of T cell. MHC class I molecules are expressed on almost all nucleated cells, whereas MHC class II molecules are only expressed on antigen presenting cells, such as macrophages and DCs.

Endogenous antigens produced by viruses replicating within a host cell are processed and coupled to MHC class I molecules, which are then expressed on the surface of the cell. These antigens are then recognised by a specific $CD8^+$ T cell/cytotoxic T lymphocyte, which enlarges into a blast cell and undergoes repeated rounds of division generating a population of effector CTLs. Effector CTLs release perforin and granulysin, which allow ions and water to enter the infected cell, causing lysis of the cell. They also release granzyme that enters these pores to induce apoptosis. CTLs do not require a co-stimulatory signal for their activation (Sepulveda *et al.* 1999).

Extracellular pathogens are engulfed by APCs which then break them down in the phagolysosome. The resulting antigens are then expressed on the surface of the APC in association with MHC class II molecules. $CD4^+$ T helper (Th) cells recognise antigen presented on MHC class II molecules. TCR binding to the MHC class II:antigen complex is the initial signal for Th activation, but a second signal is also required. The best characterised co-stimulatory molecules are B7.1 (CD80) and B7.2 (CD86). These molecules bind to CD28 on the surface of the T cell which stimulates the clonal expansion of naïve $CD4^+$ T cells (Sharpe

& Freeman 2002). This co-stimulatory signal is essential for $CD4^+$ T cell activation. Upon activation, $CD4^+$ T cells divide rapidly to form a population of effector T cells. Th cells have no cytotoxic or phagocytic activity of their own, however they are capable of influencing a variety of immune cells, and the cytokines produced by Th cells are often essential for clearance of an infection. In order to mount an effective response, the immune system must determine which cytokines are most beneficial for the host in dealing with a particular insult and generate the appropriate Th cells. The phenotype of the activated $CD4^+$ T cell is determined by the cytokine environment that exists during antigen presentation (Mosmann *et al.* 1986).

1.1.2.3. Th1/Th2 cells

Th1 and Th2 cells were originally discovered in 1986, when studies by Mosman and colleagues found that the cytokine profile of a T cell response to an antigen is indicative of which helper T cell pathway is stimulated by the APC (Mosmann *et al.* 1986). Th1 cells secrete IFN-γ, and their induction is promoted by IL-12. They function to enhance the clearance of intracellular pathogens such as *Mycobacterium tuberculosis* (Szabo *et al.* 2003). Th1 cells can activate macrophages that have ingested pathogens or that are infected, and can provide both signals required for activation of macrophages by secreting IFN-γ and by promoting expression of CD40 ligand. IFN-γ also enhances the expression of MHC class II on the macrophages. Furthermore, IFN-γ induces B cells to produce IgG2a, a subclass of antibody required for complement fixation and opsonisation, but which is also associated with a pathogenic autoantibody response in the mouse (Rosloniec *et al.* 2002). Th2 cells secrete IL-4, IL-10, IL-6, and IL-5, which are necessary for inducing the humoral response to combat parasitic infections, and promote isotype switching to IgG1 and IgE (Glimcher & Murphy 2000). Th2 cells also activate eosinophils through production of IL-5, which are important in parasite clearance (Greenfeder *et al.* 2001). IL-4 and IL-10 produced by Th2 cells

also inhibit the generation of Th1 cells, providing a balance between these two cells types, although IL-10 is also produced by other cell types, including DCs and Tr1 cells (Moser & Murphy 2000, Battaglia *et al.* 2006).

Until the mid 1990's it was thought that the balance between Th1 and Th2 cells was the major factor in determining the outcome of the adaptive immune response, as improper activation of Th2 cells can lead to asthma and allergy (Herrick & Bottomly 2003), while over activation of Th1 cells can lead to autoimmunity. But since then two additional $CD4^+$ T cell types have been discovered, namely, regulatory T cells (Treg) and IL-17 producing T cells (Th17).

1.1.2.4. Treg cells

Treg cells can be subdivided into natural and inducible Treg cells, both of which have a function in suppressing immune responses. $CD4^+CD25^+FoxP3^+$ T cells are known as natural regulatory T cells, whereas adaptive regulatory cells (Tr1 or Th3) are generated from naïve T cells in the periphery after encounter with antigen presented by an APC with a different activation status to APCs that generate Th1 or Th2 cells (Mills 2004). $FoxP3^+$ inducible Treg cells can also be generated in the periphery by conversion from naïve $CD25^-$ T cells under the influence of TGF-β.

Natural regulatory T cells develop in the thymus as shown by Sakaguchi and colleagues and then migrate to the periphery where they exert a suppressive effect on autoreactive T cells (Mills 2004). In adult mice, they are produced continuously as a fully mature population. Adaptive regulatory T cells, in contrast to natural Treg cells, do not express CD25 constitutively, and are induced when they encounter an APC in the periphery in the context of certain regulatory situations (Mills 2004). Groux and colleagues showed that they could induce a population of IL-10 producing T cells by repeatedly stimulating ovalbumin-

specific T cells with their specific antigen in the presence of IL-10. These T cells were found to be able to suppress Th1 and Th2 cell responses and Th1 cell-mediated diseases (Cottrez *et al.* 2000, Groux *et al.* 1997). These cells, termed T regulatory 1 (Tr1) cells have a poor proliferative response, secrete no IL-2 or IL-4, but produce large quantities of IL-10 and inhibit the proliferative response of bystander cells both *in vitro* and *in vivo* (Groux *et al.* 1997). The other type of inducible Treg cells so far discovered were generated in the gut by feeding self antigens (Chen *et al.* 1994). These cells, which produce large amounts of TGF-β and can prevent Th1-mediated autoimmune diseases, are known as Th3 cells.

It is now well accepted that Treg cells maintain peripheral tolerance and regulate autoimmunity. Circumstances in which this regulatory mechanism is compromised result in autoimmunity. $CD4^+CD25^-$ T cells have been implicated in protection against EAE (Olivares-Villagomez *et al.* 1998) and inflammatory bowel disease (IBD; (Annacker *et al.* 2000). Recently, it has been shown that certain immunosuppressive drugs, as well as anti-CD3 antibody, can expand suppressive Treg cells (Barrat *et al.* 2002, Belghith *et al.* 2003).

1.1.2.5. Th17 cells

Th17 cells are characterised by their production of the cytokine IL-17 (Park *et al.* 2005a, Aggarwal *et al.* 2003), but also secrete other cytokines including IL-21 and IL-22 (Wei *et al.* 2007, Ma *et al.* 2008). They are independent of Th1 and Th2 cells, and IFN-γ and IL-4 antagonise their development (Park *et al.* 2005a). IL-17 has been associated with many inflammatory diseases, such as rheumatoid arthritis and asthma (Chabaud *et al.* 1999). *In vitro,* IL-17 causes production and release of IL-8, which results in neutrophil recruitment (Laan *et al.* 1999). IL-17 receptor-deficient mice show reduced survival after pulmonary infection with *K. Pneumoniae*, and this is associated with increased bacterial and reduced

neutrophil numbers in the lung (Ye *et al.* 2001). IL-17 is also important in allergic reactions and airway hypersensitivities (Park *et al.* 2005a).

Over-activation of Th1 cells was initially thought to be responsible for the development of autoimmunity. This hypothesis was based on the fact that deletion or neutralisation of IL-12p40 in mice was associated with increased resistance to collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) (McIntyre *et al.* 1996, Leonard *et al.* 1995, Malfait *et al.* 1998). The classical view that the Th1 response mediated autoimmunity came under challenge following the demonstration that deletion of IFN-γ or the IFN-γ receptor resulted in more severe CIA (Manoury-Schwartz *et al.* 1997). Furthermore, IL-12Rβ2$^{-/-}$ mice developed earlier and more severe EAE than controls (Zhang *et al.* 2003).

The disparity between these results prompted Cua and colleagues to look at the role of IL-23 in these autoimmune diseases, as it also contains the p40 subunit of IL-12. Cua and colleagues showed that mice deficient in IL-23p19, but not IL-12p35, were protected from development of EAE and CIA (Cua *et al.* 2003, Murphy *et al.* 2003). It was then reported that IL-23 could promote development of Th17 cells. Proteolipid protein peptide primed T cells cultured with IL-23 were IL-17 producing, and were capable of inducing EAE, whereas Th1 cells were not (Langrish *et al.* 2005).

Th17 cells have been suggested to be a transient cell population, and have been shown to convert to a Th1 phenotype when restimulated through the TCR (Mathur *et al.* 2006). Double producers of IFN-γ and IL-17 have been found in EAE and in Crohn's disease (McGeachy *et al.* 2007, Annunziato *et al.* 2007). Furthermore, Th17 cells shown to be lacking in IFN-γ rapidly converted to IFN-γ-producing cells upon transfer to non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice in a mechanism thought to involve IL-12 (Bending *et al.* 2009). It is known that transforming growth factor-beta (TGF-β)

inhibits the Th1 transcription factor T-bet (Park *et al.* 2005b). Mathur and colleagues believe that this inhibition of T-bet is partly responsible for the induction of Th17 cells by the cytokines, TGF-β and IL-6 (Mathur *et al.* 2006). It has been argued that Th1 and Th17 cells are of the same T cell lineage, and that they may originate from a common 'pre-Th1' precursor cell (Bettelli & Kuchroo 2005). However, Th1 cells are unresponsive to IL-23, and IFN-γ inhibits Th17 cell development (Harrington *et al.* 2006), and so the relationship between Th1 and Th17 cells remains elusive.

1.1.2.6. The role of cytokines in T cell differentiation

The cytokine environment at the time of activation of the T cell is critical in determining its subsequent polarisation (de Jong *et al.* 2005, Kaiko *et al.* 2008). $CD4^+$ T cells initially stimulated in the presence of IL-12 and IFN-γ develop into Th1 cells (Gately *et al.* 1998), partly because IFN-γ inhibits the production of Th2 cells. T cells activated in the presence of IFN-γ induce activation of the transcription factor, T-bet. T-bet then induces further IFN-γ production by the T cell and increases its responsiveness to IL-12 by upregulating expression of the IL-12Rβ2 chain. IL-12 binds to its receptor to produce more IFN-γ via signal transducer and activator of transcription-4 (STAT-4), resulting in the production of more Th1 cells in a positive feedback loop (Dardalhon *et al.* 2008).

$CD4^+$ T cells activated in the presence of IL-4 induce Th2 cell differentiation, especially when IL-6 is also present (Diehl *et al.* 2002). Though the source of this IL-4 has yet to be conclusively determined, a recent paper suggested that basophils may be involved (Sokol *et al.* 2008). Binding of IL-4 to the IL-4 receptor mediates STAT6 activation followed by GATA3 expression, leading to IL-4 production by the T cell (Zheng & Flavell 1997, Kaplan *et al.* 1999). Once GATA3 becomes activated to a certain extent, it activates its own expression, resulting in a positive feedback loop. The transcription factors

GATA3 and T-bet are mutually antagonistic, with IL-4 suppressing T-bet activation, and IFN-γ and IL-12 suppressing GATA3.

$CD4^+$ cells activated in the presence of IL-10 induce Tr1 cell differentiation (McGuirk *et al.* 2002). Tr1 cells are characterised by the production of large amounts of IL-10 and low amounts of TGF-β. Barrat and colleagues have generated Tr1 cells using a combination of vitamin D3, dexamethasone and neutralising Th1 and Th2 cytokines and found these cells to be capable of preventing the induction of EAE in an IL-10-dependent manner (Barrat *et al.* 2002).

Th17 cells were initially thought to be dependent on the cytokine IL-23 for their development, as Th17-mediated development of autoimmunity is dependent on the presence of IL-23 (Langrish *et al.* 2005). However, subsequent studies have shown that although IL-23 appears to be required for Th17 cell-mediated immunopathology, it is not required for the induction of Th17 cells (Veldhoen *et al.* 2006, Mangan *et al.* 2006). Indeed, naïve T cells do not express IL-23R and do not differentiate into Th17 cells in response to IL-23 *in vitro* (Harrington *et al.* 2005). It was then demonstrated that TGF-β and IL-6 appear to be responsible for the differentiation of naïve T cells into Th17 cells, and that IL-23 is important in the survival and expansion of this cell type (Mangan *et al.* 2006, Veldhoen *et al.* 2006). However, IL-1 has been shown to synergise with IL-23 to stimulate IL-17 production in murine T cells, while T cells from $IL\text{-}1R1^{-/-}$ mice were unable to produce IL-17 in response to IL-23 treatment, providing evidence for a role of IL-1 in the induction of Th17 cells (Sutton *et al.* 2006). The transcription factor RAR orphan nuclear receptor (RORγt) has been shown to be required for Th17 cell development, as transduction of naïve T cells with a retroviral vector containing RORγt induces the development of Th17 cells (Ivanov *et al.* 2006).

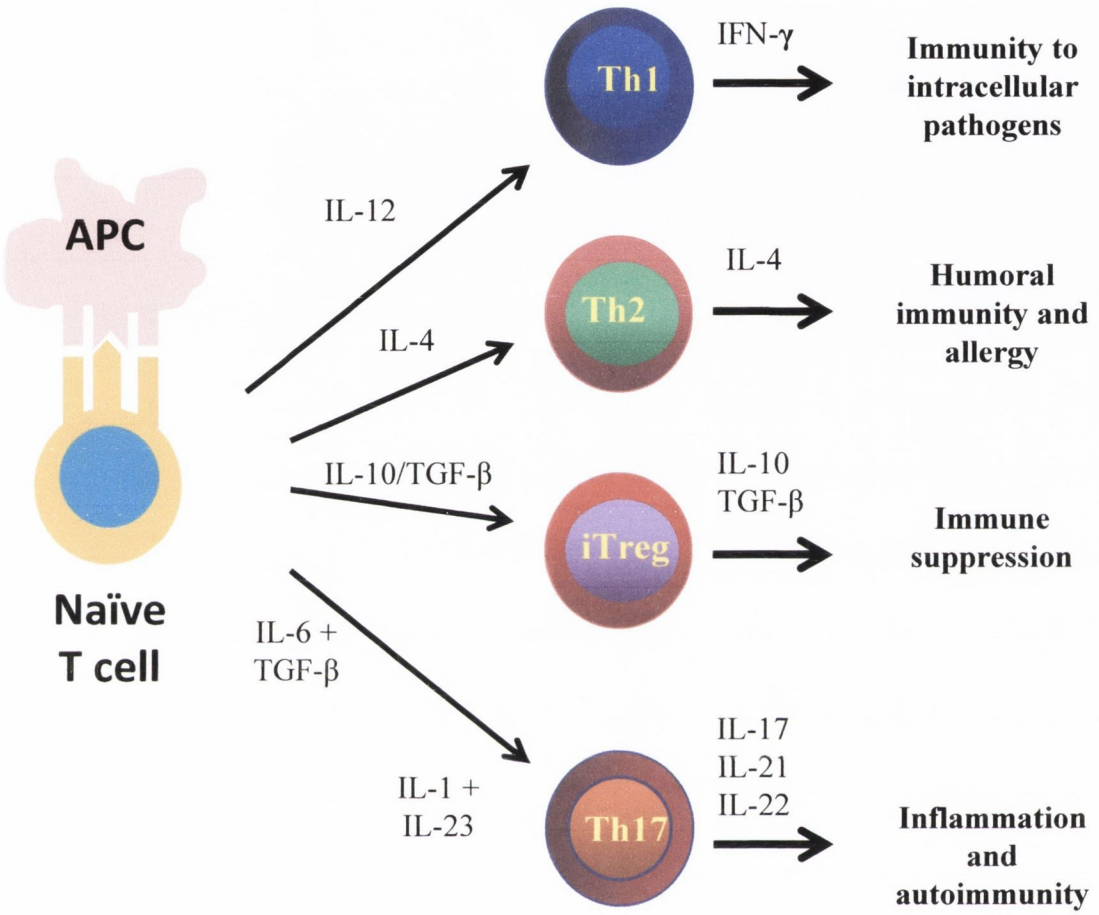


Figure 1.1. Differentiation and function of T helper subsets

Upon encountering their cognate antigens presented on APCs, naïve Th cells differentiate into effector Th1, Th2, iTreg or Th17 cells. The cytokine environment at the time of activation of the T cell is crucial in determining its subsequent polarisation. $CD4^+$ T cells activated in the presence of IL-12 differentiate into Th1 cells, which produce IFN-γ and mediate immunity against intracellular pathogens and tumours. IL-4 promotes development of Th2 cells, which secrete IL-4, IL-5, IL-10, and IL-13, mediate humoral responses and immunity against parasites, but also enhance allergy. $CD4^+$ T cells activated in the presence of IL-10 or TGF-β differentiate into inducible Treg (iTreg) cells, which mediate immune suppression. IL-6 and TGF-β promote development of Th17 cells, and their expansion is enhanced by IL-1 and IL-23. Th17 cells produce IL-17, IL-21, and IL-22, participate in inflammation and are pathogenic in a number of organ-specific autoimmune diseases.

Figure adapted from Mills, K. H. (2008). *Eur J Immunol,* **38,** 2636-49.

1.2. The central nervous system

The nervous system is a highly complex system that acts as the body's control centre and communications network. It detects changes both inside and outside of the body, interprets these changes and sends signals from the brain to respond to these changes in the appropriate manner. The nervous system can be divided into the central nervous system (CNS), and the peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord and functions to coordinate the activity of all processes in the body. The PNS connects the CNS to the outside world and sends sensory information from the body to the CNS. It activates muscles and glands in response to signals received from the CNS.

The most important cell-type found in the CNS are the neurons. These cells are responsible for communication in the brain, and function to send and store information using electrochemical signalling. There are approximately 100 billion neurons in the CNS, and the complexity of the nervous system is determined by the large number of interconnections between these neurons. However neurons only account for approximately ten percent of the total cell number of the CNS. The other 90 percent of brain cells are glial cells. Glial (literally meaning glue) cells are brain cells that do not transmit nerve impulses. They perform many important functions including engulfment of dead neurons, and provide physical and trophic support for neurons. There are three main types of glial cells in the brain: oligodendrocytes, astrocytes, and microglia.

The main function of oligodendrocytes is the myelination of axons in the CNS, a function performed by Schwann cells in the peripheral nervous system. A single oligodendrocyte can span up to 50 axons, wrapping around approximately 1mm of each and forming the myelin sheath. This myelin sheath insulates the neurons, enabling fast synaptic transmission. Demyelinating diseases such as multiple sclerosis (MS) are associated with damage to oligodendrocytes, resulting in decreased synaptic transmission.

Astrocytes are star-shaped glial cells that perform a variety of functions in the CNS. They form a matrix that provides structural support for neurons. This matrix has the added advantage of isolating synapses by taking up transmitter substances such as glutamate and gamma-aminobutyric acid that are released at the synapse, thus limiting their dispersion (Schmitt *et al.* 1996, Schmitt *et al.* 1997, Schousboe & Waagepetersen 2006). Astrocytes are critical in the induction and maintenance of the tight junctions of endothelial cells at the blood-brain barrier (BBB), which limits the entry of cells and substances into the CNS (Janzer & Raff 1987, Ballabh *et al.* 2004). They also provide neurons with nutrients by metabolising glucose into lactate, and releasing the lactate into the extracellular fluid surrounding the neurons (Lindsay 1994). More recently, adult astrocytes from the hippocampus have been found to be capable of inducing stem cells to differentiate into neurons (Song *et al.* 2002). Neurons cultured with stem cells in a similar manner did not induce neuronal differentiation, suggesting that astrocytes could be an important cell population in the promotion of adult neurogenesis *in vivo*. In this way, astrocytes have a vital function in both the generation and nourishment of neurons, and also in providing them with a structural framework.

Microglia are the principal immune cells of the brain, and function to phagocytose cellular debris and pathogens, as well as to promote inflammation and immune cell recruitment to sites of infection or tissue damage. The role of microglia and astrocytes in the CNS immune system will be discussed later.

1.2.1. The immune system in the CNS

The CNS was classically thought of as an "immune privileged" site as it is isolated from the rest of the body by the blood brain barrier (BBB), which minimises the passage of cells and macromolecules into the brain parenchyma. Medawar and colleagues found that grafts of skin tissue implanted in the brain

were accepted for a more prolonged period of time than those implanted in the periphery (Medawar 1948). However, this view is now widely regarded as being oversimplified with the discovery of resident immune cells in the CNS, and the fact that small numbers of T cells can cross the BBB into the CNS. It now seems as though the immunologically-privileged status of the CNS is actively maintained by cell-cell interactions and the release of soluble factors that suppress immune responses. It is also clear that these immune control mechanisms can be overridden, resulting in inflammation in the brain, and the development of autoimmune diseases such as MS.

1.2.2. Resident immune cells of the CNS

1.2.2.1. Astrocytes

In addition to their role in providing nutritional and structural support for neurons, astrocytes also play a role in the immune system of the CNS. Astrocytes are phagocytic cells, and though they are substantially less effective than microglia, they have been shown to actively remove apoptotic lymphocytes from the CNS by phagocytosis (Magnus *et al.* 2002). Following brain inflammation, astrocytes become activated, resulting in astrogliosis and the formation of a glial scar, which, depending on the context, may aid the repair process by producing neurotrophins to enhance neuronal and glial survival (Zafra *et al.* 1992, Zhou & Rush 1994). Glial scarring may also have a negative influence on regeneration via the inhibition of neuronal and glial growth and migration in situations of disease (Holley *et al.* 2003). It was initially suggested that astrocytes could function as APCs, following the discovery that MHC class II is expressed on their surface. In support of this hypothesis, it was shown that IFN-γ, TNF-α, and IL-1 could induce the expression of intercellular adhesion molecule 1 (ICAM-1) on human foetal astrocytes (Frohman *et al.* 1989). Astrocytes have been shown to be capable of

presenting myelin basic protein (MBP) to encephalitogenic T cell lines (Fontana *et al.* 1984). However, astrocytes do not appear to be as efficient as microglia at presenting antigen to Th1 cells, and instead may preferentially activate Th2 cells (Aloisi *et al.* 1998). Stüve and colleagues have shown that while the MHC class II transactivator (CIITA), a transcriptional cofactor, is required for CNS antigen presentation and for induction of EAE, CIITA-induced MHC class II expression on astrocytes was unable to increase susceptibility to EAE (Stuve *et al.* 2002). In addition, it has been shown that LPS can enhance IL-10 messenger ribonucleic acid (mRNA) expression in astrocytes, and that IL-10 is highly potent in suppressing LPS-induced IL-1β, suggesting a role for astrocytes in attenuating inflammation (Ledeboer *et al.* 2002).

1.2.2.2. Microglia

Microglia are classically thought of as the resident immune cells of the CNS, and belong to the mononuclear phagocyte lineage. Indeed, microglia express a number of cell surface markers associated with macrophages, including F4/80, CD11b, and CD45 (Havenith *et al.* 1998, Zhang *et al.* 2002, Carson *et al.* 1998). Microglia represent about 10 percent of the adult CNS cell population. They occur throughout the brain parenchyma where they assume a dendritic morphology with many highly ramified processes (Rock *et al.* 2004). They are continuously replenished throughout adult life by proliferation (Vilhardt 2005). The majority of microglial function goes unnoticed, as they perform general maintenance and clear cellular debris. In addition, microglia are involved in late embryonic/early postnatal brain maturation, where they enforce the programmed elimination of neural cells (Barron 1995). Microglia normally exist in a quiescent state in the healthy CNS and are characterised by a surface covered with spines (Vilhardt 2005). Microglia in this state are known as ramified microglia. Maintenance of this quiescent state is likely to be related to the cytokines present in the brain, and transforming growth factor-beta (TGF-β) and IL-10 appear to be particularly

important in this process (Ledeboer *et al.* 2002, Unsicker & Strelau 2000). In the normal CNS, microglia have no phagocytic activity, and display low levels of MHC class I and II (Aloisi 2001). Microglia have been demonstrated to constantly reorganise their processes *in vivo*, probing the CNS for signs of injury (Davalos *et al.* 2005, Fetler & Amigorena 2005). Following infection or injury to the CNS, these processes shorten and the cell body and nucleus enlarge, giving rise to an amoeboid phenotype.

1.2.2.3. Microglial activation

Microglia have evolved to recognise and respond to a large number of factors, and express many different receptors that act to initiate and modulate the immune response in the CNS. Microglia express mRNA for TLR1-9, and can be activated by a number of different PAMPs (Olson & Miller 2004). They can also respond to infection of the CNS with herpes simplex virus (Esiri *et al.* 1995). Microglia can be activated by proteins such as amyloid-beta (Aβ), resulting in their phagocytosis, and they are also responsible for phagocytosing apoptotic neurons (Koenigsknecht & Landreth 2005, Takahashi *et al.* 2005). In addition, microglia are activated by immunoglobulins and complement, serum factors that enter the CNS in response to a breakdown of the BBB (McGeer & McGeer 2002).

It was initially presumed that, once activated, microglia upregulate their expression of cell-surface molecules and proinflammatory cytokines, and therefore, microglia can be described as quiescent or activated. However, it is now believed that once activated, microglia can exist in at least two functionally distinguishable states: a phagocytic phenotype, and an antigen presenting phenotype, and that the activation state is governed by their stimulatory environment (Town *et al.* 2005). Furthermore, each of these activation states may or may not be accompanied by proinflammatory cytokine production, suggesting that several functionally-active states probably exist.

1.2.2.4. Phagocytosis

Microglia are the chief phagocytic cells of the CNS. Phagocytosis by microglia is the principal mechanism by which apoptotic cells are removed from the CNS. The signal for removal of apoptotic cells by microglia has not yet been determined, but early indications point to the involvement of microglial triggering receptor expressed on myeloid cells-2 (TREM-2). TREM-2 is an innate immune receptor expressed on DCs and microglia. Takahashi and colleagues have shown that knockdown of TREM-2 resulted in inhibited phagocytosis of apoptotic neurons by microglia, and that its overexpression resulted in increased phagocytosis and decreased proinflammatory responses (Takahashi *et al.* 2005). TREM-2 is upregulated on microglia in the spinal cord and CNS during EAE, and blockade of TREM-2 during the course of disease exacerbates disease, probably due to increased inflammation and decreased phagocytosis in the brain (Piccio *et al.* 2007). Microglia also remove pathogenic proteins, such as Aβ, from the CNS by phagocytosis, and it is thought that microglia may be involved in clearance of Aβ from the brain following immunotherapy with Aβ-specific antibodies (Bard *et al.* 2000).

1.2.2.5. Chemokine production

The chemokines produced by microglia recruit leukocytes from the blood to the site of injury in the CNS. Macrophage inflammatory protein-1 (MIP-1), interferon-inducible protein-10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) produced by microglia promote recruitment of T cells, macrophages, and dendritic cells to the CNS (Aloisi 2001). Microglia also express many of the receptors for microglial-induced chemokines, suggesting that an additional function of chemokine production is to attract more microglia to the site of injury.

Similarly to the proinflammatory cytokines produced by microglia, the synthesis of chemokines by microglia appears to be under strict control, as certain anti-inflammatory molecules, such as IL-10, down-regulate chemokine production by activated microglia *in vitro* (Marques *et al.* 2004). Overproduction of these chemokines by microglia appears to be linked to certain neuroinflammatory diseases. For example, MCP-1 has been detected *in vivo* in the reactive microglia of amyloid-beta (Aβ) containing plaques, which are a hallmark of Alzheimer's disease (AD), and MIP-1α and IP-10 are increased in lesions of MS (Balashov *et al.* 1999, Simpson *et al.* 1998). Furthermore, MCP-$1^{-/-}$ mice are resistant to the development of EAE, which was associated with decreased macrophage recruitment to the CNS (Huang *et al.* 2001).

1.2.2.6. Cytokine production

Activation of microglia through TLR ligands, such as with LPS, induces profound production of the cytokines IL-1β, IL-6, and TNF-α. These proinflammatory cytokines have been implicated in the development of CNS inflammation, partially due to their direct toxicity to neurons (Godoy *et al.* 2008). These cytokines can also upregulate expression of cell adhesion molecules on endothelial cells and astrocytes, allowing for increased infiltration of leukocytes into the CNS (Lee & Benveniste 1999).

IL-1β is a critical cytokine in the early stages of inflammation and promotes astrocyte activation, as well as further microglial activation. It has been shown that the induction of IL-1β by microglia following CNS injury was followed by an increase in GFAP expression in astrocytes, a response that was not seen in IL-1β-deficient animals (Herx & Yong 2001). In a rat model of cerebral ischemia, injection of the IL-1 receptor antagonist (IL-1Ra) into the striatum was found to protect the cortex of these rats from injury (John *et al.* 2003). IL-1β was

also found to upregulate proinflammatory cytokines, chemokines, and adhesion molecules expressed by cultured astrocytes (Hua & Lee 2000).

TNF-α is upregulated in the injured or inflamed CNS and upregulation of this cytokine appears to be associated with an increase in expression of glial fibrillary acidic protein (GFAP) by astrocytes. Like IL-1β, TNF-α can induce expression of a number of proinflammatory genes, such as those for adhesion molecules (ICAM-1, VCAM-1), and cytokines (IL-6) (Lee & Benveniste 1999). Increased expression of TNF-α has been observed in the brain following bacterial and viral infections, and in cerebral ischemia (Hanisch 2002), and it has been demonstrated that overexpression of TNF-α and its receptor in the CNS of mice resulted in the development of cerebral ischemia, characterised by endothelial cell activation and meningeal inflammation (Akassoglou *et al.* 2003).

IL-6 appears to be less of a proinflammatory cytokine than either IL-1β or TNF-α. It has been shown to promote neuronal survival and neurite outgrowth in the CNS. While IL-1β and TNF-α are both responsible for the induction of other proinflammatory genes, IL-6 only upregulates these genes in certain situations. IL-6 is also capable of down-regulating the expression of TNF-α (John *et al.* 2003). Despite this, IL-6 is still classed as a proinflammatory cytokine, and studies have shown that overexpression of IL-6 in mice results in CNS inflammation and neurodegeneration (Campbell *et al.* 1993).

Microglia are also capable of producing anti-inflammatory cytokines, such as IL-10 and IL-4 (Park *et al.* 2005c, Park *et al.* 2007). IL-4 and IL-13 appear to mediate microglial apoptosis following an inflammatory stimulus, and thus may represent an important negative feedback mechanism to regulate microglial inflammatory responses (Yang *et al.* 2002).

1.2.2.7. Antigen presentation

In the healthy CNS, microglia express very low levels of MHC Class II. Following activation, expression of MHC class II is upregulated on microglia. Microglia also express co-stimulatory molecules that are responsible for APC-mediated T cell activation, such as CD80, CD86, and CD40 (Aloisi *et al.* 2000c, Issazadeh 1998). The expression of MHC and co-stimulatory molecules, combined with their ability to phagocytose antigens and express them on their surface, enables activated microglia to operate as APCs (Aloisi *et al.* 2000b). While it has been demonstrated that microglia can present antigen to T cells *in vitro*, the role of microglia as APCs *in vivo* is more controversial.

Microglia can be differentiated from perivascular macrophages on the basis of CD45 expression. Both cell types are $CD11b^+$, but macrophages express high levels of CD45, whereas microglia express low levels of CD45 (Sedgwick *et al.* 1991). It has been demonstrated that microglia isolated from the adult CNS could not present antigen to naïve T cells, and that perivascular cells were more important in antigen presentation in the CNS (Carson *et al.* 1998). Miller and colleagues have reported that $CD11b^+$ $CD11c^+$ $CD45^{hi}$ DCs were more efficient than $CD11b^+$ $CD11c^-$ $CD45^{hi}$ macrophages in presenting myelin antigens to T cells in EAE, and that $CD11b^+$ $CD11c^-$ $CD45^{low}$ microglia were largely incapable of presenting antigen to T cells (Miller *et al.* 2007). In contrast, Juedes and colleagues have shown that $CD11b^+$ $CD45^{inter}$ microglia isolated from brains of mice with EAE were as efficient as infiltrating macrophages in presenting antigen to T cells (Juedes & Ruddle 2001). Of note, although these microglia could induce cytokine production from effector T cells, they could not induce their proliferation, and this was attributed to their production of nitric oxide.

An explanation for these apparently contradictory findings may lie in the markers used to differentiate microglia from infiltrating cells in these studies. In experiments looking at the effect of toxoplasmic encephalitis on antigen

presentation in perivascular cells and microglia, it was found that expression of CD45 correlated with disease severity (Fischer & Reichmann 2001). It was suggested in this study that microglia may upregulate their expression of CD45 in disease, and that expression of this marker is correlated with antigen presenting ability. In support of this hypothesis, it has been shown that there is enhanced expression of both CD45 and CD11c on microglia isolated from mice with EAE (Ponomarev *et al.* 2005), and so current techniques for differentiating between CNS-resident and infiltrating cells may need to be optimised further. In summary, it would appear that microglia have an important role to play in antigen presentation in the CNS, though circulating macrophages and dendritic cells may also be required to mount an effective immune response in the CNS.

1.2.2.8. Microglial activation: neurotoxic or neuroprotective?

There are conflicting reports suggesting that activated microglia may be neurotoxic or neuroprotective. Microglia can produce cytotoxic factors, such as superoxide (Zhou *et al.* 2008), IL-1β (Herx & Yong 2001), TNF-α (Jiao *et al.* 2008), and nitric oxide in response to immunologic stimuli. Microglia have been implicated in the pathogenesis of autoimmune diseases, such as AD and Parkinson's disease, and there is some evidence linking microglial production of IL-1β to Aβ plaque formation and neuronal death in AD (Griffin 2006). While there is a large body of literature on the proinflammatory effects of microglia, care must be taken before classifying these cells as neurotoxic.

There is no doubt that severe immune inflammation induced by microglia following CNS injury or in neurodegenerative disease is detrimental to neurons. However, a controlled immune response in the brain following infection or injury can lead to pathogen clearance and may also be beneficial for neuronal repair. For example, overexpression of TNF-α in the CNS has been demonstrated to protect against rabies infection of the CNS by mechanisms thought to involve both

microglial activation and leukocyte infiltration (Faber *et al.* 2005). A recent study has shown that microglial-derived TNF-α exerted a neuroprotective effect following focal cerebral ischemia, indicating that microglial production of proinflammatory cytokines can be beneficial if properly controlled (Lambertsen *et al.* 2009). Microglia have been shown to release neurotrophins, such as nerve growth factor and brain-derived neurotrophic factor, suggesting that they can increase neuronal survival (Elkabes *et al.* 1996). Microglial phagocytosis of Aβ in normal individuals is thought to prevent the build-up of toxic amyloid species in the brain, and phagocytosis of Aβ by microglia is thought to be involved in the effectiveness of immunotherapeutic treatments in mouse-models of AD (Bard *et al.* 2000, Rogers *et al.* 2002). Microglia can also phagocytose apoptotic T cells, and interferon-beta, which is used as a therapy for MS, has been shown to increase microglial phagocytosis of apoptotic T cells *in vitro*, which acts as a signal to down-regulate immune responses (Chan *et al.* 2003).

A number of *in vitro* studies have shown the neurotoxic effects of microglia on neurons, but it is now becoming increasingly clear that cultured microglia behave differently to microglia *in vivo*. The extensive ramification of microglia seen *in vivo* is rarely achieved in culture, and cultured microglia are generally more activated (Nimmerjahn *et al.* 2005). This is probably due in part to the fact that methods of isolating microglia from the brain are often traumatic insults in themselves, but more importantly, microglial activation in the healthy brain is tightly regulated by interactions with other cell types, such as astrocytes and neurons. For example, neurons express CD200, which interacts with the CD200 receptor on the surface of microglia to keep microglia in a quiescent state (Neumann 2001). Microglia from CD200-deficient mice were found to have a more activated phenotype in response to facial nerve transection, and were activated much more rapidly than microglia from wild-type controls (Hoek *et al.* 2000). Thus, it appears that while microglial activation can result in neuronal death, it is also involved in neuronal protection and repair, and a balance between

these two extremes must be maintained in order for the immune system to respond to infection and injury in the CNS, without inducing widespread neuronal death.

1.2.3. Infiltrating immune cells

1.2.3.1. Bone-marrow-derived microglia

The origin of microglia produced during development has been the subject of much debate, but there is now firm evidence that microglia originate from bone-marrow-derived cells that migrate through the BBB into the parenchyma during early embryonic stages (Ling 1979, Nakajima & Kohsaka 2001). However, monocyte migration into the CNS appears not to be restricted to embryonic stages, and has been reported to occur throughout life (Eglitis & Mezey 1997). Two different types of microglia exist in the CNS, the resident microglia, and the newly differentiated microglia that are derived from bone-marrow precursor cells (Simard & Rivest 2004). Bone-marrow-derived and CNS-resident microglia remain indistinguishable on the basis of current cell-surface markers, although they may be functionally distinct populations. It has been reported that transplantation of green fluorescent protein (GFP)-expressing stem cells into lethally irradiated mice resulted in the infiltration of GFP expressing cells into the CNS (Simard & Rivest 2004, Priller *et al.* 2001). Four months after transplantation, up to a quarter of the regional microglial population was donor derived. Closer inspection of these cells showed that they had a morphology very similar to that of microglia. A landmark study by Priller and colleagues demonstrated a role for these cells in injury (Priller *et al.* 2001). They found that induction of cerebral ischemia in these chimeric mice 4 weeks after transplant resulted in massive infiltration of GFP-labelled cells to the site of injury. However, recent work has cast doubt on the ability of bone-marrow-derived cells to cross the BBB and differentiate into microglia, and these studies suggest that

irradiation, followed by bone-marrow cell transplantation, artificially increases the number of cells infiltrating into the CNS (Ajami *et al.* 2007, Mildner *et al.* 2007).

1.2.3.2. T cells

For a long time it was thought that circulating lymphocytes could not enter the CNS unless the BBB had been compromised. This understanding of the role (or lack thereof) of the adaptive immune system in the CNS was irrevocably altered with the discovery that T cells, regardless of their antigen specificity, had the potential to penetrate the intact BBB (Wekerle *et al.* 1987). While the antigen specificity of the T cells was found to be irrelevant, their activation state was a key determinant of their ability to invade the CNS, with activated T cells being capable of crossing the BBB, while naïve T cells were excluded. It was subsequently reported that although antigen specificity was unimportant in determining entry into the CNS, only T cells capable of recognising CNS-resident antigens remained in the CNS (Hickey *et al.* 1991). The entry of T cells into the CNS has been found to be critically dependent on interactions between very late antigen (VLA)-4 and vascular cell adhesion molecule (VCAM)-1 (Baron *et al.* 1993, Roffe *et al.* 2003). Interaction of lymphocyte function-associated antigen (LFA)-1 and ICAM-1 has also been implicated in T cell migration, but the role of this interaction is not yet clear (Reiss *et al.* 1998, Bullard *et al.* 2007).

It is thought that the initial presentation of antigen to CNS-reactive T cells occurs in the cervical lymph node, as removal of the cervical lymph nodes delays the onset of EAE (Furtado *et al.* 2008). Following their activation, T cells can then cross the BBB and enter the CNS (Wekerle *et al.* 1987). Once in the CNS, activated T cells specific for CNS antigen can induce glial cell activation and BBB disruption (Smorodchenko *et al.* 2007). The subsequent effect of the T cell in the CNS may depend on its cytokine profile. Recent evidence suggests that Th1 and Th17 cells are involved in the pathogenesis of EAE. Furthermore, Th1 cells

have been implicated in encephalitis seen in patients immunised with Aβ in QS-21 adjuvant as part of a clinical trial for the treatment of AD (Kroenke *et al.* 2008, Weiner & Frenkel 2006). The role of Treg cells in protection against autoimmunity is well defined, and Treg cells have been demonstrated to suppress autoimmune responses in models of diabetes, EAE, and collagen-induced arthritis (Chatenoud & Bach 2005, Morgan *et al.* 2005, Kohm *et al.* 2002). On the other hand, the role of Th2 cells in the pathogenesis of CNS autoimmune diseases is less certain. Cao and colleagues have demonstrated a cognitive benefit of administering Aβ-specific Th2 cells to a transgenic mouse model of AD (Cao *et al.* 2009), and MBP-specific Th2 cells were shown to decrease the severity of Th1 cell-induced EAE (Cua *et al.* 1995). In addition, IL-4 has been demonstrated to be protective in mouse models of EAE, and IL-$4^{-/-}$ mice are resistant to the development of EAE (Racke *et al.* 1994, Falcone *et al.* 1998). In contrast, it has been shown in a different mouse model that MBP-specific Th2 cells were unable to inhibit Th1 cell-induced EAE, and that Th2 cells could cause EAE in immunodeficient $RAG^{-/-}$ mice (Lafaille *et al.* 1997). Thus, it would appear that the suppressive abilities of Th2 cells in autoimmune diseases are dependent on a number of as yet unknown factors, and require further investigation.

1.3. Alzheimer's disease

1.3.1. General Background

The first case of what is now known as AD was described by Alois Alzheimer in 1906 (Brettschneider *et al.* 2005). It was named by Kraeplin in 1910 based on the description of the original cases. It is the most common neurodegenerative disease, accounting for approximately two thirds of all cases. It has a prevalence of approximately 1 percent among persons from 65 to 69 years of age, but this increases dramatically with age to some 40-50% in persons 95 years of age and

over (Hy & Keller 2000). This disease results in the slow but irreversible loss of neurons, particularly in the cortex and hippocampus. It starts with lapses in episodic memory and eventually leads to a marked dementia with features including impaired judgment and memory, altered behaviour, and a general decline in cognitive function.

The vast majority of cases of AD are sporadic, and no obvious inheritance pattern is seen. Although the vast majority of cases occur among persons 60 years and older, approximately 6-7% of all cases of AD have an early onset and patients develop disease before 61 years of age (Nussbaum & Ellis 2003). Of all cases of AD, about 1 percent are familial AD (FAD), and have an autosomal-dominant pattern of inheritance. Linkage studies have indicated that mutations in three genes are responsible for a large percentage of cases of autosomal-dominant AD (Kar *et al.* 2004). These genes are the amyloid-precursor protein (APP) on chromosome 21, presenilin 1 (PS1) on chromosome 14, and presenilin 2 (PS2) on chromosome 1. Sporadic AD by contrast, shows no obvious signs of familial inheritance of any one gene, and it is likely that there exist a number of different mutations across different genes that associate with the disease (Bertram & Tanzi 2008). One such candidate is the ε4 allele of the apolipoprotein E gene (ApoE4), and possessing a single copy of this allele may increase the risk of AD by as much as 2-5 times. Other risk factors for AD include obesity, diabetes, arthrosclerosis, smoking, gender, and coronary heart disease (Luchsinger 2008, Azad *et al.* 2007, Rosendorff *et al.* 2007). Onset of sporadic AD is probably driven by a combination of environmental and genetic factors, with no one factor appearing to be dominant.

Clinical diagnosis of Alzheimer's disease rests largely on excluding other dementias (McKhann *et al.* 1984), with definitive diagnosis requiring post-mortem observation of lesions in the hippocampus and cortex. Neuronal loss, along with the formation of Aβ plaques and neurofibrillary tangles, are recognised as pathological hallmarks of the disease (Monsonego *et al.* 2001).

1.3.2. Neurofibrillary Tangles

Neurofibrillary tangles (NFT) are composed of hyperphosphorylated tau protein (Iqbal *et al.* 2005, Kosik *et al.* 1986, Wood *et al.* 1986). Tau is a normal axonal protein that is responsible for promoting microtubule assembly and stability. Tau controls these processes according to its state of phosphorylation, with an increase in phosphorylation favouring the depolymerisation of microtubules (Wen *et al.* 2005, Lindwall & Cole 1984). In AD, tau fibres consist of pairs of filaments wound into helices (paired helical fragments; (Lim *et al.* 2005). The number and localisation of NFT have been correlated with the severity of dementia, leading to the popular notion that these tangles are a key component of the clinical manifestations of AD (Arriagada *et al.* 1992a, Arriagada *et al.* 1992b). However, the mechanism of this neuronal death is currently unknown. Transgenic mice overexpressing P301L tau, a common mutation found in human AD, had severe motor and behavioural disturbances by 10 months of age (Lewis *et al.* 2000). They developed NFT and this was associated with neuronal loss, particularly in the spinal cord. It has also been suggested that tau can have pathogenic actions prior to NFT formation, and overexpression of tau in Drosophila leads to neurodegeneration, without the development of NFTs (Wittmann *et al.* 2001).

1.3.3. Neuritic Plaques

Neuritic plaques are lesions consisting of a core of Aβ deposits surrounded by reactive astrocytes, dystrophic neuritis, and activated microglia. The major amyloid peptides that are found in the plaques are β-amyloid$_{1\text{-}42}$ (Aβ_{42}) and Aβ_{40}. The majority of Aβ produced in normal metabolism is Aβ_{40}, while Aβ_{42} is the predominant form in senile plaques. The prevalence of Aβ plaques does not directly correlate with disease severity, although Naslund and colleagues have

reported a general correlation between total levels of Aβ and cognitive decline (Naslund *et al.* 2000).

1.3.4. Amyloid precursor protein (APP)

Aβ is produced from APP, a widely expressed 751-770 residue glycosylated transmembrane protein with a large hydrophilic extracellular domain, a hydrophobic 23 residue transmembrane domain and a small cytoplasmic domain (Turner *et al.* 2003). There are three major isoforms of APP, of 695, 751, and 770 residues (Kitaguchi *et al.* 1988). The 751 and 770 residues isoforms are ubiquitously expressed throughout the body and brain, whereas the expression of the 695 isoform is restricted mainly to neurons (Haass *et al.* 1991). Although the normal physiological role of APP is still unclear, there are some clues as to its function. It has been implicated in cell adhesion and motility (Breen *et al.* 1991, Sabo *et al.* 2001), however the most substantiated role for APP is in modulating neurite outgrowth and synaptogenesis. It has been reported that lack of APP results in enhanced neuritic outgrowth and thus an increased sensitivity to glutamate excitotoxicity (Priller *et al.* 2006). The importance of APP in synaptic plasticity has been demonstrated in studies using APP-transgenic mice. APP null mice show gliosis (Zheng *et al.* 1995), decreased survival of cultured neurons, and impaired LTP (Seabrook *et al.* 1999), as well as impairments in spatial learning in cognitive tests (Dawson *et al.* 1999). A recent study has shown an interaction between APP and a protein known as netrin-1, and this interaction decreased production of Aβ in the brains of APP-transgenic mice (Lourenco *et al.* 2009). Enhanced cleavage of APP, as is seen in patients with AD, may exacerbate the Aβ-induced neuronal damage seen in this disease. This cleavage may have a detrimental effect on memory and cognition, through increased production of the neurotoxic form of Aβ and through decreased availability of the neuroprotective, full-length APP.

1.3.5. Processing of APP

There are two proteolytic processing pathways of APP and its metabolic derivatives: the non-amyloidogenic pathway, which cleaves APP to form soluble APPα (sAPPα), and the amyloidogenic pathway, which produces Aβ peptides. APP is cleaved by distinct α-secretase or β-secretase pathways. The α-secretase enzyme cleaves APP between Lys-613 and Leu-614 residues to generate the neuroprotective peptide sAPPα and a 10 kDa C-terminal APP fragment that can be further processed by γ-secretase to generate the P3 peptides. The identity of the α-secretase protein is unknown, but one candidate that has been proposed as a candidate for α-secretase is TNF-α-converting enzyme, which releases TNF-α from its membrane-bound precursor (Black *et al.* 1997).

The amyloidogenic pathway, which results in the formation of the Aβ peptide, is mediated by the sequential action of β-secretase (β-APP cleaving enzyme or BACE) and γ-secretase. Cleavage of APP by β-secretase yields sAPPβ and a membrane bound C-terminal fragment. Further cleavage of this fragment by γ-secretase yields the $A\beta_{40}$ and $A\beta_{42}$ peptides (Wisniewski *et al.* 1997, Selkoe 2001). γ-secretase is considered to be a putative, multicomponent aspartyl protease containing nicastrin and presenilins (Kimberly *et al.* 2003, Esler *et al.* 2002).

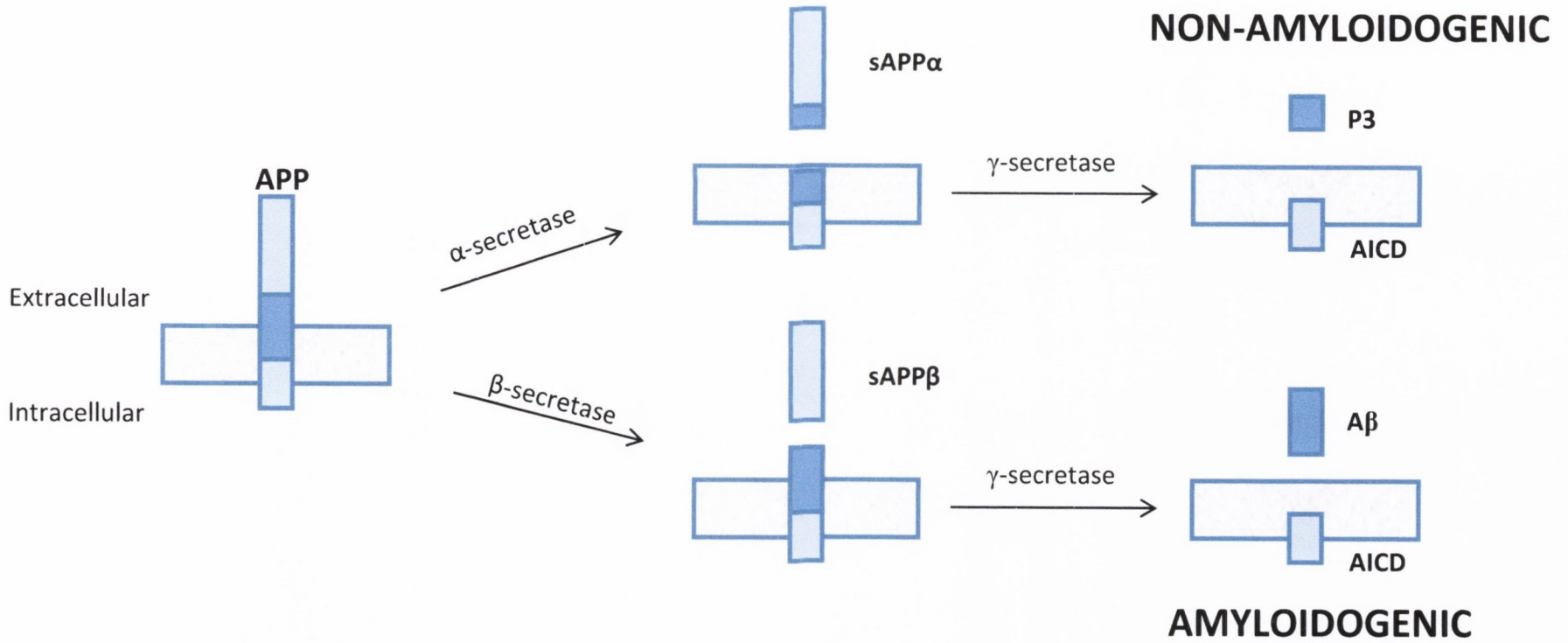


Figure 1.2. Processing pathways of APP

APP is a type I transmembrane glycoprotein that is processed by 2 distinct pathways. In normal individuals, the majority of APP is processed by the non-amyloidogenic pathway. In this pathway, APP is cleaved by α-secretase to generate soluble APP-alpha (sAPPα). The C-terminal fragment is then cleaved by γ-secretase to yield the P3 peptide and the APP intracellular domain (AICD). The amyloidogenic pathway is mediated by the sequential action of β-secretase and γ-secretase. Cleavage of APP by β-secretase releases sAPPβ. Further cleavage of this fragment by γ-secretase yields the $A\beta_{40}$ and $A\beta_{42}$ peptides

1.3.6. Amyloid-beta (Aβ)

Aβ was originally isolated from amyloid-laden meningeal arterioles and venules that are often found just outside of the brains of patients with AD or Down's syndrome (Glenner & Wong 1984b, Glenner & Wong 1984a). As it is the major constituent of the neuritic plaques found in AD, it was initially thought to be an abnormal protein. Using an Aβ-specific antibody, it was later found that Aβ is produced constitutively during normal cell metabolism (Haass *et al.* 1991). Shortly after the elucidation of the amino acid sequence of Aβ, the β-amyloid precursor protein (APP) gene was sequenced (Kang *et al.* 1987).

The majority (~90%) of Aβ peptides secreted are 40 amino acids in length, however in patients with AD this balance seems to shift more towards the 42 amino acid form ($A\beta_{42}$), which is highly hydrophobic and more toxic to neurons than the shorter peptide. Neuritic plaque amyloid was first sequenced in 1985 and was found to be primarily $A\beta_{42}$ (Masters *et al.* 1985). Soluble Aβ is primarily $A\beta_{40}$, although shorter and longer sequences exist. Attempts to study the effects of Aβ on learning and memory are complicated by the fact that it can exist in a variety of different forms, including monomers, oligomers, protofibrils, and fibrils (Walsh *et al.* 1999). Additionally, the sites of accumulation of Aβ and its pathophysiological concentrations are unknown, making it difficult to replicate these conditions using exogenous Aβ.

1.3.7. The amyloid cascade hypothesis

The central hypothesis for the cause of Alzheimer's disease is the amyloid cascade hypothesis (Hardy 1996), which states that the process of neuronal death and memory loss seen in AD is caused by an imbalance between the production and clearance of Aβ. In normal metabolism, Aβ levels are regulated by amyloid degrading-enzymes, which include neprilysin and insulin-degrading enzyme

(IDE). Each of these proteases degrades a specific type of Aβ: neprilysin and IDE degrade Aβ monomers, while other proteases cleave the fibrillogenic and oligomeric types. However, the imbalance between production and clearance seen in AD leads to a gradual accumulation in soluble Aβ, which then is thought to undergo a conformational change to a high β-sheet content, giving it a propensity to aggregate into soluble oligomers and insoluble fibrils found in Aβ plaques.

Since Aβ was first identified as the main component of neuritic plaques in AD, a number of observations have indicated that this peptide is the primary neuropathological insult in AD. Firstly, a large percentage of cases of FAD are associated with mutations in APP (Selkoe 2001). Individuals with Down's syndrome, who have 3 copies of the APP gene, develop Aβ deposits and the neuropathology associated with AD at a much earlier age than normal (Lemere *et al.* 1996). The other known genes involved in development of FAD, PS1 and PS2, appear to function as part of the γ-secretase complex in the processing of Aβ (Citron *et al.* 1997, Spasic *et al.* 2006). In addition, large amounts of Aβ are neurotoxic to cells (Goodman & Mattson 1994). It is thought that it is the oligomeric form of Aβ that is the most toxic and indeed cerebral microinjection of conditioned medium containing oligomers, but no fibrils, markedly inhibited long-term potentiation (LTP) in rats *in vivo* (Walsh *et al.* 2002). It is unclear precisely how Aβ mediates these toxic effects on neurons but suggestions include induction of apoptotic genes through inhibition of Wnt (Caricasole *et al.* 2003), and formation of ion channels in neurons (Kagan *et al.* 2002). Aβ also activates microglia, resulting in the release of proinflammatory cytokines and toxic factors such as nitric oxide from microglia (Block & Hong 2005).

Though tau appears to be responsible for at least some of the neuronal death seen in AD, studies have shown that NFT-induced neuronal death is potentiated by Aβ (Lewis *et al.* 2001, Oddo *et al.* 2004, Gotz *et al.* 2001). Injection of Aβ into the hippocampus region of P301L tau transgenic mice resulted in a five-fold increase in NFT (Gotz *et al.* 2001). Another study crossed

Aβ-overexpressing transgenic mice with P301 tau mice and found more than a seven-fold increase in NFT in the entorhinal cortex and the amygdala (Lewis *et al.* 2001). This link between tau and Aβ appears to be unidirectional, as increasing tau levels in a transgenic model of AD had no effect on Aβ deposition (Oddo *et al.* 2007). Taken together, these data imply that tau is downstream of Aβ in the cascade of events leading to neuronal death.

1.4. Models of Alzheimer's disease

1.4.1. Non-transgenic animal models of Alzheimer's disease

Initial models of AD were focused towards replicating the symptoms of AD, rather than to the pathology. A number of models were developed that involved pharmacologic blockade of the cholinergic system (Smith 1988, Everitt & Robbins 1997), but these models did not increase Aβ plaques or NFT and so offered little opportunity to identify methods which might modify the underlying causes of disease. Subsequent efforts to generate a satisfactory animal model of AD have been based on mimicking the increased Aβ load seen in disease. The majority of these models have involved intracerebral or intracerebroventricular (i.c.v.) infusions of synthetic Aβ peptides (Stephan & Phillips 2005).

The ability of Aβ infusion to replicate the neuronal death and glial cell activation seen in AD depends greatly on a number of factors, including the animal strain, the species and dose of Aβ used, and the aggregation state of the peptide. Initial studies reported that injection of SDS-isolated amyloid cores into the rat hippocampus resulted in neuronal loss in the immediate vicinity of the injected amyloid (Frautschy *et al.* 1992). Games and colleagues were unable to detect any neuronal death or glial activation following administration of $A\beta_{40}$, but this may have been due to the fact that this is a soluble rather than oligomeric or

fibrillar form of Aβ (Games *et al.* 1992). In support of this theory, intrahippocampal injection of $A\beta_{42}$ induced neuronal loss and glial cell activation in rats (Ryu *et al.* 2004).

Infusion of Aβ has also been reported to cause cognitive impairments. Aβ administration has been shown to have no effect on storage or retention of a task that has already been learned (Maurice *et al.* 1996), suggesting that Aβ in this model primarily affects working memory rather than affecting the ability to recall stored information (Stephan & Phillips 2005). Aβ has been shown to affect short-term and long-term memory. In studies looking at the effects of Aβ on short-term memory, the species of Aβ used does not appear to determine memory loss, and mice injected with either $A\beta_{40}$, $A\beta_{35}$, or $A\beta_{42}$ have been shown to exhibit deficits in memory (Flood *et al.* 1991, Maurice *et al.* 1996, Nakamura *et al.* 2001). Long-term effects of Aβ on memory appear to require an aggregated form of Aβ, which is not cleared as effectively as soluble Aβ and thus lasts for longer in the CNS. $A\beta_{1-42}$ has been shown to induce a spatial working memory deficit in rats up to 80 days after infusion of the peptide (Nakamura *et al.* 2001), and memory deficits in spatial learning following injection of aggregated, but not soluble $A\beta_{25-35}$, lend further support to this argument (Delobette *et al.* 1997).

1.4.2. Transgenic animal models of Alzheimer's disease

The identification of genes associated with the development of AD (APP, PS1, and PS2) has led to the development of genetically modified animal models of AD. These models can replicate a number of the pathological hallmarks of AD, such as Aβ plaque formation, microglia and astrocyte activation, and neuroinflammation, as well as some of the cognitive deficits seen in AD (Eriksen & Janus 2007). All of these mouse models have contributed significantly to our understanding of the disease process. However, because there are multiple factors associated with the disease, and because the process of Aβ accumulation is a

complex one, no mouse model has yet been developed that accurately models all of the pathological and behavioural symptoms of AD.

1.4.2.1. APP-based transgenic mouse models

Initial attempts at generating a mouse model of AD focused on the overexpression of the wild-type human APP gene, but with the exception of the NSE:β-APP751 mouse, these attempts were mostly unsuccessful (Higgins *et al.* 1993, Marx 1992). NSE:β-APP751 mice express the APP751 isoform under the control of the neuron-specific enolase promoter (NSE). These mice were found to have deficits in spatial memory at 12 months of age (Moran *et al.* 1995), and had an increased amyloid burden with age (Higgins & Cordell 1995). However, they did not develop neuritic plaques or NFT (Higgins & Cordell 1995), and have consequently received little attention as a transgenic model of AD.

The first transgenic mouse model to successfully develop neuritic Aβ plaques, along with associated neuropathology, was the PDAPP mouse (Games *et al.* 1995). This mouse model overexpresses human APP containing the V717F mutation, favouring the production of $A\beta_{42}$ over $A\beta_{40}$. PDAPP mice have a ten-fold overexpression of APP compared to wild-type animals. They develop a number of hallmarks of AD, including neuritic plaques, dystrophic neurites, astrogliosis, and microgliosis (Chen *et al.* 1998). Plaques are mainly localised to the hippocampus and entorhinal cortex, modelling the plaque distribution seen in AD (Reilly *et al.* 2003), although there is no evidence for accompanying neuronal loss in these areas, even at 18 months of age (Irizarry *et al.* 1997). Behavioural tests on the PDAPP mouse have shown both age-dependent and age-independent deficits in spatial memory (Dodart *et al.* 1999, Chen *et al.* 2000), with the age-dependent impairment appearing to correlate with plaque burden (Chen *et al.* 2000).

Perhaps the most widely used APP-transgenic mouse is the Tg2576 mouse model (Hsiao *et al.* 1996). This mouse model overexpresses the APP695 isoform with the Swedish double-mutation under the control of the prion protein (PrP) promoter. Aβ levels increase rapidly at 6 months of age, and Aβ plaque deposition begins at between 9 and 12 months of age (Kawarabayashi *et al.* 2001). These animals show similar patterns of Aβ expression to the PDAPP mice, with Aβ plaques appearing in the entorhinal cortex and hippocampus (Dong *et al.* 2007). Tg2576 mice also display astrogliosis, microgliosis, oxidative stress, and dystrophic neurites in areas containing plaques (Pappolla *et al.* 1998, Frautschy *et al.* 1998). Again, these mice display no neuronal loss (Eriksen & Janus 2007). They do, however, have cognitive impairments in spatial and working memory which are associated with increases in Aβ deposition (Hsiao *et al.* 1996, Zhuo *et al.* 2008).

Other APP-transgenic models of AD developed include the APP23 mouse and the TgCRND8 mouse. The APP23 mouse was the first transgenic mouse model to display neuronal loss (Calhoun *et al.* 1998), though this neuronal loss does not appear to lead to a cognitive deficit (Lalonde *et al.* 2002). TgCRND8 mice display early onset amyloid pathology, with detectable Aβ deposits at 3 months of age, and neuritic plaques observed at 5 months (Chishti *et al.* 2001). They also have significant memory impairments at an early age, allowing therapeutic experiments to be performed on young animals. However, these mice have an early lethality, with only 50% of mice surviving to 12 months of age, making longer term experiments more difficult (Chishti *et al.* 2001). These models provide extremely useful tools for the study of AD, but the length of pre-symptomatic illness has led to the creation of double transgenic APP/PS1 animals in order to accelerate both Aβ deposition and learning impairments.

1.4.2.2. APP/PS1 transgenic mouse models

Following the discovery that mutations in the presenilin genes were linked to the development of FAD, Duff and colleagues generated a transgenic mouse overexpressing either mutant (M146L) or wild-type PS1 (Duff *et al.* 1996). Overexpression of mutant, but not wild-type PS1, was found to selectively increase the amount of $A\beta_{42}$ in the brain (Duff *et al.* 1996), though these mice did not develop Aβ plaques. Crossing of these PS1 transgenic mice with the Tg2576 mouse generated progeny that had 3-5 times greater accumulation of $A\beta_{40}$ and $A\beta_{42}$ than single-transgenic littermates (Holcomb *et al.* 1998). Furthermore, these so-called PSAPP mice exhibited accelerated plaque pathology, with plaques detectable as early as 3 months of age, with concomitant microgliosis and astrogliosis. By 6-8 months, amyloid deposition was apparent in most cortical regions as well as in the hippocampus, showing a similar distribution to that seen in AD (Holcomb *et al.* 1998). Testing of PSAPP mice in the radial-arm water maze (RAWM) showed impaired spatial learning in these mice, which was correlated with an increase in Aβ load in the cortex and hippocampus (Gordon *et al.* 2001). Urbanc and colleagues demonstrated that thioflavin-S positive Aβ plaques from PSAPP mice were neurotoxic *in vivo* (Urbanc *et al.* 2002), although others have shown that this neuronal loss is relatively small (Takeuchi *et al.* 2000). This early plaque deposition, combined with spatial impairments, makes the double transgenic model of disease very attractive, as it facilitates studies that would be very difficult in the older and thus more fragile single transgenic animals.

Another double transgenic model which is becoming increasingly prevalent in therapeutic studies on AD is the APPswe/PS1dE9 mouse (Jankowsky *et al.* 2001, Jankowsky *et al.* 2004). This mouse was developed by co-injection of the APP695 isoform containing the Swedish mutation and exon-9-deleted PS1 into pronuclei, with each gene under the control of an independent PrP promoter (Jankowsky *et al.* 2001). This results in co-localisation and co-segregation of

these genes. Aβ deposition can be observed in these mice as early as 4 months of age, with substantial plaque formation in the hippocampus by 6 months of age (Garcia-Alloza *et al.* 2006). $A\beta_{1-42}$ levels are increased in this model, while $A\beta_{1-40}$ levels are unchanged (Jankowsky *et al.* 2004). Behavioural testing of these mice revealed deficits in spatial memory in the standard Morris water maze (MWM) task (Cao *et al.* 2007, Jankowsky *et al.* 2005), and also a more profound deficit in episodic-like memory in the repeated-reversal and RAWM tasks (Savonenko *et al.* 2005, Jankowsky *et al.* 2005). The age at which these behavioural deficits occur appears to depend on the task and the protocol. It has been reported that 6 month old mice were unimpaired in the Morris water maze task (Savonenko *et al.* 2005), while mice aged 8 months had impaired spatial memory in the same task (Cao *et al.* 2007, Jankowsky *et al.* 2005). A recent study has suggested that episodic-like memory is the first type of memory to be affected in APPswe/PS1dE9 mice, as it requires successful suppression of old memory traces (Reiserer *et al.* 2007).

1.5. Role of the immune system in Alzheimer's disease

1.5.1. The CNS immune system

AD was one of the first neurodegenerative diseases associated with microglial activation. Studies of brain sections from AD patients found microglia with marked expression of MHC class II on their surface to be associated with Aβ plaques (Luber-Narod & Rogers 1988, Rogers *et al.* 1988, Perlmutter *et al.* 1992). This microglial response to Aβ was initially thought to be a passive reaction to the neuronal death caused by the Aβ plaques. It is now known that Aβ can recruit and activate microglia, causing the release of neurotoxic factors from microglia such as nitric oxide, IL-1β, and TNF-α (Floden & Combs 2006, Ii *et al.* 1996). Indeed, a recent study has shown that Aβ activates the NALP-3 inflammasome, leading to

the production of proinflammatory cytokines and neurotoxic factors, and that the inflammasome is a critical factor in the recruitment of microglia to Aβ plaques in the brain (Halle *et al.* 2008). In addition to producing proinflammatory and neurotoxic factors, microglia are also capable of phagocytosing Aβ by at least two distinct mechanisms (Koenigsknecht & Landreth 2004, Bard *et al.* 2000).

Microglia are competent phagocytes and can phagocytose both amyloid aggregates and plaques *in vitro* (Paresce *et al.* 1996, Ard *et al.* 1996, DeWitt *et al.* 1998a). It has also been shown that once activated by Aβ, microglial phagocytosis remains elevated after removal of Aβ from the culture medium (Kopec & Carroll 1998). Injection of Aβ cores isolated from AD patients into the cortex and hippocampus of rats has been shown to induce microglial phagocytosis of the injected Aβ (Frautschy *et al.* 1992). Furthermore, soluble Aβ injected into the rat striatum is phagocytosed by microglia within 1 day of injection, though fibrillar Aβ persists for longer (Weldon *et al.* 1998). In line with these findings, microglia cultured with Aβ fibrils have been shown to ingest the Aβ, which is then localised to the phagosome (Frackowiak *et al.* 1992). It should be noted however, that while microglia rapidly internalise Aβ, they appear not to be very efficient in degrading it and consequently may be overwhelmed by the large amounts of Aβ produced in AD (Paresce *et al.* 1997, Chung *et al.* 1999).

However, it is clear that despite their association with Aβ plaques *in vivo*, microglia fail to efficiently phagocytose Aβ plaques in either the AD brain or in mouse models of AD. A number of hypotheses have been proposed as to why microglia cannot efficiently phagocytose these plaques. It is possible that the large aggregates of fibrils found *in vivo* cannot be completely enclosed by microglial pseudopodia, and thus cannot be degraded (D'Andrea *et al.* 2004). Stalder and colleagues reported that in APP23 transgenic mice, microglia were unable to effectively phagocytose Aβ plaques, despite their close physical association with them (Stalder *et al.* 2001). Three-dimensional reconstruction showed that, while the microglia had internalised bundles of amyloid, these bundles remained

connected to extracellular amyloid deposits, preventing phagocytosis of the plaques. It has also been reported that Aβ-containing microglia accumulated around the blood vessels following injection of Aβ cores into the cortex and hippocampus (Frautschy *et al.* 1992). Aβ was detected along the vessel walls, leading the authors to speculate that perhaps microglia were moving the Aβ to the vasculature rather that degrading it. Streit and colleagues have proposed the idea that microglia may become dysfunctional with age, and thus cannot effectively phagocytose Aβ plaques (Streit 2004). Consistent with this hypothesis, there is a large increase in the number of apoptotic microglia in the AD brain (Lassmann *et al.* 1995). In addition, it has been shown that while neonatal and adult microglia could both phagocytose Aβ oligomers in vitro, adult microglia had a decreased ability to phagocytose Aβ fibrils (Floden & Combs 2006).

The mechanism whereby microglia phagocytose Aβ has been the subject of much debate, and CD14 appears to be necessary for the interaction of Aβ and microglial cells (Bate *et al.* 2004). Koenigsknecht and colleagues have provided evidence that microglia interact with Aβ through a distinct mechanism involving a complex consisting of the B-class scavenger receptor CD36, $\alpha_6\beta_1$ integrin, and integrin-associated protein (CD47; (Koenigsknecht & Landreth 2004). There is also evidence that microglia phagocytose Aβ plaques by a mechanism involving engagement of Fc antibody receptors (FcR) by Aβ-specific antibodies bound to the plaques (Bard *et al.* 2000, Schenk *et al.* 1999), and this is consistent with in vitro studies which show enhanced phagocytosis of Aβ-IgG conjugates (Paresce *et al.* 1996, Brazil *et al.* 2000). However, the requirement for FcR in the removal of Aβ deposits has been contested (Das *et al.* 2003b). Alternatively, plaques may be phagocytosed by a complement-mediated mechanism (Wyss-Coray *et al.* 2002). It would thus appear that microglia can phagocytose Aβ by a number of different mechanisms, and that this may be related to the activation state of the microglial cell, as well at the cytokine milieu present at the time of microglial activation. Indeed, it has been shown that microglial phagocytosis of fibrillar Aβ can be inhibited by the addition of proinflammatory cytokines (Koenigsknecht &

Landreth 2005), and that this is reversed by co-incubation with anti-inflammatory cytokines.

As well as the beneficial effects of phagocytosing plaques in AD, microglia can have a neurotoxic role in the disease process. Microglia treated with Aβ produced factors which could then kill neurons *in vitro* (Giulian *et al.* 1996). Importantly, removal of microglia from these cultures almost totally eliminated the toxic effects of Aβ on cultured neurons, suggesting that microglia, or the substances they produce, can mediate the neurotoxic effects of Aβ. Fibrillar Aβ-stimulated microglia also produce reactive oxygen species and nitric oxide (Akiyama *et al.* 2000). Initial evidence that microglia play a role in inflammation in the CNS of AD patients came from studies showing that IL-1β, IL-6, and TNF-α expression is upregulated in these individuals, and that it was predominantly associated with AD plaques (Griffin *et al.* 1989, Wood *et al.* 1993, Cacabelos *et al.* 1994). These cytokines were subsequently found to be produced predominantly by activated microglia (Lue *et al.* 2001b, Griffin *et al.* 1995). Microglia isolated from brain tissue of AD and non-demented patients showed increased secretion of IL-1β, IL-6, TNF-α, and IL-8 following treatment with Aβ (Lue *et al.* 2001a). Exposure of cultured neonatal microglia to Aβ, particularly in the presence of inflammatory mediators, such as LPS and IFN-γ, results in increased IL-1β, IL-6, and TNF-α production (Gasic-Milenkovic *et al.* 2003, Meda *et al.* 1995, Combs *et al.* 2001). Intraperitoneal injection of LPS was subsequently shown to induce IL-1β expression, microglial activation, and tau phosphorylation, but not Aβ deposition in a triple-transgenic model of AD (Kitazawa *et al.* 2005).

A number of recent reports have cast doubt on the hypothesis that inflammation is solely detrimental in AD, and it may even have a beneficial role in certain circumstances (Wyss-Coray 2006, Shaftel *et al.* 2008). Intracerebral injection of LPS, which activates microglia to produce IL-1β and IL-6, has been shown to reduce Aβ plaques in mouse models of AD (DiCarlo *et al.* 2001, Herber

et al. 2007, Herber *et al.* 2004). This mechanism of plaque removal appears to require microglial activation, as treatment of these mice with dexamethasone reduced microglial activation and reversed the LPS-induced plaque clearance (Herber *et al.* 2007). In addition, overexpression of IL-1β in the hippocampus of APPswe/PS1dE9 mice led to a decrease in Aβ load which was associated with an increased number of microglia in contact with Aβ plaques (Shaftel *et al.* 2007b). This evidence points towards a potential beneficial role of neuroinflammation in AD, although it is important to note that LPS has also been reported to increase Aβ deposition (Sheng *et al.* 2003, Qiao *et al.* 2001). Thus, depending on the circumstances, it would appear that microglial inflammation can be beneficial in AD.

Activation of microglia by Aβ is associated with increases in chemokine production, attracting more microglia to the sites of Aβ deposition in the brain (Rogers *et al.* 2002). Microglia cultured from postmortem AD brains showed increased expression of the chemokines MCP-1, and MIP-1α following exposure to Aβ (Lue *et al.* 2001b). A recent study by El Khoury and colleagues has shown that Tg2576 mice deficient in CCr2, the receptor for MCP-1, accumulated Aβ earlier in life and died prematurely (El Khoury *et al.* 2007). This was found to be correlated with a decreased accumulation of microglia at the sites of plaque formation, thus indicating a protective role for microglia in plaque clearance. Additionally, it has been shown that bone-marrow derived monocytes from the periphery migrated across a blood-brain barrier model in response to Aβ, implicating a role for these cells in AD (Fiala *et al.* 1998).

1.5.2. Bone-marrow-derived microglia

It has been demonstrated that newly differentiated bone-marrow-derived microglia express higher levels of proteins required for antigen presentation, and may therefore be more efficient at clearing Aβ plaques than microglial cells

(Simard & Rivest 2004). Recently, a number of studies have examined the role of bone-marrow-derived cells in the brain during the development and progression of AD. Initial studies reported that transplantation of bone-marrow cells from GFP expressing mice into lethally irradiated APPswe/PS1dE9 transgenic mice resulted in increased recruitment of GFP expressing cells to the CNS relative to wild-type controls (Malm *et al.* 2005). This effect was seen in young, but not aged mice. Local inflammation caused by intrahippocampal injection of LPS significantly increased the recruitment of bone-marrow-derived cells in aged APP-transgenic mice and decreased hippocampal Aβ burden (Malm *et al.* 2005). In an attempt to establish whether these bone-marrow-derived cells are beneficial or detrimental in the development of AD, Simard and colleagues generated APPswe/PS1-transgenic animals expressing thymidine kinase protein under the control of the CD11b promoter. When these cells are treated with gancyclovir, monocytes are eliminated when they undergo cellular division. Elimination of BM-derived cells from the CNS resulted in a significant increase in Aβ plaque size in APP-transgenic animals (Simard *et al.* 2006). They also showed that the number of bone-marrow-derived cells in the CNS decreases at 9 months, a time when plaque size expands rapidly. Thus, the development of plaques in AD may be due to an age-dependent decrease in the number of bone-marrow-derived microglia in the CNS (Soulet & Rivest 2008). These results should be interpreted with caution however, as recent studies have called into question the functional relevance of these models of cell infiltration. Recently, Mildner and colleagues identified LY-$6C^{hi}$ $CCR2^{+}$ monocytes as precursors of microglia (Mildner *et al.* 2007). They demonstrated that when the BBB remains intact, only negligible numbers of bone-marrow-derived cells entered the CNS except under special circumstances, such as irradiation of the CNS. As the BBB is reported to be intact in AD patients, the role of these cells in the clearance of Aβ plaques may have been overestimated in previous experiments.

1.5.3. T cells

Recent experiments have uncovered links between T cell activation and the development of AD. Self-reactive T cells are not necessarily deleted during negative selection in the thymus (Gammon & Sercarz 1989), and indeed some weakly autoreactive T cells are positively selected (Sakaguchi *et al.* 2003). Although such self-reactive T cells can mediate autoimmune diseases, such as MS, they may also have a beneficial role in immune regulation and maintenance of normal tissue homeostasis. T cells were subsequently found to be capable of crossing the intact BBB (Wekerle *et al.* 1987), raising the possibility that they could be involved in CNS diseases, such as AD. The first study on such T cells was by Trieb and colleagues, who showed that lymphocytes from healthy controls, but not patients with AD, proliferated in response to $A\beta_{1\text{-}40}$ (Trieb *et al.* 1996). They concluded that this T cell response to Aβ could be important in the normal clearance of the peptide, and that this mechanism may be impaired in patients with AD. Studies of humoral responses to Aβ found that anti-Aβ antibody titers are decreased with age and are strongly decreased in patients with AD (Du *et al.* 2001, Brettschneider *et al.* 2005). A study in humans showed an increased T cell response to Aβ in the elderly (Monsonego *et al.* 2003b). This increased immunoreactivity appeared to be unique to the Aβ antigen, as the same patients that had increased responses to Aβ had decreased responses to anti-CD3/CD28 and to glatiramer acetate stimulation. Amino acids 20, 22, and 23, of the Aβ peptide were found to be particularly important in inducing T cell proliferation, as their mutation significantly decreased T cell proliferation. Interestingly, amino acids 22 and 23 are the sites of missense mutations in humans that lead to early onset of AD, providing further indirect evidence that impaired T cell responses are associated with AD. More recent studies have implicated a switch in the T cell response towards Treg cell response in AD. Loewenbrueck and colleagues studied T cell responses *ex vivo* using PBMCs, and found that AD was accompanied by a decrease in $CD4^+$ T cell derived IFN-γ and IL-2 production, with a concomitant increase in IL-10 production (Loewenbrueck *et al.* 2008). Furthermore, the

frequency of $CD4^+Foxp3^+$ Treg cells has been shown to increase with age, and $CD4^+CD25^{high}$ Treg cells from patients with AD had increased suppressive abilities (Rosenkranz *et al.* 2007). There is thus increasing evidence that alterations in T cell number and/or function play a role in the pathogenesis of AD.

1.5.4. Interaction of T cells and microglia

Upregulation of MHC class II and adhesion/co-stimulatory molecules on microglia activated in the course of immune-mediated CNS diseases suggests that this cell type may acquire APC function and play a role in T cell stimulation (Aloisi *et al.* 2000b, Kreutzberg 1996). Naïve T cells do not normally cross the BBB and, in general, they tend to circulate through lymphoid organs rather than through non-lymphoid tissues (Aloisi *et al.* 2000b). However, once activated, they gain the ability to penetrate the intact BBB (Wekerle *et al.* 1987). It would seem most likely that antigen is initially presented to CNS-reactive T cells outside of the CNS, and that these T cells subsequently cross the BBB and are restimulated upon interaction with APCs in the CNS (Becher *et al.* 2006). In AD MCP-1 is upregulated by BBB-associated glial cells, and this chemokine has been implicated in trafficking leukocytes across the BBB (Ma *et al.* 2002). Aβ can freely cross the BBB (DeMattos *et al.* 2001), and thus priming of Aβ-specific T cells can occur outside the brain. While there is no clear T cell response in the brains of AD patients, it may be the case that the numbers of Aβ-specific T cells are low in AD, and thus difficult to detect. Alternatively, these T cells may become activated in the periphery and express soluble mediators that exert their effects in the CNS. It is thus vitally important to understand the interaction of T cells with microglia, to assess their likely effects in the CNS of patients with AD.

To mimic the encounter between ramified microglia and pre-activated T cells, Aloisi and colleagues co-cultured an enriched population of $CD45^{low}CD11b^+$ microglia isolated from the adult mouse CNS with Th1 and Th2

cell lines from OVA transgenic mice (Aloisi *et al.* 1998). They found that Th1 cells induced expression of MHC class II, CD40, and CD54 on the surface of microglia, whereas Th2 cells failed to induce significant upregulation of these molecules (Aloisi *et al.* 2000a, Aloisi *et al.* 1998). T cell activation was found not to be required for expression of CD86 on microglia. Microglia isolated from the CNS of mice induced IFN-γ and IL-2 production by Th1 cells. Treatment of microglia with IFN-γ upregulated MHC class II, CD40, and CD54 expression, and conferred upon them the ability to present antigen to Th2 cells (Aloisi *et al.* 2000a). Microglia found to be co-localised with Aβ plaques in AD patients have been shown to express high levels of MHC class II on their cell surface (Luber-Narod & Rogers 1988, Rogers *et al.* 1988, Perlmutter *et al.* 1992). Das and colleagues investigated the effects of an $A\beta_{42}$ immunisation of Tg2576 mice with different MHC or HLA backgrounds on the generation of B and T cell responses (Das *et al.* 2003a). They found that the presence of certain MHC class II molecules, or combinations thereof, could influence the type of immune response generated against $A\beta_{42}$.

Binding of CD40 to its ligand, CD154, seems to be important in the interaction of microglia and T cells. CD40 is a member of the TNF receptor superfamily and is expressed on a wide variety of cells, including microglia (van Kooten & Banchereau 1997), while CD154 is expressed primarily on activated T cells, but is also expressed on astrocytes (Townsend *et al.* 2005). Tan and colleagues demonstrated that CD40-CD154 interaction in the presence of $A\beta_{42}$ increased production of TNF-α by microglia, and also enhanced microglial-induced neuronal injury over that induced by Aβ alone (Tan *et al.* 1999). Blockade of this interaction, by using mice lacking the CD40 or the CD154 gene, resulted in reduction of Aβ levels in APP-transgenic mice (Tan *et al.* 1999, Laporte *et al.* 2006). Furthermore, the interaction of CD40 and CD154 was found to decrease phagocytosis of Aβ by microglia (Townsend *et al.* 2005). In a study examining the role of CD80 and CD86 in the interaction of T cells and microglia, CD80 expression was found to be upregulated on microglia following treatment

with Th1 cells, while constitutive expression of CD86 was decreased (Wolf *et al.* 2001). Th2 cells were found to have no effect on expression of CD80 or CD86. In a separate study, Th1 cells were found to increase the expression of ICAM-1 on the surface of microglia, which was reversed by the addition of Th2 cells (Gimsa *et al.* 2001). These studies collectively indicate that T cells are able to interact with microglia, and can differentially modulate their activation state.

Town and colleagues have postulated that once activated, microglia exist in at least two functionally discernable states: a phagocytic phenotype which they have termed "innate activation", and an antigen presenting phenotype termed "adaptive activation", and that the activation state is governed by their stimulatory environment (Town *et al.* 2005). They found that microglia challenged with FITC-labelled Aβ exhibited a time dependent phagocytic response to Aβ, which was not associated with IL-1β, TNF-α, or IL-6 production. The addition of CD40L reversed this response, and was also capable of increasing secretion of TNF-α (Townsend *et al.* 2005, Town *et al.* 2005). The Th1-type cytokines IFN-γ and TNF-α were found to inhibit Aβ phagocytosis, while the Th2-type cytokines IL-4 and IL-10 boosted the phagocytic response (Town *et al.* 2005). MHC class II, co-localised with Aβ, was detected on the surface of the microglial cell and treatment of microglia with Aβ in combination with CD40L markedly increased concentrations of IFN-γ, IL-6, and TNF-α (Town *et al.* 2005). It has been hypothesised that when microglia encounter Aβ in a proinflammatory, co-stimulatory environment they shift from a phagocytic phenotype to one that is closer to an APC. In support of this theory, it has been shown that IL-10, IL-4, and IL-13 all suppressed Aβ and LPS-induced proinflammatory cytokines to varying extents (Szczepanik *et al.* 2001). Importantly, while proinflammatory cytokines can inhibit phagocytosis through the CD47, CD36, $\alpha_6\beta_1$ integrin complex, they were found to be unable to inhibit phagocytosis elicited by antibody-mediated activation of FcR (Koenigsknecht & Landreth 2005). This may explain why Aβ vaccination therapy has proven to be so effective in clearing Aβ deposits in transgenic models of AD. In contrast, it has been demonstrated that

astrocyte-derived IL-4 inhibited microglial phagocytosis of Aβ *in vitro* (Stoll & Jander 1999). In addition, Butovsky and colleagues have shown that activation of microglia by aggregated Aβ impaired MHC class II expression and rendered microglia cytotoxic, and that IFN-γ or IL-4 could overcome this toxicity (Butovsky *et al.* 2005b). These authors argue that an initial Th1 response to Aβ may be beneficial in recruiting microglia and other immune cells to the site of injury, provided it is followed by a complementary anti-inflammatory response (Butovsky *et al.* 2005b). It would thus appear that microglia have the potential to be detrimental or beneficial in AD, and their activation state depends on both the surrounding cytokine milieu, and the cell types present in their vicinity.

1.5.5. The role of cytokines in the pathogenesis of Alzheimer's disease

1.5.5.1. IL-1β

Initial evidence that IL-1β was involved in the pathogenesis of AD came from studies showing increased expression of IL-1 in activated microglia surrounding AD plaques (Griffin *et al.* 1989). It has since been shown that activated microglia expressing IL-1β are associated with Aβ plaques in Tg2576 transgenic mice (Benzing *et al.* 1999). Polymorphisms in the IL-1β gene have been linked to increased risk of developing AD (McGeer & McGeer 2001). IL-1β is produced by microglia and macrophages, via activation of the inflammasome, in response to treatment with Aβ *in vitro* (Murphy *et al.* 1998, Lorton *et al.* 1996, Meda *et al.* 1999, Halle *et al.* 2008). Furthermore, an increase in IL-1β production with microglia is associated with increased production of APP (Brugg *et al.* 1995). *In vivo*, intracerebroventricular injection of Aβ into IL-Ra deficient mice enhanced neuronal damage and microglial activation above that induced in wild-type mice, suggesting that uncontrolled production of IL-1β results in increased neuropathology in AD (Craft *et al.* 2005). In contrast, recent data support a role

for IL-1β in plaque clearance in AD. Chronic overexpression of IL-1β in the brains of APPswe/PS1dE9 mice was found to reduce plaque number (Shaftel *et al.* 2007b). This model of chronic IL-1β expression was found to result in increased MCP-1 production by microglia, increased BBB leakage, and significant infiltration of neutrophils, T cells, and macrophages, suggesting that it one or more of these cell types may be having an effect on plaque clearance in this instance (Shaftel *et al.* 2007a).

1.5.5.2. IL-6

Polymorphisms in the IL-6 gene have been associated with an increased risk of developing AD (McGeer & McGeer 2001). Moreover, plasma concentration of IL-6 in AD patients has been shown to be significantly higher than in non-demented controls (Shibata *et al.* 2002). IL-6 has been detected adjacent to Aβ plaques in brain sections from AD patients (Strauss *et al.* 1992). Overexpression of IL-6 in the brain under the GFAP promoter resulted in neurodegeneration and cognitive decline (Campbell *et al.* 1997). Treatment of human postmortem microglia with Aβ results in production of IL-6 (Lue *et al.* 2001b). Furthermore, IL-6 enhanced expression of neuronal APP *in vitro* (Del Bo *et al.* 1995).

1.5.5.3. TNF-α

TNF-α is produced by a number of different cell types, including T cells, NK cells, macrophages, astrocytes, and microglia (Vassalli 1992). In support of a role for TNF-α in the pathogenesis of AD, elevated levels of TNF-α have been found in the sera of AD patients when compared with non-demented controls (Fillit *et al.* 1991), and other studies have shown that increased levels of TNF-α in the cerebrospinal fluid (CSF) precede the development of AD (Tarkowski *et al.* 2003, Tarkowski *et al.* 1999). Polymorphisms in the TNF-α gene have also been shown

to be associated with AD (Perry *et al.* 2001). TNF-α has been shown to enhance Aβ production and reduce its degradation in Tg2576 mice (Yamamoto *et al.* 2007), while deletion of the tumour necrosis factor type 1 death receptor (TNFR1) in APP23 mice results in inhibition of Aβ plaque formation and improvements in memory, suggesting that TNF-α-induced signalling mediates plaque formation and memory loss (He *et al.* 2007). Aβ induces production of TNF-α from rat microglia *in vitro*, and TNF-α augments Aβ-induced apoptosis in human neuronal cells (Jiao *et al.* 2008, Blasko *et al.* 1997). These studies prompted researchers to look at the effect of the TNF-α antagonist, etanercept as a potential treatment for Alzheimer's disease. These studies have shown that weekly perispinal administration of etanercept induced rapid (within 2 h) improvements in patients short-term memory and verbal fluency, though there is currently no mechanism postulated for these improvements (Tobinick & Gross 2008b, Tobinick & Gross 2008a).

1.5.5.4. IFN-γ

The predominant cellular sources of IFN-γ are T cells and NK cells, but IFN-γ is best known for its strong association with a Th1 response. It is a potent inducer of proinflammatory cytokine secretion by microglial cells, and it activates microglia into fully competent APCs by upregulating their expression of MHC class II and co-stimulatory molecules (Aloisi 2001, Nguyen & Benveniste 2000b, O'Keefe *et al.* 1999, O'Keefe *et al.* 2002). In the context of AD, IFN-γ acts synergistically with Aβ to produce nitric oxide and TNF-α from microglia (Goodwin *et al.* 1995, Meda *et al.* 1995). Furthermore, treatment of microglia with a combination of IFN-γ and Aβ leads to neuronal cell injury *in vitro* (Bate *et al.* 2006, Li *et al.* 2004).

Whether IFN-γ is protective or pathogenic in Alzheimer's disease is currently a matter of considerable debate. Yamamoto and colleagues generated an

APP-overexpressing mouse which lacked the IFN-γ receptor type I and found that these mice had reduced microglial activation and Aβ plaques at 14 months of age (Yamamoto *et al.* 2007). Furthermore, this study showed that IFN-γ suppressed Aβ degradation by microglia and increased Aβ production from neurons and astrocytes by upregulating expression of β-secretase, suggesting that IFN-γ is directly involved in the pathogenesis of AD (Yamamoto *et al.* 2007, Cho *et al.* 2007). Indeed, mice expressing IFN-γ in the brain under the MBP promoter were found to have increased microglial and astroglial activation, hypomyelination, and defective cerebellar development, resulting in death at 2-4 weeks of age (Corbin *et al.* 1996). IFN-γ has also been reported to inhibit phagocytosis of Aβ *in vitro* (Town *et al.* 2005). A particularly interesting study found that while microglia treated with IFN-γ were able to take up Aβ, it inhibited the degradation of the peptide, thus leading to an overall decrease in the phagocytosis of Aβ (Yamamoto *et al.* 2008). In contrast, IFN-γ has been demonstrated to induce neurite outgrowth *in vitro* (Song *et al.* 2005). Expression of lower levels of IFN-γ under the MBP promoter than that reported by Corbin and colleagues were found to enhance neurogenesis in both wild-type and APP-transgenic mice (Baron *et al.* 2008). Additionally, expression of low levels of IFN-γ in the CNS of transgenic mice, protected them from cuprizone-induced demyelination (Gao *et al.* 2000). It would thus appear that the dose and timing are critical in whether IFN-γ is neurotoxic or neuroprotective. It should be noted that while numerous studies have found that IFN-γ inhibits microglial phagocytosis of Aβ (Town *et al.* 2005, Yamamoto *et al.* 2008, von Bernhardi *et al.* 2007, Koenigsknecht & Landreth 2005), none have shown induction of phagocytosis by this cytokine.

1.5.5.5. TGF-β

Perhaps the most studied cytokines in research into AD are those of the transforming growth factor-beta (TGF-β) family. The TGF-β family consists of several multifunctional cytokines that play key roles in immune cell activation,

cell growth and differentiation, and repair (Li *et al.* 2006). In the periphery, TGF-β can suppress the proliferation of T and B cells, and inhibit the production of TNF-α and IFN-γ (Bright & Sriram 1998, Li *et al.* 2006). Initial studies on TGF-β expression in the CNS found that TGF-β2 was produced by microglial cells associated with Aβ plaques in brains of patients with AD (Peress & Perillo 1995). Subsequent studies found TGF-β1 to be associated with senile plaques in the dentate gyrus and entorhinal cortex (Peress & Perillo 1995, van der Wal *et al.* 1993). Additionally, TGF-β2 levels were found to be increased threefold in brains of AD patients (Flanders *et al.* 1995).

Based on this data showing increases in TGF-β both in AD, and in APP-transgenic mice (Salins *et al.* 2008), it was thought that perhaps TGF-β was involved in the pathogenesis of the disease. In support of this, TGF-β1 has been reported to substantially increase the production of APP by microglia and astrocytes (Gray & Patel 1993, Monning *et al.* 1994). It has also been shown that overexpression of TGF-β1 in APP-transgenic mice accelerated the rate of deposition of Aβ (Wyss-Coray *et al.* 1997). Town and colleagues genetically impaired TGF-β and downstream Smad2/Smad3 signalling by overexpressing dominant-negative TGF-β receptor 2 in APP-transgenic mice, and found attenuation of Aβ plaque formation and reversal of memory impairments in these animals (Town *et al.* 2008). In contrast to these findings, TGF-β was found to inhibit Aβ induced neuronal apoptosis *in vitro* (Kim *et al.* 1998). Reducing neuronal TGF-β signalling in APP-transgenic mice resulted in Aβ accumulation and neuronal degeneration (Tesseur *et al.* 2006). Furthermore, Wyss-Coray and colleagues demonstrated that expression of a low level of TGF-β1 in the brains of a mouse model for AD dramatically reduced Aβ load in the hippocampus and neocortex, and that this was associated with microglial activation (Wyss-Coray *et al.* 2001). TGF-β would therefore appear to be a highly important cytokine in the context of Aβ regulation.

1.5.5.6. IL-10

IL-10 is an anti-inflammatory cytokine that is produced by a wide variety of cell types, including Th2 and Treg cells, macrophages, astrocytes, and microglia. Like TGF-β, it can inhibit the APC function of microglia by down-regulating their expression of MHC class II (O'Keefe *et al.* 1999). A number of studies have examined the association of polymorphisms in the IL-10 gene with the risk of developing AD, with conflicting results. It has been reported that IL-10 polymorphisms are associated with increased risk of developing AD in studies on Italian (Bagnoli *et al.* 2007), and Chinese (Ma *et al.* 2005) patients. In contrast, other studies have shown no linkage between polymorphisms in IL-10 and the development of AD (Culpan *et al.* 2006, Depboylu *et al.* 2003). IL-10 has been shown to decrease proinflammatory cytokine production by microglia *in vitro* (Szczepanik *et al.* 2001). It can also act on TNF-α-stimulated microglia to increase microglial chemotaxis to Aβ, and to enhance its endocytosis (Iribarren *et al.* 2007).

1.5.5.7. IL-4

Like IL-10, IL-4 is an anti-inflammatory cytokine produced by Th2 cells, and is also produced by mast cells and B cells. IL-4 inhibits proinflammatory cytokine production by macrophages and IFN-γ production by Th1 cells (Paul & Seder 1994). *In vitro*, IL-4 inhibits the production of NO by microglia (Chao *et al.* 1993), and also inhibits IFN-γ-induced MHC class II and CD40 (Nguyen & Benveniste 2000a, O'Keefe *et al.* 1999). Additionally, it has been shown that IL-4 reverses IFN-γ-induced impairment in phagocytosis by microglia (Koenigsknecht-Talboo & Landreth 2005).

1.6. Current therapies for Alzheimer's disease

There is currently no known cure for AD, and current drugs used to treat the disease can at best slow its progress and temporarily stabilise the symptoms. Drugs that are currently approved for the treatment of AD include acetylcholinesterase inhibitors, which are designed to keep levels of the neurotransmitter, acetylcholine high in the brain, even after the neurons that produce it are destroyed. The other main drug used in treatment is memantine, an N-methyl-D-aspartate (NMDA) antagonist, which works by blocking excessive NMDA receptor activity without disrupting its normal activity, thus preventing some of the excitotoxic cell death seen in AD (Chen & Lipton 1997). Other drugs are used to manage the mood disorder and psychosis seen in later stages of the disease, but none can modify the pathogenic disease process. Current studies are focused on modifying the underlying disease process rather than the symptoms.

Most of the approaches currently being taken involve anti-tau and anti-amyloid therapies. One such drug is methylthioninium chloride, or Rember™. This drug has recently been reported to dissolve tau paired helical fragments isolated from the brain of AD patients, and patients receiving the drug were found to have no reduction in regional cerebral blood flow, in contrast to AD patients receiving a placebo (Staff *et al.* 2008). Therapies involving reducing Aβ plaque formation or enhancing plaque clearance have received a lot of attention due to the fact that the amyloid cascade is widely purported to cause disease. Inhibitors of γ-secretase are currently in clinical trials and a recent report suggests that this treatment results in a reduction in plasma Aβ (Fleisher *et al.* 2008), although levels of Aβ in the brain parenchyma and cognitive function have not yet been measured. β-secretase inhibitors are also currently in development, however progress has been slow, owing to the difficulty of generating a specific inhibitor (Ghosh *et al.* 2008). Another treatment strategy currently receiving considerable attention is anti-Aβ immunotherapy.

1.7. Immunotherapy

The term immunotherapy incorporates an array of strategies of treatment based on the concept of modulating the immune system to achieve a prophylactic and/or therapeutic goal. This may be done in one of two ways, passive immunotherapy and active immunotherapy. Passive immunotherapy involves the *in vitro* activation or generation of specific immune molecules or cells, which are then administered to the patient to fight disease. For example monoclonal antibodies which are generated to recognise and attack specific targets can be administered to patients, resulting in destruction or neutralisation of the target. However, these passive immunotherapies do not stimulate the patient's own immune system and are thus short-lived responses. Active immunotherapy stimulates the body's own immune cells to mount a specific response against the particular target. This has the added advantage of generating memory immune cells against the target, thus protecting the host from subsequent insults. Vaccines such as the measles, mumps, and rubella vaccine which is a mixture of live attenuated viruses are examples of active immunotherapy.

1.7.1. Immunotherapy of Alzheimer's disease

1.7.1.1. Active Immunotherapy

Studies into the possibility of using immunotherapy to treat AD began following the development in 1995 of the PDAPP-transgenic mouse model of AD (Games *et al.* 1995). These mice show an enhanced production of $A\beta_{42}$ and develop amyloid plaques in the cortex and the hippocampus by middle age. The first pre-clinical study on active immunotherapy against AD was by Schenk and colleagues in 1999, who found that repeated immunisation of PDAPP mice over an extended period with $A\beta_{42}$ emulsified in complete Freund's adjuvant (CFA) resulted in

prevention of Aβ plaque formation in young animals and reduction in the plaque burden in older animals (Schenk *et al.* 1999). Immunohistochemical staining with anti-mouse immunoglobulin antibodies showed that any remaining plaques were often co-localised with IgG in the immunised mice. Cells phenotypically resembling microglia and expressing MHC class II were found to be associated with the remaining Aβ plaques. The authors suggested that active immunisation induced production of anti-Aβ antibodies, which bound to the Aβ plaques in the brain, triggering their removal by monocytic/microglial cells via their F_c receptors. Further studies by other laboratories showed that active immunisation with Aβ could prevent the cognitive decline seen in animal models of AD (Morgan *et al.* 2000, Chen *et al.* 2000, Janus *et al.* 2000). These findings were then extended to a study in a non-human primate, the caribbean vervet, which showed clearance of $A\beta_{42}$ immunoreactive plaques following immunisation with $A\beta_{42}$ (Lemere *et al.* 2004). Aβ-specific antibodies were detected in the CSF of immunised vervets, but not in control animals. Following immunisation, Aβ levels were decreased in the CSF and increased in the plasma. Lemere and colleagues also found no evidence of microglial activation in the vervet brain. None of these studies reported any evidence of T cell infiltration into the brains of immunised animals.

1.7.1.2. Clinical Trials

Promising preclinical results in several species led to the initiation of a randomised placebo-controlled Phase II clinical trial. An Aβ synthetic peptide (AN-1792) was administed to AD patients in conjunction with the Th1 adjuvant, QS-21. Although the Phase I clinical trial showed that multiple injections of AN-1792 and QS-21 were tolerated, in the Phase II trial 6% of treated patients presented with symptoms consistent with meningoencephalitis, leading to an abrupt discontinuation of the trial (Orgogozo *et al.* 2003).

Despite these setbacks, there were several promising findings from this clinical trial. Immunisation with Aβ in adjuvant was found to reduce plaque burden in the CNS in 6 of the 8 patients that have been autopsied to date (Holmes *et al.* 2008, Nicoll *et al.* 2003, Ferrer *et al.* 2004), although other features of AD such as CAA, neurofibrillary tangles, and neuropil threads were unaffected. Additionally, microglia staining for HLA-DR and CD68 were found to be associated with Aβ in areas where Aβ appeared to be cleared from the brain (Boche & Nicoll 2008). It has been suggested that clearance of Aβ in these cases was due to a non-specific effect of brain inflammation, rather than a specific immune-cell-mediated clearance (Akiyama & McGeer 2004), however this would seem to be unlikely as a pathology report from a patient that did not develop encephalitis also showed plaque clearance (Masliah *et al.* 2005). Another interesting finding was that in regions of plaque clearance, dystrophic neurites were absent, implying that perhaps they were removed by microglial phagocytosis when plaques were cleared (Boche & Nicoll 2008). Hock and colleagues followed up a cohort of 30 patients and reported that patients with plaque reactive Aβ-specific antibodies showed significantly slower rates of cognitive decline than those that did not have detectable Aβ-specific antibodies (Hock *et al.* 2003). However, this study analysed only a small subset of patients from this clinical trial, and another study found no apparent correlation between antibody response and either clinical score or incidence of meningoencephalitis (Orgogozo *et al.* 2003). Another study of the patients from this trial revealed that cognitive increases associated with Aβ-specific antibodies were linked to decreases in brain volume (Fox *et al.* 2005). This is thought to be due to amyloid removal and associated cerebral fluid changes. The majority of patients immunised in this trial had Aβ-specific IgG antibodies in their serum, but more than half also had Aβ-specific antibodies in their CSF, indicating a compromise of the BBB in many of these individuals. Aβ immunisation is now linked with a compromised BBB in both human and mouse (Mathews & Nixon 2003).

The meningoencephalitis seen in a subset of patients from the clinical trial is thought to be due to T cell infiltration. The BBB is no longer intact in a number of these individuals, and T cell infiltrates have been shown in the brain of immunised patients (Mathews & Nixon 2003, Ferrer *et al.* 2004). It is possible that AD patients with a high frequency of Aβ-specific T cells develop severe inflammation when immunised with Aβ formulated with QS-21. It should be noted, however, that a pathology report of a patient from the trial that did not develop meningoencephalitis also showed some T and B cell infiltration (Masliah *et al.* 2005). In addition, titers of Aβ-specific antibodies were found not to correlate with the development of meningoencephalitis, suggesting that it is not necessarily directly linked to the presence of Aβ-specific T cells. Presumably Aβ-specific T cells contributed to the production of Aβ-specific antibodies, but it is not yet clear whether they contributed to the severe brain inflammation seen in these clinical trials. It has also been suggested that prolonged exposure to QS-21 induced the development of pathogenic myelin-specific T cells (Monsonego & Weiner 2003). This hypothesis is supported by MRI scans showing lesions in white matter of the brain where Aβ is not normally expressed. Possible reasons for the lack of T cells seen in Aβ immunised mice are: (i) animals were boosted with IFA, which could result in increased Th2 cell induction, as opposed to the Th1 cells probably produced upon QS-21 immunisation; (ii) Aβ may not have been sufficiently immunogenic to generate T cell responses in the mouse strains tested.

Given the relative success of immunisation on clearance of Aβ plaques, and the cognitive improvements seen in mice immunised with Aβ, it is somewhat surprising that a more substantial cognitive benefit was not seen in clinical trials. There are a number of possible explanations for this disparity. Firstly, there was only a relatively small decline in cognitive function in the placebo group, possibily obscuring any beneficial effects of the immunisation on cognition (Holmes *et al.* 2008). Secondly, Aβ immunisation has been shown to cause a transient increase in CAA, and immunised patients were found to have more microhaemorrhages and microvascular lesions than controls (Boche *et al.* 2008).

This may result in cognitive impairments that offset the beneficial effect of Aβ clearance from the parenchyma. Finally, it may be the case that in order to deliver functional cognitive benefits, patients need to be immunised at an earlier stage in the disease, before widespread neuronal damage occurs. Support for this hypothesis comes from a recent study of active immunisation in a large (n = 150) sample of mice. Chen and colleagues found that immunisation of mice with Aβ in MPL provided no overall cognitive benefit despite moderate plaque clearance (Chen *et al.* 2007a). However, when they correlated Aβ levels with behavioural performance, they found a significant correlation between Aβ levels and cognitive performance in preventative, but not in therapeutic studies. Taken together, the data suggest that Aβ is still an important therapeutic target in AD.

1.7.1.3. Alternative adjuvants

A potential factor put forward to explain the failure of the Aβ vaccine to induce substantial cognitive improvements is the use of the adjuvant QS-21, which preferentially induces a Th1 type response (Cribbs *et al.* 2003). In addition, Pride and colleagues have reported that the addition of the emulsifier, polysorbate 80, to the injection formula upon initiation of phase II clinical trials induced a highly polarised Th1 response (Pride *et al.* 2008). Regardless, it would seem that a potent proinflammatory response was induced in the CNS of patients enrolled in clinical trials, and thus it may be beneficial to use an adjuvant that induces a more anti-inflammatory immune response. A study on immunisation of mice with Aβ and a number of different adjuvants found that QS-21 and CFA induced strong antibody responses to Aβ, whereas alum induced a lower response (Cribbs *et al.* 2003). Based on the subtypes of antibody generated it was concluded that QS-21 and CFA induced primarily Th1 type responses, whereas alum induced a Th2 response. Furthermore, spleen cells from mice immunised with either QS-21 or CFA produced significant amounts of IFN-γ or TNF-α in response to Aβ, whereas immunisation with alum generated spleen cells that produced IL-4 primarily.

In support of the hypothesis that alum might be a suitable adjuvant to use in the therapy of AD, it has been shown that immunisation of Tg2576 mice with the synthetic Aβ peptide K6Aβ1-30 in alum reduced Aβ plaque burden without the microhemorrhages seen following passive immunotherapy, although this reduction in Aβ was much more modest than that seen with immunisation with Aβ in CFA (Asuni *et al.* 2006). This may be related to the lack of microglial activation seen in these animals, as microglia are known to be involved in plaque clearance (Bard *et al.* 2000). Immunisation in alum was also found to improve cognition in Tg2576 mice.

Subcutaneous administration of Aβ with a monophosphoryl lipid A /trehalose dicorynomycolate adjuvant resulted in high titers of Aβ antibodies of the IGg2b and IgG1 type, although restimulation of spleen cells from immunised animals with Aβ resulted in the production of moderate levels of IFN-γ (Maier *et al.* 2005). Immunisation with Aβ and mutant *Escherichia coli* heat-labile enterotoxin (LT R192G) produced lower levels of Aβ-specific antibodies, but produced almost no IFN-γ in response to Aβ stimulation, suggesting that this may be a better adjuvant to use for vaccination. However, neither adjuvant generated lymphocytes capable of producing IL-4 or IL-10 in response to Aβ stimulation.

1.7.1.4. Alternative routes of immunisation

An alternative approach taken to reduce the inflammatory effect of Aβ immunisation, while still promoting plaque clearance, is to use different routes of administration of the peptide. Mucosal administration of proteins can decrease autoimmune inflammation, and oral administration of MBP has previously been shown to suppress EAE (Higgins & Weiner 1988). Administration of Aβ intranasally was found to have a beneficial effect on plaque burden in the PDAPP mouse (Weiner *et al.* 2000). Antibodies to Aβ were induced and plaques were

partially cleared. This immunisation protocol also resulted in decreased microglial activation, IgG1 and IgG2b antibody production, and infiltration of small numbers of mononuclear cells secreting IL-4, IL-10, and TGF-β into the brain parenchyma. Importantly, there was no IFN-γ detected in the brains of these animals. Intranasal immunisation with $A\beta_{40}$ for 7 months led to a 50-60% reduction in cerebral Aβ burden in PDAPP mice (Lemere *et al.* 2000). However, due to the fact that mucosally delivered antigens are not very immunogenic, the level of Aβ-specific antibody produced in these experiments was relatively low. This process was later optimised by immunising animals with Aβ in LT(R192G) which resulted in a 16-fold increase in antibody titres over that seen in mice given Aβ intranasally without adjuvant (Lemere *et al.* 2002). Immunisation of APPswe/PS1dE9 transgenic mice with a single i.p. injection of Aβ followed by chronic intranasal administration of Aβ with LT(R192G) resulted in development of increased levels of Aβ-specific antibodies and a 75% decrease in the number of plaques (Lemere *et al.* 2003).

Another potential route of immunisation that has been studied in this context is transcutaneous immunisation. Strong humoral and cellular responses have been induced following transcutaneous immunisation, which is largely due to the diverse populations of resident APCs and other immune cells in the various dermal layers. It has been shown that transcutaneous immunisation of APPswe/PS1dE9 mice with $A\beta_{42}$ with cholera toxin (CT) induced significant decreases in Aβ levels in the brain (Nikolic *et al.* 2007). This was associated with increased levels of both Aβ protein and Aβ-specific antibody in the serum (primarily IgG1), which may indicate an antibody-induced efflux of Aβ from the brain. This was not found to be associated with T cell infiltration or cerebral microhemorrhage. Stimulation of spleen cells from these mice with Aβ produced significantly more IFN-γ, IL-2, and IL-10 than mice immunised with CT alone.

1.7.1.5. Synthetic forms of Aβ

An additional active immunotherapy approach being studied is the use of fragments of Aβ that stimulate B cells preferentially, thus inducing the production of antibodies against Aβ without the concomitant T cell response. Sigurdsson et al. immunised Tg2576 mice with a fragment of Aβ (1-30) coupled to 6 poly Lys residues, and found a reduction in Aβ plaque burden (89%) and soluble Aβ burden (57%) in the brain (Sigurdsson *et al.* 2002). They also found no microglia in the brain expressing IL-1β. Immunisation with $K6A\beta_{1\text{-}30}$[E18E19], which has a mutation in the T cell epitope of Aβ, induced primarily an IgM response, in contrast to the IgG response seen with $A\beta_{30}$ or $A\beta_{42}$ (Sigurdsson *et al.* 2004). Plasma levels of IgM correlated inversely with amyloid plaque burden, and it was suggested that IgM binds to Aβ in the periphery, resulting in an efflux of Aβ from the brain parenchyma.

$A\beta_{15}$ contains the dominant B cell epitopes that bind to Aβ plaques, and no T cell epitopes (Monsonego & Weiner 2003). Immunisation of mice with $A\beta_{15}$ in combination with the Th cell pan HLA DR epitope (PADRE) in Quil A induced robust Aβ-specific antibody production, which was not accompanied by lymphocyte or macrophage infiltration (Ghochikyan *et al.* 2006). $A\beta_{15}$ alone did not induce T cell proliferation by spleen cells from mice immunised with PADRE, indicating that T cell responses induced against PADRE provide the T cell help necessary for a strong anti-Aβ antibody response (i.e. no Aβ-specific T cells were induced) (Agadjanyan *et al.* 2005). This vaccine was recently tested in Tg2576 mice, and researchers found a reduction in the number of Aβ plaques, which was correlated with an increase in the concentration of $A\beta_{42}$-specific antibody (Petrushina *et al.* 2007). However, Quil A is a rumoured Th1-type adjuvant, and thus current studies are looking at the complement component C3d as an alternative adjuvant (Movsesyan *et al.* 2008). Kim and colleagues found that intranasal immunisation of C57BL/6 mice with a deoxyribonucleic acid (DNA) plasmid encoding $(A\beta\ 1\text{-}6)_{11}$ followed by a booster immunisation with an

adenovirus vector containing $(A\beta\ 1\text{-}6)_{11}$ induced anti-Aβ antibody titers in 4 out of 7 mice (Kim *et al.* 2007). Their hypothesis was that these heterologous prime-boost strategies often induce very strong immune responses. This vaccination regime induced Aβ-specific antibodies that were predominantly of the IgG1 isotype, and increased levels of IL-10 in the serum. Another approach employs virus-like particles (VLPs). Antigens conjugated to VLPs can induce antibody responses against both self- and foreign-targets at low doses of antigen and without the use of exogenous adjuvants. Immunisation of C57BL/6 mice with $A\beta_{40}$-VLP induced antibodies primarily of the IgG1 subtype, in contrast to the IgG2c antibodies induced by immunisation with $A\beta_{40}$ in CFA (Chackerian *et al.* 2006). Notably, there was no significant T cell response induced against Aβ.

The goal of all of the above studies was to induce Aβ-specific antibodies, without the concomitant T cell response that gave rise to the meningoencephalits seen in clinical trials. Another approach has been to administer these Aβ-specific antibodies directly. This eliminates problems with patients not producing antibody, a common problem with active immunisation, and it also allows for a finer control of the antibody subtype being delivered to patients.

1.7.1.6. Passive immunisation

Direct administration of Aβ-specific antibodies has the advantage that it would bypass immune senescence and would not be expected to lead to encephalitis, which appears to be T cell-mediated. The levels of Aβ-specific IgG antibody in APP-transgenic mice are reduced following immunisation as compared with their wild-type littermates (Monsonego *et al.* 2001). This is thought to be due to a state of T cell tolerance specific for Aβ peptide. Furthermore, a number of studies have shown decreased levels of Aβ-specific antibodies in serum and CSF from AD patients compared with elderly controls (Weksler *et al.* 2002, Brettschneider *et al.* 2005, Du *et al.* 2001). Whether this reduction in antibody levels is of any clinical

significance has yet to be thoroughly investigated, although Elan have developed a monoclonal antibody (bapineuzumab) against Aβ which is currently entered into phase III clinical trials in December 2007.

Aβ-specific antibodies have been shown to dissolve aggregated Aβ and also inhibit Aβ induced cytotoxicity in vitro (Solomon *et al.* 1997). Bard and colleagues showed that passively administered antibodies against Aβ reduced plaque deposition in the PDAPP mouse (Bard *et al.* 2000). Mice (8-10 month old) were passively immunised weekly with Aβ-specific antibody for 6 months. The results reveal that this antibody was able to cross the BBB and bound to Aβ plaques in the CNS. Using an *ex vivo* assay in which primary microglial cells were cultured with sections of PDAPP mouse brain, they showed that administration of Aβ-specific antibody induces phagocytosis of the Aβ plaques by microglial cells, and went on to show that the microglial phagocytosis of Aβ was Fc-mediated. Antibodies against Aβ which lacked the Fc portion of the antibody bound to Aβ plaques, but were unable to trigger microglial phagocytosis. Studies have shown that passive immunisation with antibodies that preferentially bind to oligomers of Aβ improve memory in APP-transgenic mice (Lee *et al.* 2006). It has also been reported that passive immunotherapy of APP-transgenic mice decreased Aβ plaque burden in the brain, but rapidly increased 1000-fold the concentration of Aβ in the plasma (DeMattos *et al.* 2001). They concluded that Aβ was removed from the brain not by Fc-mediated phagocytosis, but by the antibodies sequestering the Aβ present in the serum, leading to efflux of Aβ from the brain.

Three mechanisms have been postulated to explain the clearance of Aβ plaques from the brain by these autoantibodies (Weiner & Frenkel 2006). The first involves direct clearance of Aβ by the antibody, leading to dissolution of Aβ plaques, which is supported by evidence from Solomon and colleagues showing these antibodies can dissolve Aβ plaques in vitro (Solomon *et al.* 1997). The second mechanism involves Fc-mediated phagocytosis of the Aβ-antibody

complexes by microglial cells. Consistent with this, treatment of APP-transgenic mice with dexamethasone following administration of Aβ-specific antibodies down-regulated microglial activation to a roughly the same extent as they inhibited removal of Aβ deposits (Wilcock *et al.* 2004a). Like Bard and colleagues, they also found that administration of Aβ antibodies lacking the Fc portion of the antibody did not result in plaque clearance, indicating that phagocytosis of Aβ is Fc-mediated. The final mechanism postulates that addition of antibody results in an efflux of Aβ from the brain to the plasma, which is supported by data showing that administration of Aβ antibodies to APP-transgenic mice resulted in a decrease of Aβ plaque burden in the brain, accompanied by an increase in Aβ levels in the plasma (DeMattos *et al.* 2001).

There are a number of challenges associated with implementing passive immunotherapy on a large scale in humans. These include the difficulty in administering multiple injections of high concentrations of Aβ-specific antibody every 13 weeks (the amount of time these antibodies appear to remain effective), as well as the high cost of a humanised monoclonal antibody. Three studies of passive immunotherapy in transgenic mouse models have observed increased brain microhemorrhages (Wilcock *et al.* 2004b, Pfeifer *et al.* 2002, Racke *et al.* 2005). In autopsy cases from the clinical trial, patients were reported to have evidence of microhemorrhages, possibly caused by an overabundance of Aβ in the plasma induced by Aβ-specific antibodies (Ferrer *et al.* 2004). Indeed, a recent study shows an increase in the number of blood vessels containing $A\beta_{42}$ in the brain (Boche *et al.* 2008), and brain imaging studies show focal changes in the cerebral white matter of immunised patients (Weller *et al.* 2009). This raises the possibility that antibody therapy may induce a transient increase in the incidence of CAA, thereby impairing the blood supply to other areas of the brain. Thakker and colleagues have shown that, in contrast to systemic administration of antibody, i.c.v. injection of low doses of Aβ antibody induces parenchymal and vascular Aβ clearance, with associated improvements in memory (Thakker *et al.* 2009).

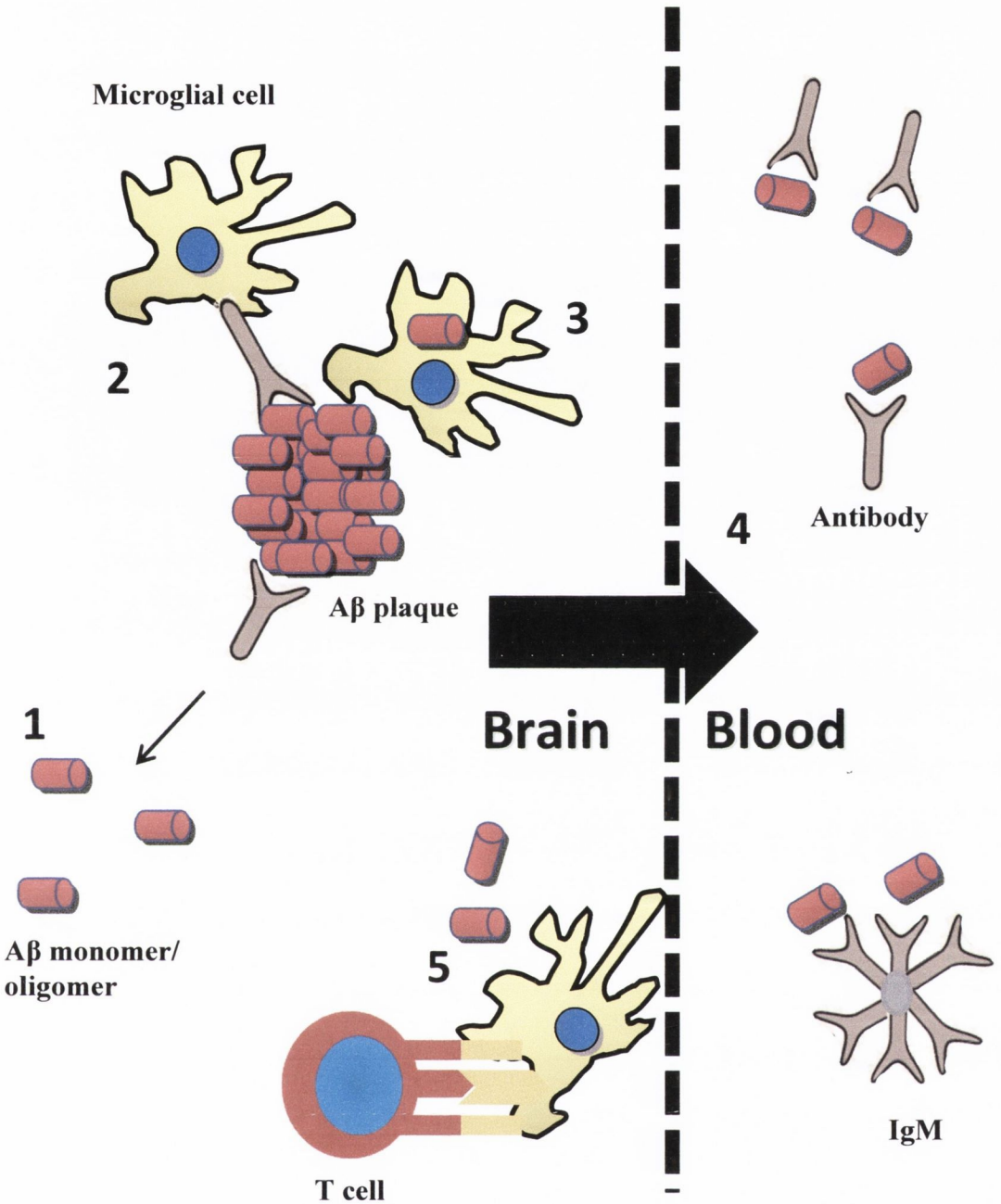


Figure 1.3. Potential mechanisms of immunomodulation of Aβ pathology

There are a number of possible mechanisms by which activation of the immune system might reduce Aβ in the brain. (1) Dissolution of Aβ fibrils by antibodies against the N-terminus of Aβ. (2) Fc receptor-mediated phagocytosis of Aβ by microglia. (3) Non-Fc-mediated phagocytosis of Aβ by microglia. (4) IgG and IgM-mediated clearance of circulating Aβ resulting in an efflux of Aβ from the brain to the periphery. (5) T cell-induced activation of microglia may alter Aβ levels in the brain.

Adapted from Wisniewski, T. (2008). *Lancet Neurol,* **7,** 805-11.

1.7.1.7. Role of microglia in immunotherapy

There is an increasing body of literature that links activation of microglia with a reduction in Aβ plaque burden in transgenic mouse models of AD. The initial immunisation studies using Aβ reported enhanced microglial activation in the region of Aβ plaques (Schenk *et al.* 1999). Initial studies on passive immunotherapy for AD also reported Aβ clearance to be associated with Fc-mediated phagocytosis (Bard *et al.* 2000). Furthermore, blocking of complement activation reduced microglial cell activity in APP-transgenic mice and this was associated with increased Aβ loads (Wyss-Coray *et al.* 2002). It has been reported that overexpression of the macrophage colony-stimulating factor (M-CSF) receptor on microglia increases phagocytosis of opsonised Aβ by upregulating microglial cell expression of Fc receptors for IgG (Mitrasinovic & Murphy 2003), and that microglia activated in this way have neuroprotective properties (Mitrasinovic *et al.* 2005). Treatment of APP-transgenic mice with the anti-inflammatory drug dexamethasone reduced microglial activation which was associated with decreased plaque clearance (Wilcock *et al.* 2004a). It should be noted however that other studies have found that Fab fragments, which lack the Fc portion of the antibody, can reduce Aβ plaque formation (Bacskai *et al.* 2002). Immunisation of Fcγ-receptor knockout APP-transgenic mice with Aβ has also been shown to reduce plaque formation (Das *et al.* 2003b). Taken together, these results indicate that, while microglia certainly contribute to plaque removal in AD, there are also alternative mechanisms of plaque removal that are independent of microglial uptake.

1.7.1.8. Role of bone-marrow-derived cells in immunotherapy

Recently, a number of studies have begun to study the role of bone-marrow-derived cells in Aβ plaque clearance. Elimination of blood-derived microglia from the CNS was found to result in a significant increase in Aβ plaque size in APP-

transgenic animals (Simard *et al.* 2006). Studies from other laboratories point towards a role for bone-marrow-derived dendritic-like cells in clearance of Aβ plaques. Immunisation of APPswe/PS1dE9 mice with glatiramer acetate, which cross-reacts weakly with CNS auto-antigens, reduced the number of Aβ plaques in the hippocampus (Butovsky *et al.* 2006). This was later reported to be associated with infiltration of both T cells and $CD11c^+$ cells (Butovsky *et al.* 2007). These bone-marrow-derived $CD11c^+$ cells may acquire their DC-like phenotype either in the periphery, or in the diseased sites in the brain, by encountering immunisation-induced T cells.

1.7.1.9. Role of T cells in immunotherapy

The meningoencephalitis observed in patients in clinical trials of the AN1792/QS21 vaccine is believed to be due to a T cell response to Aβ (Monsonego & Weiner 2003, Weiner & Frenkel 2006, Ferrer *et al.* 2004). A study in humans has shown an increased T cell response to Aβ in the elderly (Monsonego *et al.* 2003b). Although such self-reactive T cells can mediate autoimmune diseases such as MS, they may also have a beneficial role in immune regulation and maintenance of normal tissues. In the phase II clinical trials of Aβ vaccination, most patients developing high titers of Aβ antibody did not develop meningoencephalitis, implying that a T cell response alone is insufficient for the development of AD. In addition, a pathology report from a patient that did not develop encephalitis showed T and B cell infiltration (Masliah *et al.* 2005).

Although these self-reactive T cell populations are present in humans, they were not detected in mice immunised with Aβ. The reasons for this are unclear but perhaps it depends on the inflammatory environment present in the CNS at the time of immunisation. Microglia were found to be capable of inducing proliferation of Aβ-specific T cells, but only when they were pre-incubated with IFN-γ (Monsonego *et al.* 2003a). APP-transgenic mice overexpressing IFN-γ were

found to have a proinflammatory environment in the brain, and vaccination of these animals with Aβ resulted in the development of encephalitis (Monsonego *et al.* 2006). T cell infiltrates were localised to the sites of Aβ plaques in the brain and were found to be associated with clearance of Aβ. This suggests that the meningoencephalitis observed in clinical trials may be due to a Th1-cell response against Aβ.

It is important to note that T cell responses may be beneficial in aiding the clearance of Aβ, possibly through their activation of microglial cells. It was reported that immunisation of APP-transgenic mice with glatiramer acetate in protollin induces clearance of Aβ from the brain without encephalitis (Frenkel *et al.* 2005). This effect occurred independently of Aβ-specific antibody. The authors suggest that this immunisation induces glatiramer acetate-specific Th1 cells which migrate to the brain and produce IFN-γ to activate microglial induced clearance of Aβ. It has also been observed that intrahippocampal administration of LPS leads to Aβ plaque clearance in APP-transgenic mice (DiCarlo *et al.* 2001). This may be due in part to T cell induced activation of microglia.

Despite their potentially clinically beneficial role in activating microglial cells, Th1 cells appear to cause encephalitis in patients with AD. Thus, it would appear to be preferable to develop a therapeutic strategy that favours anti-inflammatory responses. Adoptive transfer of Aβ-specific Th2 cells to APP-transgenic mice was recently shown to improve performance in a test of working memory (Cao *et al.* 2009). Interestingly, a separate experiment found that transfer of Th1-enriched cells provided no cognitive benefit, suggesting that perhaps anti-inflammatory T cells are more beneficial in the therapy of AD (Ethell *et al.* 2006). It should be noted that transfer of Th2 cells induced no reduction in parenchymal plaques. Nonetheless, these findings are consistent with reports showing that Aβ-specific antibodies can improve cognitive performance in APP-transgenic mice. There was no benefit afforded to mice infused with OVA-specific T cells.

1.8. Study aims

The aims of these studies were as follows:

- Establishment of immunisation and *ex vivo* culture methods for the generation of Aβ-specific Th1, Th2, and Th17 cell subsets.
- Development of a model of microglial activation in AD using i.c.v. injection of Aβ.
- Assessment of the modulatory effects of Aβ-specific T cell subsets on Aβ-induced microglial activation *in vitro* and *in vivo*.
- Evaluation of the impact of prototypic T cell cytokines on microglial activation.
- Determination of the role of Aβ-specific T cell subsets in ameliorating/exacerbating plaque formation, cognitive impairments, and inflammatory cell activation seen in a transgenic mouse model of AD.

Chapter 2

Materials and Methods

2.1. Solutions

2.1.1. Cell culture media

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera, UK) was supplemented with 8 % heat inactivated (56°C for 60 min) foetal calf serum (FCS) (Gibco, UK), 100 mM L-glutamine (Gibco, UK), and 100 µg/ml penicillin/streptomycin (Gibco, UK). Dulbecco's Modified Eagles Medium (DMEM, Gibco, UK) was supplemented with 10 % heat inactivated (56°C for 60 min) FCS and 100 µg/ml penicillin/streptomycin.

2.1.2. Phosphate-buffered saline (PBS) 20X

320 g sodium chloride (NaCl)

46.4 g di-sodium hydrogen phosphate (Na_2HPO_4)

8 g potassium dihydrogen phosphate (KH_2PO_4)

8 g potassium chloride (KCl)

Dissolved in 2 L dH_2O, pH 7.0

2.1.3. Ammonium chloride lysis solution (0.87 %)

4.35 g ammonium chloride (NH_4Cl)

Dissolved in 500 ml ddH_2O

2.1.4. ELISA wash buffer

500 ml 20X PBS

9.5 L dH_2O

5 ml Tween 20

2.1.5. Phosphate Citrate Buffer

10.2 g citric acid anhydrous ($C_6H_8O_7$)

36.9 g di-sodium hydrogen orthophosphate dodecahydrate ($NaHPO_4.12H_2O$)

Dissolved in 1 L of dH_2O, pH 5.0

2.1.6. ELISA substrate solution

25 ml phosphate citrate buffer

7 µl H_2O_2

1 o-phenylenediamine dihydrochloride (OPD) tablet (Sigma-Aldrich, UK)

2.1.7. ELISA stopping solution (1M)

26.6 ml 18.8 M H_2SO_4

473.4 ml dH_2O

2.1.8. Fluorescence-activated cell sorting (FACS) buffer

500 ml PBS (Dulbecco's)

10 ml FCS

0.5 g sodum azide (NaN_3)

2.1.9. Saturated sodium chloride solution

50 g sodium chloride (NaCl)

500 ml 80 % ethanol, stir overnight before use

2.1.10. Congo red staining solution

2.5 g congo red (Sigma-Aldrich, UK)

500 ml saturated sodium chloride solution

Stir overnight and filter through a 0.22 μm membrane filter before use

2.1.11. 2 mM stock thioflavin T solution

500 ml dH_2O

0.319 g ThT

Filter through a 0.22 μm membrane filter before use

2.1.12. 50 mM stock glycine solution

7.51 g glycine

Dissolve in 1 L dH_2O, pH 8.5

2.1.13. Homogenisation buffer

0.292 g sodium chloride

1 g sodium dodecyl sulphate (SDS)

Dissolve in 100 ml dH_2O, pH 10

Add: 10 μl/ml protease inhibitor cocktail

5 μl/ml phosphatase inhibitor I cocktail

5 μl/ml phosphatase inhibitor II cocktail

2.1.14. Kit buffer

200 ml 1X tris wash buffer (TWB, Meso-Scale Discovery, USA)

Add: 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich, UK)

5 μl/ml phosphatase inhibitor I cocktail (Sigma-Aldrich, UK)

5 μl/ml phosphatase inhibitor II cocktail (Sigma-Aldrich, UK)

2.1.15. Guanidine buffer

7.51 g glycine

Dissolve in 1 L dH_2O, pH 8

2.1.16. Sodium chloride-sodium citrate (SSC) buffer (20X)

88.2 g trisodium citrate ($Na_3C_6H_5O_7$)

175.32 g sodium chloride

Dissolve in 1 L dH_2O, pH 7-8

2.1.17. Buffer X

5 ml 20X SSC

200 µl 0.5 M ethylene diamine tetraacetic acid (EDTA)

2 ml 1M tris-hydrochloride (Tris-HCl, pH 7-8)

92.8 ml H_2O

2.2. Methods

2.2.1. Preparation of primary glial cell culture

Primary mixed glia were isolated from postnatal one-day old C57BL/6 mice. Mice were sacrificed, and the whole brain was dissected out and cross-chopped bi-directionally using a sterile scalpel. The brain was incubated in 3 ml DMEM (Gibco, UK) supplemented with 10% FCS (Gibco, UK), penicillin (100 U/ml; Gibco, UK) and streptomycin (100 U/ml; Gibco, UK) for 10 min at 37°C. Tissue was triturated, filtered through a sterile nylon mesh filter (40 μm; BD Biosciences, USA), and centrifuged at 1200 rpm for 3 min at 20°C. The pellet was resuspended in DMEM. Resuspended mixed glia (2 x 10^6 cells/ml) were plated onto the centre of each well of a 6-well plate (250 μl per well) and allowed to adhere for 2 h at 37°C in a humidified 5% CO_2 environment (Nuaire Flow CO_2 incubator, Jencons, UK). Wells were then flooded with 1500 μl of pre-warmed DMEM. Media was changed every 2-3 days and glia were grown for 10-14 days before treatment.

2.2.2. Cell counts

Cell counts were performed by diluting cells (normally 1:50 or 1:10) with trypan blue (Sigma, UK). An aliquot (10 μl) of the cell suspension was loaded onto a disposable haemocytometer (Hycor Biomedical, UK). The number of viable cells, which appear white under a light microscope, were counted.

2.2.3. Amyloid-beta

2.2.3.1. Preparation of Aβ

Aβ (Biosource, USA) was dissolved in high-performance liquid chromatography-grade water to provide a 6 mg/ml stock solution, which was diluted to 1 mg/ml using sterile PBS, and allowed to aggregate for 48 h at 37°C according to the manufacturer's instructions. Aβ was used immediately or stored at -20°C until required. For use in footpad immunisations, Aβ was dissolved in high-performance liquid chromatography-grade water to provide a 12 mg/ml stock solution, diluted to 2 mg/ml using sterile PBS and allowed to aggregate for 48 h at 37°C.

2.2.3.2. Testing of Aβ Aggregation

The presence of fibrillar Aβ in prepared aliquots was demonstrated by assessing the binding of thioflavin T (ThT, Sigma-Aldrich, UK). Glycine (final concentration 27 mM; Sigma-Aldrich, UK) was prepared by adding 20 ml of 50 mM stock glycine to 17 ml H_2O. A final solution of ThT (100 μM) was prepared by adding 1 ml of stock ThT to 19 ml of H_2O. An aliquot of freshly reconstituted Aβ, an aliquot of aggregated Aβ, and an aliquot of PBS were thawed on ice. Samples were vortexed and 5 μl was added in triplicate to a black ELISA plate (Labsystems, Finland). Glycine solution (185 μl), and ThT solution (10 μl) were added to each well and the plates were read immediately using a fluorometer (Molecular Devices, USA) with the excitation filter set to 435 nm and the emission filter set to 485 nm. As thioflavin T exhibits enhanced fluorescence upon binding to amyloid fibrils, aggregation of Aβ was measured by an increase in fluorescence intensity.

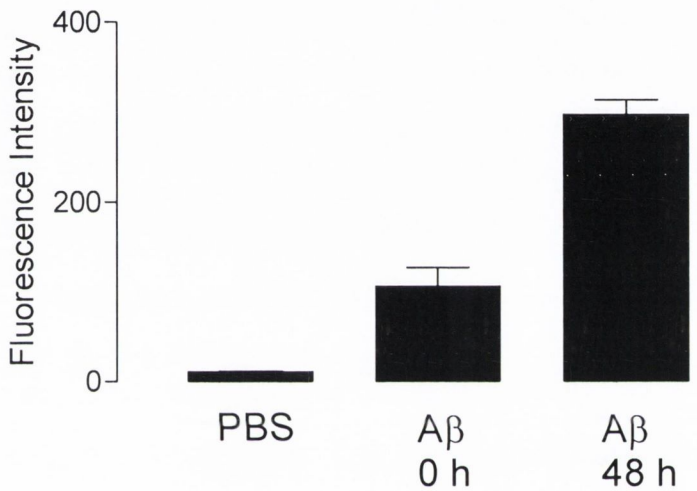


Figure 2.1. Measurement of Aβ aggregation by thioflavin T assay

Aβ was aggregated as described in Section 2.2.3.1. A sample of Aβ (10 µl) was removed prior to aggregation and after 48 h of aggregation. PBS served as a negative control. The aggregation of Aβ was measured as described in Section 2.2.3.2, and the aggregation was expressed as units of fluorescence intensity.

2.2.3.3. Endotoxin detection in Aβ preparation

The endotoxin concentration in aggregated samples of Aβ was measured using the Pyrogene® Recombinant Factor C Endotoxin Detection System (Cambrex, USA). The Aβ sample to be tested was added to an endotoxin-free glass tube, and vortexed vigorously for 10 min before proceeding. The sample to be tested was diluted serially (1:10) using LAL reagent water, and vortexed for 5 min between dilutions. A standard curve was prepared (0 EU/ml – 10 EU/ml), and 100 µl of samples and standards were added in triplicate to a 96-well plate. The plate was pre-incubated in the plate reader (Multiskan RC, Labsystems, Finland) at 37°C for 10 min. Working reagent (100 µl; assay buffer:substrate, 1:4.5 ratio) was added to each well, and the fluorescence was recorded at time zero. The plate was incubated in the plate reader at 37°C for 1 h and the fluorescence was recorded. The standard curve was calculated by plotting log Δ relative fluorescence unit (RFU) against log endotoxin concentration and the endotoxin concentration of the samples was calculated by reference to this standard curve. Values are expressed as endotoxin units per ml (EU/ml).

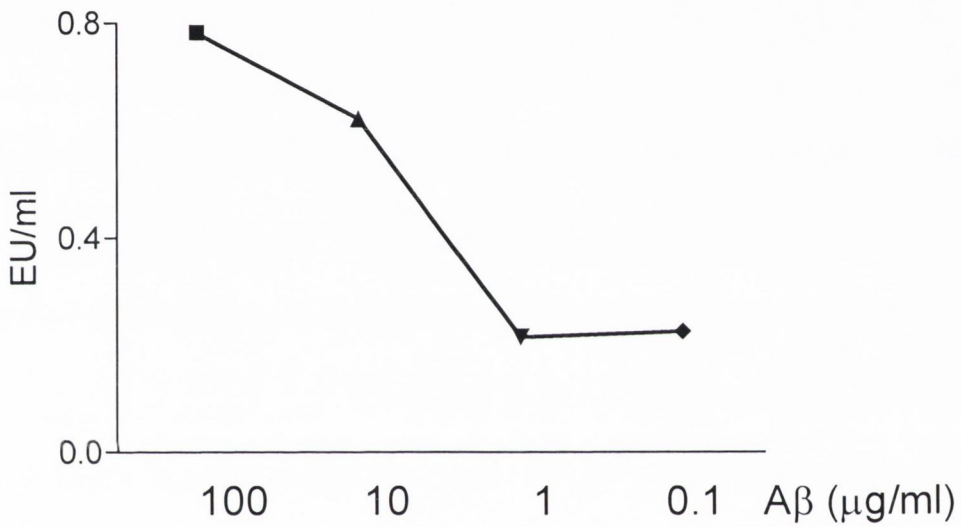


Figure 2.2. Measurement of endotoxin content by LAL assay

Aβ was aggregated as described in Section 2.2.3.1. A 200 μl aliquot of Aβ was removed and tested for endotoxin concentration as described in Section 2.2.3.3. Values are expressed as endotoxin units per ml (EU/ml).

2.2.4. Treatment of cultured glia

All treatments were diluted to the required concentration in the appropriate pre-warmed, supplemented media and all solutions were filtered through a syringe with a cellulose acetate membrane filter (0.2 μM Supor membrane Acrodisc syringe filters, Pall Corporation, USA).

Lipopolysaccharide (LPS, Alexis, USA) was obtained as a stock solution at 1 mg/ml and was diluted to a final concentration of 100 ng/ml in media. Cells were treated with LPS for 24 h. Aβ (Biosource International, USA) was diluted to a final concentration (usually 40 μg/ml) in media. Cells were treated with Aβ for 24 h. Recombinant IL-4, IL-17, IL-10 and IFN-γ (all from R&D Systems, USA) were reconstituted with PBS containing 1% bovine serum albumen (BSA) and diluted to a final concentration of between 1 ng/ml and 200 ng/ml in DMEM medium.

Supernatants were removed into fresh tubes and stored at -80°C until required for analysis of cytokine concentration by ELISA. Cells were harvested for analysis by polymerase chain reaction (PCR) by washing once in ice-cold PBS and adding 353.5 μl of cell lysis mastermix (Nucleospin RNA II, Macherey-Nagel, Germany) for 1 min at 4°C. Samples were stored at -80°C prior to RNA extraction. Cells were harvested for flow cytometry by washing in pre-warmed PBS and adding 400 μl of trypsin-EDTA for 5 min at 37°C.

2.2.5. Animals

Specific pathogen-free C57BL/6 mice were purchased from Harlan UK Ltd., Bicester, UK. Breeding pairs of APPswe/PS1dE9 mice were purchased from the Jackson Laboratory, Maine, USA, and bred in an SPF unit in house. All animal

strains were maintained according to the regulations and guidelines of the Irish Department of Health. All animals were maintained under veterinary supervision at an ambient temperature of 22°–23°C and under a 12-hour light-dark cycle and food (normal laboratory chow) and water were available *ad libitum*. Animal experimentation was performed under a license granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act 1876 and the European Community Directive, 86/609/EEC, and every effort was made to minimise stress to the animals.

2.2.6. Genotyping of APPswe/PS1dE9 mice

2.2.6.1. Isolation of genomic DNA from ear punches

Ear punches taken from wild-type and transgenic mice were either stored on ice and processed the same day or stored at -20°C for longer periods. Digestion buffer was prepared fresh by adding 50 µl of 10% SDS and 5 µl of proteinase K (20 mg/ml; Sigma-Aldrich, UK) to 450 µl buffer X. Digestion buffer (500 µl) was added to each ear punch and tubes were incubated for 3 h at 55°C. The solution was gently mixed once per hour by flicking the tube. DNA was extracted from ear punches by adding 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1; Sigma-Aldrich, UK). Tubes were mixed by inversion for 5 min and centrifuged at 15,000 rpm for 5 min. Using a blue tip with the end cut off so as not to shear the DNA, the upper layer of the solution was removed to a new Eppendorf tube. The DNA was precipitated by adding 500 µl of isopropanol, and the tubes were mixed by inversion for 1 min. Samples were centrifuged at 8,000 rpm for 1 min, and the DNA pellet was washed with 500 µl of 70% ethanol. The majority of the ethanol was removed by pipetting. With a small amount of ethanol left in the bottom of the tubes (~10 µl), the genomic DNA was resuspended in 10 mM Tris-HCl (pH8.2). Any remaining ethanol was then evaporated by heating the open

Eppendorf tubes at 65°C for 10 min. To ensure that the DNA was completely solubilised, samples were stored overnight at 4°C. DNA concentration was quantified using a NanoDrop Spectrophotometer (ND-1000 v3.5, NanoDrop Technologies Inc., USA).

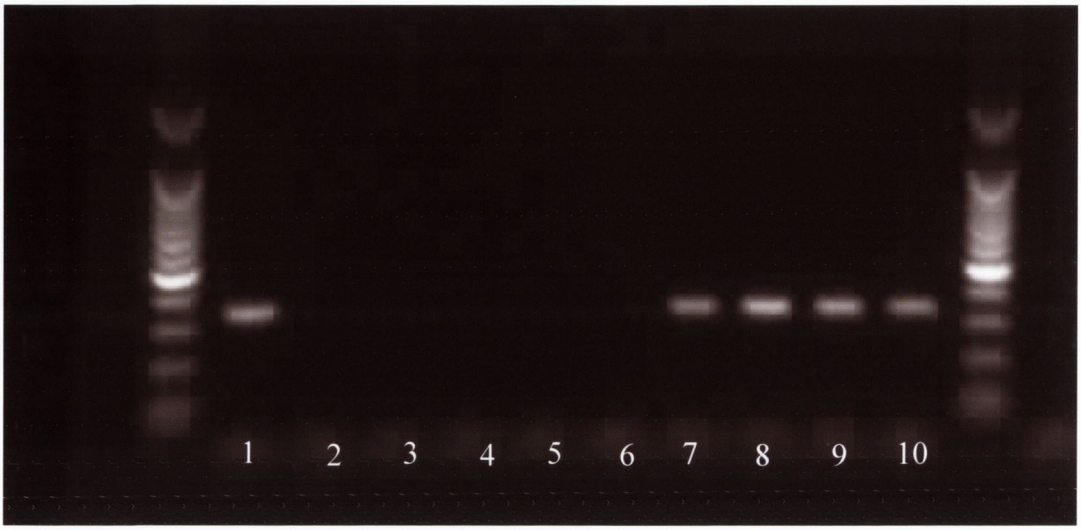
2.2.6.2. Polymerase chain reaction for APPswe and PS1dE9 genes

The presence of the APPswe and PS1dE9 mutations in wild-type and transgenic mice was assessed using PCR. Mastermix was prepared by addition of DNase-free water (17.3 µl), 10X reaction buffer (2.5 µl; 500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, pH 9, Promega, USA), magnesium chloride (1.5 µl; 25 mM, Promega, USA), nucleotide mix (0.2 µl; 25 mM, Bioline, UK), Jumpstart™ Taq DNA polymerase (0.5 µl; Sigma-Aldrich, UK), and sense and antisense primers (0.5 µl; 100 µM, MWG Biotech, Germany) to a sterile tube (see Table 2.1 for primer sequences). The mastermix for the PS1dE9 gene also contained sense and anti-sense primers for the PrP gene as an internal control. DNA (2 µl) and mastermix (23 µl) were added to fresh tubes, which were then placed in a thermocycler (MJ Research Peltier Thermal Cycler-200, Biosciences, Ireland). The amplification process consisted of an initial denaturing step of 94°C for 3 min, followed by 35 cycles comprising of a denaturing step of 94°C for 30 sec, an annealing step of 67°C for 1 min, and an extension step of 72°C for 1 min. Upon completion of 35 cycles of amplification, a final extension step of 72°C for 10 min was applied to ensure complete extension of PCR products. Equal volumes of PCR product from each sample (10 µl) and a 100 base pair ladder (Promega, USA) were mixed with loading buffer (2 µl; Promega, USA), and loaded onto a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Samples were separated by application of 90 V for 120 min. PCR products were visualised under a ultraviolet light and photographed using a ultraviolet transiluminator (Labworks, Ultra Violet Products, Bioimaging Systems, USA).

Table 2.1. Primers used for DNA amplification

Target gene	Primer Sequence	Size (bp)
PrP (control)	fwd 5'-CCTCTTTGTGACTATGTGGACTGATGTCGG-3'	750
	rev 5'-GTGGATAACCCCTCCCCCAGCCTAGACC-3'	
APPswe	fwd 5'-GACTGACCACTCGACCAGGTTCTG-3'	350
	rev 5'-CTTGTAAGTTGGATTCTCATATCCG-3'	
PS1dE9	fwd 5'-AATAGAGAACGGCAGGAGCA-3'	608
	rev 5'-GCCATGAGGGCACTAATCAT-3'	

(A)



(B)

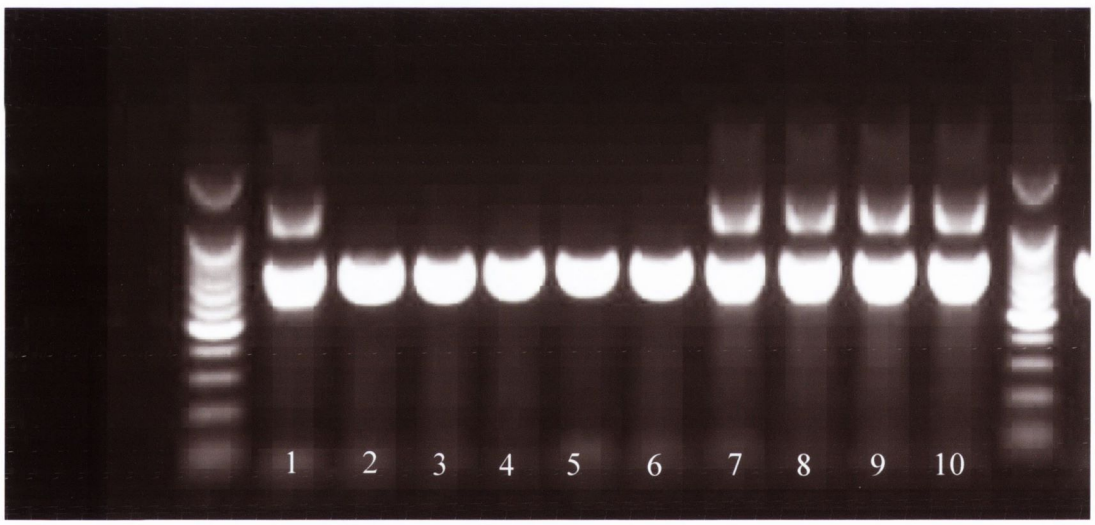


Figure 2.3. Genotyping of APPswe/PS1dE9 mice

Mice were genotyped for the presence the APPswe (A) and PS1dE9 (B) mutations as described in Section 2.2.6.2. Mice 1, 7, 8, 9, and 10 contain both transgenes, while mice 2-6 are wild-type animals. The lower band on gel (B) present in all lanes corresponds to the PrP control gene.

2.2.7. Immunisations

2.2.7.1. Subcutaneous immunisations

C57BL/6 mice were immunised by subcutaneous (s.c.) injection into the flank using a total volume of 200 µl. Mice were injected with PBS, Aβ (100 µg/mouse) in CFA (1:1 ratio Aβ:CFA), Aβ (100 µg/mouse) and CpG (25 µg/mouse, Sigma-Aldrich, UK), Aβ (100 µg/mouse) and CT (100 ng/mouse), or Aβ (100 µg/mouse) in alum (Superfos Biosector, Denmark). The mice were boosted after 21 days as indicated in the results. Mice were sacrificed 7 days after the booster immunisation and antigen-specific immune responses were assessed.

2.2.7.2. Intraperitoneal injections

C57BL/6 mice were injected intraperitoneally (i.p.) with Aβ (100 µg/mouse) and CpG (25 µg/mouse) in a total volume of 200 µl. The mice were boosted after 21 days as indicated in Section 2.2.10.2. Mice were sacrificed 7 days after the booster immunisation and antigen-specific immune responses were assessed.

For immunisation experiments using pertussis toxin (PT), C57BL/6 mice were injected i.p. with PT (150 ng/mouse; Kaketsuken, Japan) in a total volume of 200 µl.

2.2.7.3. Subcutaneous immunisation into the footpad

C57BL/6 mice and non-transgenic littermates to the APPswe/PS1dE9 mice were injected with Aβ (75 µg/mouse) and CpG (25 µg/mouse) into the rear footpads with a total volume of 50 µl (25 µl per foot). The mice were boosted after 21 days

as indicated in Section 2.2.10.3. Mice were sacrificed 7 days after the booster immunisation and antigen-specific immune responses were assessed. This route of immunisation was used to develop T cell lines.

2.2.8. Detection of murine T cell responses *ex vivo*

Immunised mice were sacrificed by cervical dislocation, usually one week after immunisation. Serum was prepared from immunised and control naïve animals and analysed for total antigen-specific total immunoglobulin G (IgG), as well as the IgG1 and IgG2a subclasses. Whole spleens and local lymph nodes were removed from mice and cell suspensions prepared for the detection of cellular immune responses. Single cell suspensions were prepared by passing spleens or lymph nodes through a sterile nylon mesh filter (70 μm). Spleen cells were centrifuged at 1200 rpm for 5 min and red blood cells were lysed by resuspending cells in 1 ml of sterile 0.87% ammonium chloride solution for 2 min at room temperature (RT). Cells were flooded with RPMI-1640 medium (Gibco, USA) and centrifuged again at 1200 rpm for 5 min, before being resuspended in fresh medium. Spleen mononuclear cells (2×10^6) or lymph node cells (2×10^6) were cultured in triplicate wells of 96-well 'U bottomed' microtitre plates at 37°C in a humidified 5% CO_2 environment with various concentrations of antigen or with phorbol myristate acetate (25 μg/ml PMA; Sigma, USA) and anti-mouse CD3 (1 μg/ml; BD Biosciences, USA) or medium only as positive and negative controls respectively. Supernatants were removed after 72 h for cytokine analysis by ELISA.

2.2.9. Determination of proliferative responses by T cells *in vitro*

T cells were stimulated *ex vivo* for 72 h as outlined in Section 2.2.8. Fresh medium (200 μl) was added to the cells which were pulsed with 0.5 μCi of [^{3}H]-

thymidine (Amersham Biosciences, USA) in 25 µl of complete RPMI for the final 18 h of culture. After incubation, cells were harvested onto glass fibre filters (Wallac, UK) using an automatic cell harvester (Wallac, UK). The filters were dried using a microwave and sealed in plastic sample bags with 5 ml of non-aqueous scintillation fluid (BetaScint, Wallac, UK). [^{3}H]-thymidine incorporation was assessed using a Beta-plate scintillation counter (Wallac, UK). Results are expressed as mean counts per min (cpm) of [^{3}H]-thymidine incorporation for triplicate cultures of spleen cells or lymph node cells.

2.2.10. Generation of T cell Lines

2.2.10.1. Generation of Aβ-specific T cell lines following subcutaneous immunisation

C57BL/6 mice were immunised s.c. with Aβ and CpG, or Aβ in alum. After 21 days, the mice were boosted with Aβ in CFA or Aβ in alum respectively. After 28 days, the spleens were harvested and restimulated *ex vivo* with Aβ (25 µg/ml) in the presence of cytokines and antibodies depending on the type of line being developed. Th1 cell lines were developed using spleen cells (2 x 10^6 cells/ml) from mice immunised with Aβ + CpG/CFA and were incubated with IL-12 (10 ng/ml) and anti-IL-4 (10 µg/ml) at the initiation of the culture. Th2 cell lines were developed using spleen cells from mice immunised with Aβ in alum and were incubated with IL-4 (10 ng/ml) and anti-IFN-γ (10 µg/ml) at the initiation of culture. After 4 days, IL-2 (5 ng/ml) was added to the T cell cultures. After a further 7 days, surviving T cells (1 x 10^5 cells/ml) were restimulated with irradiated APCs (2 x 10^6 cells/ml) and Aβ (25µg/ml). IL-2 (5 ng/ml) was added after 4 days and cells were cultured for a further 6 days. T cells were harvested at the end of this second cycle and used for adoptive transfer experiments.

2.2.10.2. Generation of Aβ-specific T cell lines following intraperitoneal immunisation

C57BL/6 mice were immunised i.p. with Aβ and CpG as described in Section 2.2.7.2. After 21 days the mice were boosted with Aβ and CpG. After a further 7 days the spleens were harvested and restimulated *ex vivo* with Aβ (25 μg/ml) in the presence of cytokines and antibodies depending on the type of line being developed. Th1 cells were incubated with IL-12 (10 ng/ml) at the initiation of the culture. Th2 cell lines were incubated with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml) at the initiation of culture. Th17 cell lines were incubated with IL-1 (10 ng/ml), IL-23 (10 ng/ml), and anti-IFN-γ at the initiation of culture. After 4 days, IL-2 (5 ng/ml) was added to the Th1 and Th2 cell cultures, while Th17 cells were incubated in the presence of RPMI only. After a further 7 days, surviving T cells (1×10^5 cells/ml) were restimulated with irradiated APCs (2×10^6 cells/ml) and Aβ (25μg/ml). IL-2 (5 ng/ml) was added after 4 days and cells cultured for a further 6 days. T cells were harvested at the end of this second cycle and used in co-culture experiments with mixed glia.

2.2.10.3. Generation of Aβ-specific T cell lines following subcutaneous immunisation into the footpad

C57BL/6 mice and non-transgenic littermates to the APPswe/PS1dE9 mice were immunised in the footpad with Aβ and CpG as described in Section 2.2.7.3. After 21 days the mice were boosted with Aβ and CpG. After a further 7 days the spleens were harvested and restimulated *ex vivo* with Aβ (25 μg/ml) in the presence of cytokines and antibodies depending on the type of line being developed. Th1 cells were incubated with IL-12 (10 ng/ml) at the initiation of the culture. Th2 cell lines were incubated with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml) at the initiation of culture. Th17 cell lines were

incubated with IL-1 (10 ng/ml), IL-23 (10 ng/ml), and anti-IFN-γ at the initiation of culture. After 4 days, IL-2 (5 ng/ml) was added to the Th1 and Th2 cell cultures, while Th17 cells were incubated in the presence of RPMI only. After a further 7 days, surviving T cells (1 x 10^5 cells/ml) were divided into 2 groups. One group were restimulated with irradiated APCs (2 x 10^6 cells/ml) and Aβ (25 μg/ml) for 4 days followed by 7 days of culture with IL-2. The second group were harvested at the end of this first cycle for use in co-culture experiments with mixed glia.

2.2.10.4. Preparation of irradiated APCs

Whole spleens from C57BL/6 mice were placed in 50 ml tubes (Greiner Bio-One, USA) containing ice-cold complete RPMI. These 50 ml tubes were placed into the irradiation chamber of a Nordian Gammcell 3000 Elan irradiator and irradiated at a dose of 30 Gy, which prevents cell proliferation. A single cell suspension was prepared, the red blood cells were lysed in 0.87% ammonium chloride solution, and the cells were counted as described in Section 2.2.2.

2.2.11. Coculture of T cells and glia

Following culture of Aβ-specific T cells, the different wells of each T cell subset were pooled and washed in complete RPMI. Cells were counted and added to mixed glial cultures at a ratio of 0.5:1 (2.5 x 10^5 T cells: 5 x 10^5 glia). The cells were cultured for 24 h, supernatants were removed for cytokine analysis by ELISA, and cell surface marker expression on microglia was assessed by flow cytometry.

2.2.12. Intravenous injection of T cells

For experiments involving transfer of Aβ-specific Th1 or Th2 cells into C57BL/6 mice injected intracerebroventricularly (i.c.v.) with Aβ, 1 x 10^5 cells/mouse were injected into the lateral vein in the tail. For experiments involving transfer of Aβ-specific Th1, Th2, or Th17 cells into APPswe/PS1dE9 mice, 15 x 10^6 cells/mouse were injected into the lateral tail vein. The T cells were injected into the tail vein in a total volume of 300 µl.

2.2.13. Intracerebroventricular injection of Aβ

Mice were anaesthetised by i.p. injection of a mixture of ketamine and xylazine (60 mg/kg ketamine, Vetalor, UK; 6 mg/kg xylazine, Rompun, UK), and were maintained by an additional inhalation anaesthetic of isoflurane (1-2%) using the Vapex 3 system (VetTech Solutions Ltd, UK). The absence of a pedal reflex was used to confirm deep anaesthesia. Anaesthetised mice received a unilateral, i.c.v. injection of either sterile water or Aβ (5 µg/mouse), at coordinates 0.3 mm posterior to bregma, 1.3 mm lateral to the midline, and 3 mm ventral to the skull surface. Mice were then injected s.c. with carprofen (5 mg/kg; Rimadyl, USA) as a post-operative analgesic. Mice were monitored during recovery and kept under a heat lamp until they were active. The behaviour of the mice was monitored closely for 48 h following treatment.

2.2.14. T cell transfer study protocols

2.2.14.1. Transfer of Aβ-specific T cells to mice injected intracerebroventricularly with Aβ

C57BL/6 mice (2-4 months) were injected i.c.v. with Aβ (5 μg/mouse). After 24 h, Aβ-specific Th1 or Th2 cells (1 x 10^5 cells/mouse) were injected intravenously (i.v.) into these mice. Mice were sacrificed 24 h after T cell transfer, and brains were processed for analysis by flow cytometry.

2.2.14.2. Transfer of Aβ-specific T cells to APPswe/PS1dE9 mice

To evaluate muscle strength and motor co-ordination in APPswe/PS1dE9 mice (6-7 months), these mice were tested on the wire-hang test, inverted screen test, and using footprint analysis. After 7 days, Aβ-specific Th1, Th2, and Th17 cells (15 x 10^6 cells/mouse) were injected i.v. into these mice. APPswe/PS1dE9 mice were tested for spatial memory in the Morris water maze 3 weeks after administration of Aβ-specific T cells. Two days after the completion of behavioural testing wild-type and APPswe/PS1dE9 mice were anaesthetised with sodium pentobarbital (40 μl, Euthatal, Merial Animal Health, UK) and perfused intracardially with ice-cold PBS (20 ml). The brains were rapidly removed and bisected on ice along the midline. The right hemisphere was processed for later immunohistochemical staining. The hippocampus of the left hemisphere was snap-frozen for analysis by PCR, part of the cortex was snap-frozen for analysis of Aβ concentration by multi-spot ELISA, and mononuclear cells were isolated from the rest of the left hemisphere for analysis by flow cytometry.

2.2.15. Behaviour

2.2.15.1. Wire-hang test

To evaluate muscle strength and co-ordination in wild-type and APPswe/PS1dE9 mice, front- and hind- paw strength was tested on the wire-hang test. The apparatus consisted of a horizontal steel wire (26 cm long, 0.2 cm diameter), suspended between 2 wooden poles (19.5cm high) above a padded surface. The test consisted of 2 one-minute trials with an inter-trial interval of 20 min. On the first trial the mice were suspended from the wire by all 4 paws. On the second trial, only the front 2 paws of the mice were placed on the wire. The mice were released and the length of time that they held onto the wire (latency) was recorded. Any mice that crossed the wire to the poles at either end were given a maximum score of 60 sec.

2.2.15.2. Inverted screen test

The inverted screen test was used to evaluate muscular strength in wild-type and APPswe/PS1dE9 mice. Mice were placed on a wire-mesh screen (43 x 43 cm), which was slowly inverted and held 60 cm above a padded surface. The length of time that mice held onto the screen was recorded. The trial lasted until the mouse fell from the screen or the 60 sec cutoff time was reached.

2.2.15.3. Footprint analysis

Footprint analysis was used to assess motor coordination and balance in wild-type and APPswe/PS1dE9 mice. The apparatus consisted of an open-top runway (50 cm long, 10 cm wide, and 10 cm high) with an enclosed dark box at one end. A sheet of white paper (A3) was laid underneath the runway and goal box. The front

and rear paws of the mouse were painted with red and black non-toxic poster paint respectively. The mouse was immediately placed inside the runway on the white sheet of paper, at the end opposite the goal box. Each mouse was given 60 sec to run across the white paper to the goal box. Data were not collected until performance was stable and the mouse ran rapidly and voluntarily to the goal box. The apparatus was cleaned thoroughly with 70% ethanol before beginning the next trial. Stride length (cm), hind-base width (cm), and front-base width (cm), of each mouse were assessed (Fig. 2.4.).

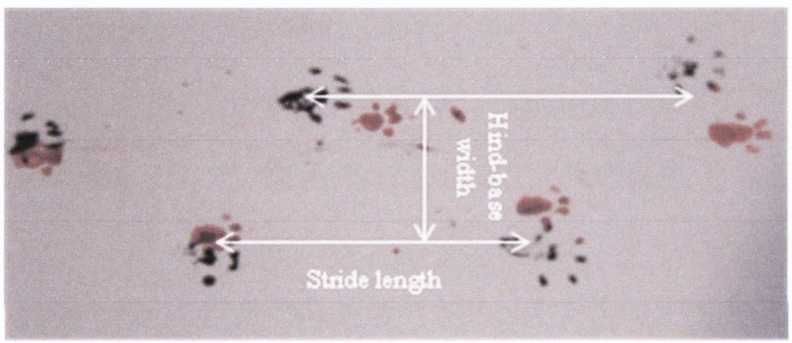


Figure 2.4. Parameters measured in footprint analysis

2.2.15.4. Morris water maze apparatus

The Morris water maze (MWM) was set up in a sound-proof room with adequate fluorescent lighting. The water maze consisted of a white circular tank 120 cm in diameter and 60 cm in height. It was filled up to 24 cm with water, the temperature of which was regulated to between 20 and 22 °C. A Perspex platform, 15 cm in diameter and 23 cm in height, was submerged 1 cm below the water surface and 13 cm from the edge of the pool wall in the southeast quadrant. A grid

was etched into the surface of the platform to ensure that the animals could grip onto it once they had located it. Two white walls and 2 white screens (110 cm in height by 120 cm in width) surrounded the water maze at an equal distance of 30 cm and each bore 2 distinct visual cue cards (30 cm x 30 cm approx.). A camera was fixed to the ceiling above the water maze and connected to the computer-based tracking programme (Water 2020, HVS Image, UK). The pool was divided into 4 quadrants (northwest, northeast, southwest, southeast) with the aid of an imaginary line produced by the Water 2020 software.

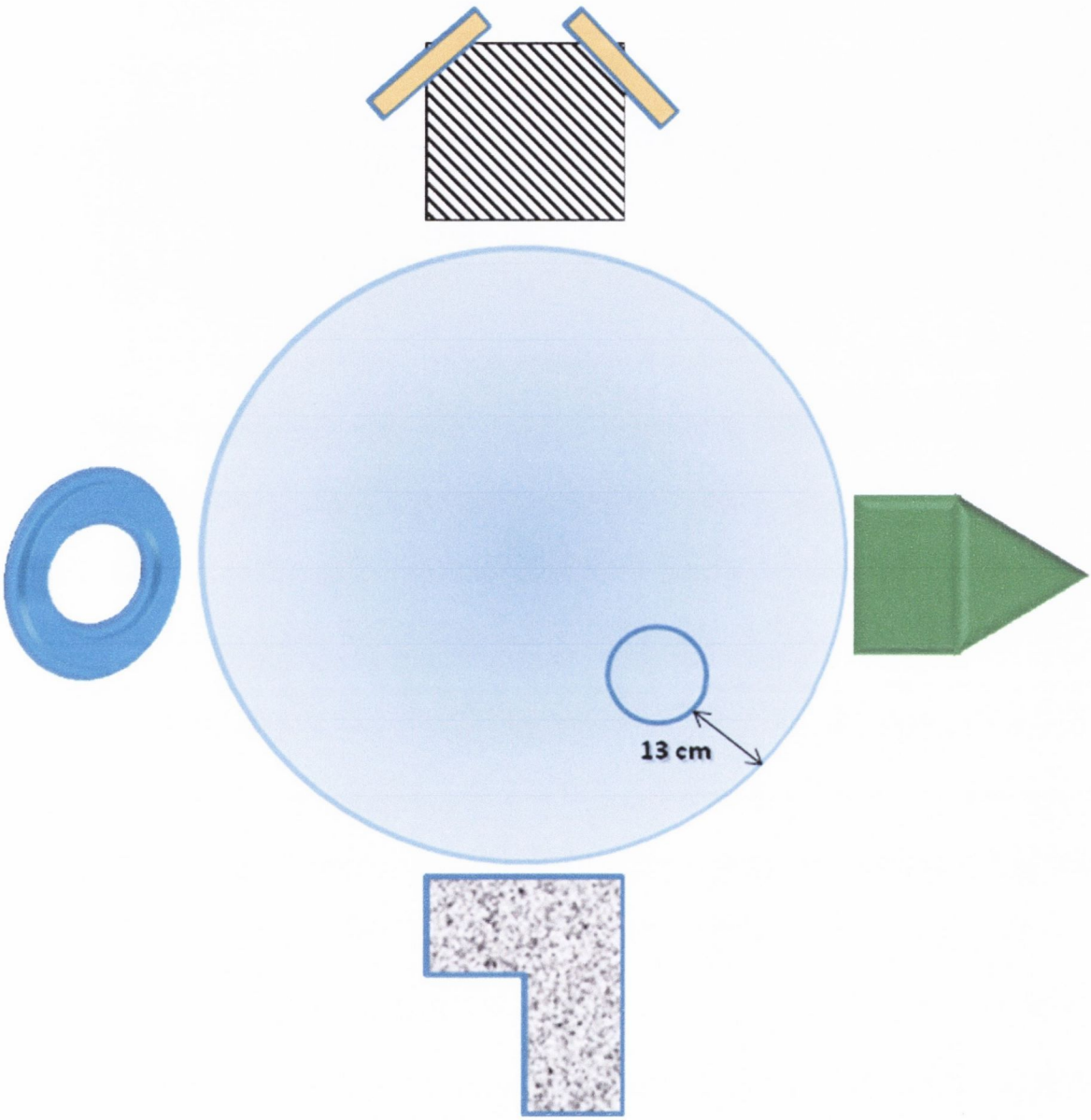


Figure 2.5. Water maze with platform and visual cues

2.2.15.5. Morris water maze habituation

Before recording any training data, animals were first subjected to a single habituation session in which the water was clear and the platform was clearly marked with a striped flag. No visual cues were used during the habituation session. Each animal was placed into the northwest quadrant of the pool facing the wall and guided to the platform by the experimenter using a plastic rod. All animals found the platform within 1 min and remained on it for 20 sec to become familiar with the trial objective and the surrounding area.

2.2.15.6. Morris water maze training

Training began 24 h after habituation and was carried out for 5 consecutive days, at the same time each day. During the training sessions, the visual cues were set in place and the water was made opaque with the use of a white tempura paint powder (Crafty Devils, UK). Each training day consisted of 4 one-minute trials with an inter-trial interval of 5 min during which the animal was returned to its home cage. The home cage was heated to approximately 30 °C by means of a hot water bottle placed underneath the base of the cage. Each animal was gently lowered by the tail into the pool facing the pool wall. During each trial all animals were released from semi-random start points (north, south, east, and west, with each start point being used once per day) and the order of these start points was randomised daily by means of a random sequence generator programme (random.org). The order in which the mice were placed in the water maze was also randomised each day. If an animal found the platform within 60 sec, the duration of the trial was recorded, and the animal was left on the platform for 20 sec to become familiar with the location of the platform relative to the proximal visual cues. Animals that failed to find the platform within 60 sec were placed on the platform by the experimenter, and left there for 20 sec.

2.2.15.7. Morris water maze probe trial

The probe trial was carried out the day after the final training day. This consisted of a single one-min trial in which the platform was removed from the water maze. For the probe trial, animals were placed into the centre of the water maze. The percentage of time and pathlength each animal spent swimming in the quadrant previously containing the platform was measured. The number of times each animal swam across the area previously containing the platform was also measured.

2.2.16. Cell isolation from CNS tissue

2.2.16.1. Total cell isolation from CNS tissue

C57BL/6 mice were sacrificed by cervical dislocation followed by decapitation. The brain was removed rapidly and bisected along the midline. The hippocampus was removed and placed in Hank's balanced salt solution containing 3 % FCS (HBSS/FCS, Gibco, UK). Tissue was dissociated through a sterile nylon-mesh filter (70 μm), washed with HBSS/FCS and centrifuged at 170 g for 10 min atRT. The resulting pellet was resuspended in a solution containing collagenase D (1 mg/ml, Roche, Ireland) and DNAse I (10 μg/ml, Sigma-Aldrich, UK) and incubated on a rotary wheel for 1 h at 37°C to facilitate breakdown of tissue. The pellet was washed with HBSS/FCS and cells were counted to determine the optimal dilution for each sample. Cells were washed twice by centrifuging at 1200 rpm for 5 min and re-suspending in 2 ml fluorescence-activated cell sorting (FACS) buffer. Cells were stained for analysis by flow cytometry.

2.2.16.2. Mononuclear cell isolation from CNS tissue

APPswe/PS1dE9 mice and wild-type controls were anaesthetised with sodium pentobarbital (40 µl, Euthatal, Merial Animal Health, UK) and perfused intracardially with ice-cold PBS (20 ml). The brain was removed rapidly and processed as described in Section 2.2.14.2. The portion of the left hemisphere of the brain taken for analysis by flow cytometry was placed in a 15 ml Falcon tube containing 2 ml HBSS/FCS. Tissue was dissociated through a sterile nylon-mesh filter (70 µm), washed with HBSS/FCS and centrifuged at 170 g for 10 min at RT. The resulting pellet was resuspended in a solution containing collagenase D (1 mg/ml) and DNAse I (10 µg/ml), and incubated on a rotary wheel for 1 h at 37°C to facilitate breakdown of tissue. Cells were washed in HBSS/FCS and resuspended in 9 ml of 1.088g/ml Percoll (Sigma-Aldrich, UK). This was underlayed with 5 ml of 1.122 g/ml Percoll, and overlayed with 9 ml each of 1.072 g/ml Percoll, 1.030 g/ml Percoll, and 1X PBS. Percoll gradients were centrifuged at 1250 g for 45 min at 20°C. Mononuclear cells were removed from the 1.088:1.072 and 1.072:1.030 g/ml interfaces and washed twice in HBSS/FCS. Cells were stained for analysis by flow cytometry.

2.2.17. Flow cytometry

Surface marker expression on cells prepared *ex vivo* from the brain and on mixed glial cultures was analysed by flow cytometry using a DAKO $CyAN_{ADP}$ flow cyometer. The flow cytometer was calibrated using Flow-Check Fluorospheres (Beckman Coulter, USA). Mononuclear cells were resuspended in 100 µl FACS buffer. Cultured mixed glial cells were harvested by washing with 1X PBS post-stimulation, followed by addition of trypsin-EDTA (400 µl; Sigma-Aldrich, UK). Plates were placed in the incubator for approximately 3 min to remove the cells from the wells, and the trypsin was inactivated by placing the cells in 2 ml DMEM. Cells were washed twice by centrifuging at 1200 rpm for 5 min and re-

suspending in 2 ml FACS buffer. The low-affinity IgG receptors (FcγRIII) were blocked by incubating with CD16/CD32 FcγRIII block (1:100 dilution; BD Biosciences, USA) for 10 min at RT. The cells were washed in FACS buffer, resuspended in 100 µl of FACS buffer, and incubated with the appropriate FACS antibodies (Table 2.2) for 20 min at RT in the dark. Excess staining antibody was removed by washing each tube twice in FACS buffer (2 ml/tube). Immunofluorescence analysis was performed using Summit software (Dako, Denmark) and the results analysed using FlowJo software (Tree Star, USA).

For intracellular staining, cells were harvested, washed, blocked, and stained with antibodies specific for cell surface molecules as described above. The cells were prepared for intracellular staining using a cell permeabilisation kit (Dako, Denmark). To fix the cells, 100 µl of IntraStain Reagent A was added to each tube. The cells were incubated for 15 min at RT in the dark. The cells were washed twice in FACS buffer. To permeabilise the cells, 100 µl of Intrastain Reagent B was added to each tube along with the intracellular antibodies, and cells were incubated for 20 min at RT in the dark. Following two washes in FACS buffer, immunofluorescence analysis was performed using Summit software (Dako, Denmark) and the results analysed using FlowJo software (Tree Star, USA).

Table 2.2. Antibodies used in flow cytometry

Surface Marker	Fluorescent Label	Dilution Factor	Supplier
CD11b	Alexa Fluor 647	1/400	BD Biosciences
I-A/I-E	FITC/PE	1/500	BD Biosciences
CD40	FITC/PE	1/100	BD Biosciences
CD80	PE	1/200	BD Biosciences
CD86	FITC	1/200	BD Biosciences
CD4	PE-Cy7	1/100	eBioscience
CD3	APC-Alexa Fluor 750	1/100	BD Biosciences
TNF-α	FITC	1/100	eBioscience
IL-6	PE	1/20	eBioscience
IFN-γ	FITC	1/30	BD Biosciences
IL-17	PE	1/30	BD Biosciences
IL-10	FITC	1/30	BD Biosciences

2.2.18. Protein Assay

Protein concentrations in CNS tissue prepared from APPswe/PS1dE9 mice were determined using a bicinchoninic acid protein assay kit (Pierce, The Netherlands). To make up the working reagent, 500 µl of Reagent B was added to 25 ml of Reagent A. A standard curve was prepared (0 – 2000 µg/ml) using a range of concentrations of BSA diluted in the same buffer as the samples to be tested. Aliquots (25 µl) of sample/standard was added in triplicate to a 96-well plate. Working reagent (200 µl) was added to each well and the plate was then incubated for 30 min at 37 °C to allow the colour to develop. The optical density values were determined by measuring the absorbance at 590 nm using a microtitre plate reader (Multiskan RC, Labsystems, Finland) and protein concentrations in the test samples were evaluated with reference to the standard curve.

2.2.19. Determination of cytokine concentrations by ELISA

2.2.19.1. IL-1β, IL-10, IL-17, and TNF-α ELISA

The concentrations of IL-1β, IL-10, IL-17, and TNF-α were measured using commercially available ELISA kits (R&D Systems, USA). High binding 96-well microtitre plates (Greiner Bio-One, USA) were coated overnight (minimum 18 h) at 4°C with 50 µl/well of rat anti-mouse IL-1β (4 µg/ml), IL-10 (2 µg/ml), IL-17 (2 µg/ml), and TNF-α (0.8 µg/ml) capture antibody in PBS. Plates were washed 3 times in wash buffer (0.05% Tween 20 in PBS), and non-specific binding sites were blocked by adding 150 µl of blocking buffer (1% BSA in PBS) for 2 h at RT. Plates were washed and 50 µl/well of test supernatant as well as serially diluted recombinant mouse IL-1β standard (0-1000 pg/ml), IL-10 standard (0-2000 pg/ml), IL-17 standard (0-1000 pg/ml), or TNF-α standard (0-2000 pg/ml) in 1% BSA/PBS were added. Plates were incubated overnight at 4 °C, washed, and

incubated with 50 µl/well of biotinylated goat anti-mouse IL-1β (100 ng/ml), IL-10 (400 ng/ml), IL-17 (200 ng/ml), or TNF-α (150 ng/ml) in 1% BSA for 2 h at RT. After washing, 50 µl/well of horseradish peroxidase (HRP)-conjugated streptavidin (1:200 in 1% BSA; R&D Systems, USA) was added and plates were incubated for 30 min at RT in the dark. The plates were washed and 50 µl/well of ELISA substrate solution was added. The colour reaction was allowed to develop and the enzyme reaction was stopped with 25 µl/well 1 M H_2SO_4 once the standard curve had developed sufficiently. The optical density values were determined by measuring the absorbance at 493 nm using a microtiter plate reader (Multiskan RC, Labsystems, Finland) and cytokine concentrations in the test samples were evaluated with reference to the standard curve prepared using recombinant mouse IL-1β, IL-10, IL-17, or TNF-α of known concentration.

2.2.19.2. IL-6, IL-4, IL-5, and IFN-γ ELISA

Concentrations of IL-6, IL-4, IL-5, and IFN-γ were determined by ELISA using commercially available paired antibodies (BD Biosciences, USA). High binding 96-well microtitre plates were coated overnight (minimum 18 hr) at 4°C with 50 µl/well of purified anti-mouse IL-6 (1 µg/ml), IL-4 (1 µg/ml), IL-5 (1 µg/ml), or IFN-γ (1 µg/ml) capture antibody in PBS. Plates were washed 3 times in wash buffer (0.05% Tween 20 in 500 ml PBS), and non-specific binding sites were blocked by adding 150µl of blocking buffer (3% BSA in PBS, or 10% dried milk in PBS) for 2 h at RT. Plates were washed again in wash buffer and 50 µl/well of test supernatant as well as serially-diluted recombinant mouse IL-6 standard (0-5000 pg/ml), IL-4 standard (0-2500 pg/ml), IL-5 standard (0-2500 pg/ml) or IFN-γ standard (0-10 ng/ml) in PBS were added. Following incubation overnight at 4°C, the plates were washed and incubated with 50 µl/well of biotinylated rat anti-mouse IL-6 (1 µg/ml), IL-4 (1 µg/ml), IL-5 (1 µg/ml), or IFN-γ (1 µg/ml) for 1 h at RT. The plates were washed and incubated with 50 µl/well of horseradish peroxidase (HRP)-conjugated streptavidin (1:1000 in PBS; BD Biosciences,

USA) for 30 min at RT in the dark. After washing, 50 µl/well of ELISA substrate solution was added. The colour reaction was allowed to develop and the enzyme reaction was stopped with 25 µl/well 1 M H_2SO_4 once the standard curve had developed sufficiently. The optical density values were determined by measuring the absorbance at 493 nm using a microtitre plate reader (Multiskan RC, Labsystems, Finland) and cytokine concentrations in the test samples were evaluated with reference to the standard curve prepared using recombinant mouse IL-6 or IFN-γ of known concentration.

Table 2.3. Origin and specificities of antibodies used in ELISA

Antibody Specificity	Clone	Purified/ Conjugate	Supplier
Mouse IFN-γ	RA-6A2	Purified	BD Biosciences
Mouse IFN-γ	XMG1.2	Biotin	BD Biosciences
Mouse IL-4	11B11	Purified	BD Biosciences
Mouse IL-4	BVD6-24G2	Biotin	BD Biosciences
Mouse IL-5	TRFK5	Purified	BD Biosciences
Mouse IL-5	TRFK4	Biotin	BD Biosciences
Mouse IL-6	MP5-20F3	Purified	BD Biosciences
Mouse IL-6	MP5-32C11	Biotin	BD Biosciences
Mouse IL-1β	-	Purified	R & D Systems
Mouse IL-1β	-	Biotin	R & D Systems
Mouse IL-10	-	Purified	R & D Systems
Mouse IL-10	-	Biotin	R & D Systems
Mouse IL-17	-	Purified	R & D Systems
Mouse IL-17	-	Biotin	R & D Systems
Mouse TNF-α	-	Purified	R & D Systems
Mouse TNF-α	-	Biotin	R & D Systems
Mouse Total IgG	-	Biotin	CalTag
Mouse IgG1	-	Biotin	CalTag
Mouse IgG2a	-	Biotin	BD Biosciences

2.2.20. Quantification of serum IgG and IgG subclass

Serum samples were prepared from peripheral blood removed from the thoracic cavity of mice after sacrifice. Blood was allowed to clot for 2 h at RT and stored at 4°C overnight. Serum was transferred into fresh tubes and stored at -20°C. The level of $A\beta_{42}$ specific IgG antibodies in the sera of naïve and treated animals was determined by ELISA. $A\beta_{42}$ (5 µg/ml in PBS) was added to flat-bottomed 96-well microtitre plates and incubated overnight in a laminar flow hood at RT. Excess antigen was removed by washing with wash buffer (0.05% Tween 20 in PBS) and non-specific binding sites were blocked by incubating the plates with 200 µl/well PBS supplemented with 10 % (w/v) of dried milk (Marvel) for 2 h at RT. Serum samples were serially diluted in PBS and 50 µl of sample was added to each well of the plates to determine the endpoint titres, starting at a dilution of 1:20 or 1:50. Serial dilutions of normal mouse serum served as a blank. The plates were incubated overnight at 4°C. After washing, plates were incubated for 2 h at RT with 50 µl/well of a biotin-conjugated rat anti-mouse IgG monoclonal antibody (1:10,000 dilution in PBS; Caltag, USA), with specificity for all IgG subtypes, or 50 µl/well of a 1:4,000 dilution of either IgG2a or IgG1 (1:1000 dilution, BD Biosciences, USA). Plates were washed and incubated for 30 min at RT with 50 µl/well of HRP-conjugated streptavidin (1:1,000 dilution in PBS; BD Biosciences, USA). The plates were washed and 50 µl of ELISA substrate solution was added to each well. The enzymatic reaction was stopped with 25 µl of 1 M H_2SO_4. The absorbance was measured at 492 nm. Results are expressed as $\log_{10}$ endpoint antibody titres as determined by extrapolation of the linear portion of the dilution curve to the OD_{492nm} value of the control serum.

2.2.21. Real-time polymerase chain reaction (PCR)

2.2.21.1. Isolation of RNA from brain tissue

Messenger RNA (mRNA) was extracted from snap-frozen hippocampal tissue obtained from wild-type and APPswe/PS1dE9 mice treated with Th1, Th2, or Th17 cells, and from *in vitro* glial cultures. Tissue from *in vivo* studies was homogenised in 350 µl of cell lysis buffer (NucleoSpin RNA II, Macherey-Nagel, Germany) using a Polytron homogeniser (Kinematica, Switzerland) for extraction of RNA. To remove cultured glial cells, 350 µl of cell lysis buffer was added to each well, samples were left on ice for 5 min, and glia were removed from the plate by trituration. To remove any insoluble tissue and reduce viscousity, lysate was added to the membranes of NucleoSpin Filters and filtered by centrifugation at 11,000 g for 1 min. Filters were discarded and 350 µl of ethanol (70%) was added to the filtrate. The filtrate was mixed and loaded onto the membrane of a NucleoSpin RNA II column. Tubes were centrifuged at 8,000 g for 30 sec, allowing the RNA to bind to the membrane. The silica membrane was desalted by adding 350 µl of membrane desalting buffer to the membrane, and samples were centrifuged at 11,000 g for 1 min to dry the membrane. DNA was digested by adding 95 µl of DNase reaction mixture directly onto the centre of the NucleoSpin RNA II column and incubating the tubes at RT for 15 min. The DNase was inactivated by washing samples in 200 µl of RA2 buffer and tubes were centrifuged at 11,000 g for 30 sec to dry the membrane. The silica membrane was washed with 600 µl RA3 buffer and centrifuged at 11,000 g for 30 sec. The membrane was washed again with 250 µl of RA3 buffer and tubes were centrifuged at 11,000 g for 2 min to completely dry the membrane. Filters were placed into nuclease-free collecting tubes and 40 µl of RNase free H_2O was added to the membrane. RNA was eluted by centrifuging samples at 11,000 g for 1 min, and RNA concentration was quantified using a NanoDrop Spectrophotometer (ND-1000 v3.5, NanoDrop Technologies Inc., USA).

2.2.21.2. Reverse transcription for cDNA synthesis

Total mRNA was reverse-transcribed into complementary DNA (cDNA) using a high-capacity cDNA archive kit (Applied Biosystems, Germany) according to the protocol provided by the manufacturer. RNA (0.5 µg) was added to fresh tubes containing the appropriate volume of nuclease-free H_2O to make a final volume of 10 µl. A 2X mastermix was prepared containing the appropriate volumes of 10X RT buffer, 25X dNTPs, and 10X random primer multiscribe reverse transcriptase (50 U/µl). The mastermix (10 µl) was added to the RNA and nuclease free H_2O. Tubes were incubated for 10 min at 25°C followed by 2 h at 37°C on a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences, Ireland).

2.2.21.3. Real-time PCR

Real-time PCR dye-labelled primers were delivered as "TaqMan® Gene Expression Assays" for the mouse genes listed in Table 2.4 (Applied Biosystems, Germany). cDNA was diluted 1:5 with RNase free H_2O and 4.5 µl of sample was added to each well (18 ng/well). This was mixed with 4.5 µl of TaqMan Universal PCR Fast Mastermix (Applied Biosystems, Germany) and 0.5 µl each of the target primer and the endogenous control primer, for a total volume of 10 µl per well. Human 18S RNA (Applied Biosystems, Germany) was used as the endogenous control. Real-time PCR was performed using Applied Biosytems ABI Prism 7300 Fast Track Sequence Detection System v1.4.1 in 96-well format with a 10 µl reaction volume per well. Each sample was measured in duplicate in a single real-time PCR run. Samples were initially brought to 95°C for 20 sec before being run for 40 cycles of 95°C for 3 sec followed by 60°C for 30 sec.

2.2.21.4. PCR quantification

The expression of each target gene was determined using the efficiency-corrected comparative CT method. Target genes in different samples were compared to a reference gene (18S RNA). These values were normalised to the control sample and the relative differences in expression of the target gene between the samples were expressed as a ratio.

Table 2.4. Gene expression assay numbers of PCR primers used in this study

Gene Name	Gene Description	TaqMan Gene Expression Assay Number	Genbank Accession Number
IL-1	Interleukin-1 beta	Mm00434228_m1	NM_008361.3
TNF-α	Tumour Necrosis Factor-alpha	Mm00443258_m1	NM_013693.2
CD40	CD40	Mm00441891_m1	NM_170701.2
CD68	CD68	Mm03047343_m1	NM_009853.1

2.2.22. Determination of Aβ concentration in tissue samples

2.2.22.1. Extraction of soluble and insoluble Aβ from brain tissue

Soluble and insoluble Aβ were extracted from snap-frozen cortical tissue obtained from wild-type and APPswe/PS1dE9 mice treated with Th1, Th2, or Th17 cells. Tissue for Aβ extraction was homogenised in 5 volumes (w/v) of homogenising buffer (SDS/NaCl in dH_2O with added proteases, pH10). The samples were centrifuged at 15,000 rpm for 40 min at 4°C. Pellets were stored at -80°C for isolation of insoluble Aβ.

Supernatants were used to extract SDS-soluble Aβ. Protein concentrations in supernatants were equalised to 4 mg/ml with homogenisation buffer using a bicinchoninic acid protein assay (see Section 2.2.18). The protein concentration was kept above 2 mg/ml to ensure that SDS was diluted to less than 0.1% on the plate. Equalised supernatants were aliquoted and neutralised by the addition of 10% (v/v) 0.5 M Tris-HCl (pH 6.8). Samples were stored at -20°C for later detection of soluble Aβ.

Pellets were used for the extraction of insoluble Aβ. Pellets were disrupted by ultrasonication at 23 kHz for 2 x 30 sec in guanidine buffer (5.0 M guanidine-HCl/50 mM Tris-HCl, pH8.0, Sigma-Aldrich, UK), incubated for 4 h on ice, and centrifuged at 15,000 rpm for 30 min at 4°C. Supernatants were equalised to 0.4 mg/ml with guanidine buffer, aliquoted, and stored at -20°C for later detection of insoluble Aβ.

2.2.22.2. Detection of SDS-soluble and insoluble Aβ by multi-spot ELISA

Concentrations of Aβ in SDS-solubilised and guanidine-treated samples were determined using "MSD® 96-well multi-spot 4G8 Aβ triple ultra-sensitive assay" kits (Meso Scale Discovery, USA). Multi-spot Aβ 3-plex plates were blocked by adding 150 µl of 1% Blocker A (Meso Scale Discovery, USA) for 1 h at RT with shaking. Plates were washed 3 times in 1X tris wash buffer (TWB). Detection antibody solution (25 µl; Meso Scale Discovery, USA) was added to each well.

SDS-solubilised samples were diluted to 200 µg/ml in kit buffer and 25 µl of sample was added in duplicate to the appropriate wells of a 96-well plate (5 µg/well). Guanidine-treated samples were diluted to 0.4 µg/ml in TWB and 25 µl of sample was added in duplicate to the appropriate wells of the 96-well plate (10 ng/well). Serially-diluted recombinant human $A\beta_{38}$ (0-3,000 pg/ml), $A\beta_{40}$ (0-10,000 pg/ml), and $A\beta_{42}$ (0-3,000pg/ml) in 1% Blocker A solution were added to appropriate wells of the 96-well plate, which was incubated for 2 h at RT. Plates were washed again in wash buffer, and 150 µl of 2X MSD read buffer (Meso Scale Discovery, USA) was added. The plate was read immediately using a Sector Imager plate reader (Meso Scale Discovery, USA) and Aβ concentrations in test samples were evaluated with reference to the standard curve prepared using recombinant $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$.

2.2.23. Determination of Aβ concentration in serum

Concentrations of Aβ in serum were determined using "MSD® 96-well multi-spot 4G8 Aβ triple ultra-sensitive assay" kits. Multi-spot Aβ 3-plex plates were blocked by adding 150 µl of 5% Blocker A for 1 h at RT with shaking. Plates were washed 3 times in 1X TWB. Serum samples to be tested (25 µl), and serially diluted recombinant human $A\beta_{38}$ (0-3,000 pg/ml), $A\beta_{40}$ (0-10,000 pg/ml), and $A\beta_{42}$ (0-3,000pg/ml) in 1% Blocker A solution were added to appropriate

wells of the 96-well plate. Guanidine buffer (25 µl) was added to each well and samples were incubated for 2 h at RT with shaking. Plates were washed in TWB, and 25 µl of detection antibody was added for 1.5 h at RT with shaking. Plates were washed again in TWB and 150 µl of 2X MSD read buffer was added. The plate was then read immediately using a Sector Imager plate reader and Aβ concentrations in the test samples were evaluated with reference to the standard curve prepared using recombinant $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$.

2.2.24. Immunohistochemistry

2.2.24.1. Preparation of tissue sections for immunohistochemistry

Slices for immunochemistry were prepared from tissue obtained from wild-type and APPswe/PS1dE9 mice treated with Th1, Th2, or Th17 cells. For cryostat sectioning, the left hemisphere of each brain was placed onto cork discs coated in OCT compound (R.A. Lamb, UK). The brain was covered with OCT compound and snap-frozen in isopropanol which had been pre-chilled on dry ice. Brains were stored at -80°C until required for sectioning. Pre-subbed slides were used for sectioning as they provided a suitable surface to which the section could adhere. On the day of sectioning the half brain was allowed to equilibrate to -20°C for 2 h. Saggital sections (10 µm) were cut, and periodically stained with a 1% toluidine blue solution until the hippocampus was visible on the section. Toluidine blue was added for 30 sec and sections were viewed by light microscopy (Nikon Labophot, Nikon Instech Co., Japan). When the hippocampus became visible, 60 sections (10 µm) were cut from each animal onto 20 pre-subbed slides (3 sections per slide), making sure that one section from the outside, middle, and inside of the brain hemisphere was added to each slide. Sections were allowed to dry for 20 min and stored at -20°C until required.

2.2.24.2. Immunohistochemical staining for Aβ using Congo red

Brain sections were stained for Aβ plaques using the Congo red staining method. Sections were left at RT for 30 min to allow the temperature to equilibrate. Following fixation in ice-cold ethanol for 5 min, brain slices were washed twice for 5 min in PBS. During the washes, 2 ml of 1 M NaOH solution was added to 200 ml of saturated NaCl to produce an alkaline solution. Slices were incubated in alkaline-saturated NaCl for 20 min at RT. During this incubation, 200 ml of Congo red solution was filtered through a syringe using a cellulose acetate membrane filter (0.2 μM Supor membrane Acrodisc syringe filters, Pall Corporation, USA). Congo red (200 ml) and 1 M NaOH (2 ml) were mixed to generate an alkaline solution. The brain slices were incubated in alkaline Congo red solution for 30 min at RT. Slices were rinsed briefly (2 dips) in dH_2O to remove excess stain and slides were incubated in a 1% (w/v) methyl green solution (Sigma-Aldrich, UK) for 30 sec to counterstain the sections. Following washing in running dH_2O for 1 min to remove excess stain, sections were dehydrated by dipping 6 times each in 95% ethanol, 100% ethanol, and 100% ethanol. Sections were dried briefly to remove excess alcohol, and incubated for 5 min each in 3 xylene dishes. Coverslips were mounted onto the slides using the xylene-based mountant, DPX (Electron Microscopy Sciences, USA), and allowed to dry in a fume hood overnight.

2.2.24.3. Quantification of cerebral amyloid angiopathy

Congo red positive Aβ plaques were counted under the same light and magnification settings in 3 representative sections from each animal and results were expressed as the number of plaques per section.

2.2.25. Statistical Analysis

Statistical analysis was performed using the computer based statistical package GraphPad Prism. Student's t-test and one-way ANOVA using the Newman Keuls post-hoc test were used where appropriate to determine statistical differences.

Chapter 3

Modulation of glial activation by adjuvant-polarised T cells

3.1. Introduction

Alzheimer's disease is a neurodegenerative disease characterised by neuronal loss, neurofibrillary tau tangles, and neuritic plaques. One of the major theories postulated to explain the mechanism of neurodegeneration leading to AD is the amyloid cascade hypothesis. In this model, the imbalance between the production and clearance of Aβ gives rise to an accumulation of the peptide in the brain, leading to plaque formation, microglial activation, astrogliosis, and ultimately neuronal loss, resulting in cognitive decline. There is strong evidence to support a key role for Aβ in the development of AD (Weiner & Frenkel 2006), and a number of therapies in development are focused on clearance of Aβ from the brain.

Immunisation with recombinant or purified native antigens from pathogens usually requires co-administration with an adjuvant to boost the immune response, especially with poorly immunogenic antigens. It was postulated that immunisation with Aβ in combination with an adjuvant would induce a systemic adaptive immune response to Aβ, resulting in reduction of the amyloid burden in the brain, and slowing the deterioration of memory seen in AD. Schenk and colleagues reported that immunisation of PDAPP transgenic mice with $A\beta_{42}$ in CFA dramatically decreased the Aβ burden in the hippocampus (Schenk *et al.* 1999). Subsequent experiments showed that this Aβ immunisation protocol was able to protect APP-transgenic mice from the memory impairment normally seen

in these animals as they age (Janus *et al.* 2000, Morgan *et al.* 2000). These results were then replicated in a non-human primate, the Caribbean vervet, which showed clearance of Aβ from the brain following immunisation (Lemere *et al.* 2004). In clinical trials however, immunisation of patients with Aβ in combination with the Th1-type adjuvant, QS-21, led to 6% of participants developing meningoencephalitis (Orgogozo *et al.* 2003). It is important to note that meningoencephalitis was not seen in early immunisation experiments using APP-transgenic mice.

The reason for the development of this meningoencephalitis is unclear, but it may be due to the induction of Aβ-specific Th1 cells by the vaccine (Schenk *et al.* 2004). QS-21 is an adjuvant known to stimulate a strong Th1 cell response, as well as inducing CTLs against exogenous antigens (Sun *et al.* 2009), and indeed T cells have been detected in the brains of immunised patients (Wilcock & Colton 2008). Immunisation of mice with Aβ in QS-21 induced high titers of Aβ-specific IgG2a antibodies, indicative of a Th1 response, and spleen cells from QS-21-immunised mice produced significant levels of IFN-γ in response to Aβ (Cribbs *et al.* 2003). However, there is no conclusive evidence that Th1 cells were responsible for the development of meningoencephalitis in immunised patients, and it is possible that this side effect of therapy may be due to other factors, such as increased efflux of Aβ from the parenchyma to the cerebral vasculature resulting in CAA, or an over-activation of microglia induced by antibody-opsonised Aβ (Boche & Nicoll 2008). It is thus important to characterise the effects of Aβ-specific T cells in the brain to understand the role they play in both the disease process and in immunotherapy studies.

The adjuvant used to induce plaque clearance in animal models of AD, CFA, is a water-in-oil emulsion containing heat-killed *Mycobacterium tuberculosis*. It is a potent inducer of antigen-specific Th1 cells (Sano *et al.* 1999, Shibaki & Katz 2002), and is an established inducer of Th1-dependent delayed hypersensitivity reactions (Carson & Raz 1997). Furthermore, antigen

restimulation of spleen cells from mice immunised with Aβ in CFA was found to produce significant levels of IFN-γ and TNF-α (Cribbs *et al.* 2003). Another adjuvant that promotes Th1 responses is CpG. Unmethylated CpG motifs are present in bacterial DNA, but not in the DNA of mammals (Krishnamachari & Salem 2009, Roman *et al.* 1997). It can be protective in viral and bacterial infections, and has been found to protect BALB/c mice against *Leishmania* infection by skewing the immune response from one that is Th2-dominated towards a Th1 response (Walker *et al.* 1999).

In order to prevent the encephalitis seen in clinical trials of vaccination, it may be preferable to generate a Th2-dominated anti-inflammatory response. Alum is an aluminium salt with immune adjuvant activity. It promotes a Th2-type immune response, and immunisation of mice with Aβ in alum generated primarily IL-4-producing Th2 cells (Cribbs *et al.* 2003). Furthermore, immunisation of Tg2576 mice with a synthetic Aβ peptide in alum reduced Aβ levels and improved cognitive performance, without the associated microhaemorrhages seen with passive administration of Aβ-specific antibodies (Asuni *et al.* 2006). It is thus of interest to study the effects of Th2-promoting adjuvants on microglial activation as well as plaque clearance. Th17 cells are associated with the pathogenesis of a number of immune diseases, such as rheumatoid arthritis and multiple sclerosis (Chabaud *et al.* 1999, Langrish *et al.* 2005), and thus they may also have a role in the pathogenesis of AD, though this has not been systematically examined.

Microglial activation appears to play a central role in AD, but whether this activation is beneficial or harmful remains unclear. Activated microglia expressing MHC class II and CD40 were found in the vicinity of Aβ plaques in brain sections from AD patients (Perlmutter *et al.* 1992, Togo *et al.* 2000). Increased expression of IL-6 and IL-1β has also been reported in the brains of AD patients (Wood *et al.* 1993). It has also been shown that treatment of microglia *in vitro* with Aβ induces production of nitric oxide, IL-1β, and TNF-α (Floden &

Combs 2006, Halle *et al.* 2008, Ii *et al.* 1996). In addition, microglia isolated from the brains of AD patients produced the proinflammatory cytokines IL-1β, IL-6, and TNF-α following stimulation with Aβ (Lue *et al.* 2001b). Intracerebroventricular injection of Aβ enhances production of IL-1β and TNF-α, as well as expression of MHC class II and CD86 (Clarke *et al.* 2007, Lyons *et al.* 2007). Treatment of microglia with Aβ renders them more toxic to neurons *in vitro* (Butovsky *et al.* 2005a, Giulian *et al.* 1996), and this has led to the suggestion that microglial activation is detrimental in AD. However, there is no clear evidence for microglial-induced neurotoxicity *in vivo*. It has been demonstrated that microglia phagocytose Aβ *in vitro* (Koenigsknecht & Landreth 2005, Paresce *et al.* 1996), and Aβ injected into the hippocampus of rats is rapidly phagocytosed by microglia (Frautschy *et al.* 1992). Additionally, immunisation of PDAPP mice with Aβ in CFA was found to reduce Aβ plaque burden in a mechanism involving enhanced phagocytosis by microglia (Bard *et al.* 2000). It will be important to characterise the interaction between T cells and microglia to determine if this plays a role in brain inflammation or plaque clearance seen in clinical trials of AD.

The aims of this chapter were to:

- Investigate the potential of using different adjuvants to generate polarised, Aβ-specific Th1, Th2, and Th17 cells.
- Examine the effects of Aβ on cytokine production and co-stimulatory molecule expression by microglia *in vitro* and *in vivo*.
- Assess the effect of Aβ-specific T cell subsets on Aβ-induced microglial activation *in vitro* and *in vivo*.

3.2. Results

3.2.1. Effect of CFA and CpG on the induction of Aβ-specific T cells and antibodies

Due to tolerance, self-antigens such as Aβ are poorly immunogenic, and thus require the use of adjuvants to break tolerance and generate an immune response. In order to ascertain whether adjuvants could be used to generate an Aβ-specific T cell response *in vivo*, a protocol was used that was adapted from one used for the induction of EAE with myelin oligodendrocyte glycoprotein (MOG). Previous data from this laboratory have shown that this protocol of administration induces T cells, particularly Th1 and Th17 cells, specific for MOG. C57BL/6 mice were injected s.c. into the back with either CFA or CpG (25 μg/mouse) along with Aβ (100 μg/mouse). PT (150 ng/mouse) was administered i.p. on the same day. The mice were boosted 21 days later with Aβ in IFA or CFA respectively. Seven days after the boost, blood (from which serum was prepared) and spleen cells were harvested from immunised mice. The sera were analysed for Aβ-specific antibody responses by ELISA. Spleen cells were restimulated *ex vivo* with Aβ (10-50 μg/ml) for 72 h. After 3 days in culture, supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentrations by ELISA, as a measure of Th1, Th2, Treg, and Th17 responses, respectively. Cells were pulsed with [^{3}H]-thymidine for 18 h and proliferation was assessed using a liquid scintillation counter.

The data show that the immunisation protocol of Aβ plus CpG followed by a booster immunisation with Aβ in CFA induced significant cell proliferation in response to Aβ above that induced by the other immunisation protocols (Fig. 3.1). Spleen cells from mice immunised with Aβ in CFA and then Aβ in IFA produced significant concentrations of IFN-γ, and there was also an increase in IL-17

concentration, though this did not reach statistical significance (Fig. 3.2). Spleen cells from mice immunised with Aβ plus CpG and then Aβ in CFA produced significantly higher concentrations of IFN-γ and IL-17 than spleen cells from mice immunised with Aβ plus CFA and then Aβ in IFA.

Analysis of IgG concentration showed that immunisation with Aβ in CFA and then Aβ in IFA produced significant levels of Aβ-specific IgG in the serum (Fig. 3.3). In contrast, sera from mice immunised with Aβ plus CpG and then Aβ in CFA were found to have no increase in Aβ-specific IgG when compared with control animals. The data presented here indicate that Aβ in CFA followed by Aβ in IFA induced a mixed Th1/Th17 phenotype, with a concomitant antibody response. Immunisation with Aβ and CpG followed by Aβ in CFA induced a stronger Th1/Th17 response, and proliferative response to Aβ, but did not produce Aβ-specific antibodies.

3.2.2. Pertussis toxin has little effect on the phenotype of Aβ-specific T cells

The data shown in Figs. 3.1 - 3.3 demonstrate that different adjuvants can modulate the adaptive immune response to Aβ to varying extents. However, these experiments all involved an i.p. injection of PT in addition to the adjuvant, and it has been shown that PT can modulate the T cell response to antigen. Indeed, PT stimulated dendritic cells have been shown to preferentially induce the production of IFN-γ-producing Th1 cells (Hou *et al.* 2003). To investigate the role of PT injection in mediating these T cell and antibody responses, C57BL/6 mice were injected s.c. into the back with Aβ (100 μg/mouse) in combination with CFA or CpG (25 μg/mouse), with or without i.p. injection of PT (150 ng/mouse). The mice were boosted 21 days later with Aβ in IFA. Seven days after the boost, sera and spleen cells were harvested from immunised mice.

Analysis of Aβ specific IFN-γ and IL-17 production after 3 days of culture showed that the addition of PT induced a modest increase in Aβ-specific IL-17 and IFN-γ concentrations in mice immunised with Aβ in CFA (Fig. 3.4A and B). At this time-point, it had no effect on production of IFN-γ or IL-17 in mice immunised with Aβ and CpG (Fig. 3.4A and B). After 12 days in culture, concentrations of IFN-γ produced by these T cells increased (Fig. 3.4C and D). Addition of PT enhanced IFN-γ concentration in both immunisation groups at this time-point (Fig. 3.4C). Immunisation with Aβ and CpG and then Aβ in IFA produced higher levels of IFN-γ than immunisation with Aβ in CFA and then Aβ in IFA. Immunisation of animals with Aβ and CpG, followed by Aβ in IFA failed to induce production of IL-17. This is in contrast to the previous experiment, where immunisation with Aβ and CpG followed by Aβ in CFA induced significant levels of IL-17, and suggests that the boost immunisation with CFA may be inducing IL-17-producing cells in the spleens of these mice.

Analysis of Aβ-specific antibodies showed that immunisation with Aβ in CFA followed by Aβ in IFA produced a stronger Aβ-specific IgG response than immunisation with Aβ plus CpG followed by Aβ in IFA (Fig. 3.5). It was also found that immunisation with Aβ in CFA followed by Aβ in IFA induced a significant increase in IgG1 antibody titer (Fig. 3.6). Immunisation with Aβ plus CpG followed by Aβ in IFA was found to have no effect on IgG1 or IgG2a antibody titers. The data also show that the addition of PT had no effect on the levels of Aβ specific antibodies produced in response to either immunisation protocol. The data shown indicate that i.p. injection of PT had little effect on Aβ-specific T cell responses or antibody production, and this injection was thus omitted from subsequent immunisation protocols.

3.2.3. Determination of optimal conditions for Aβ treatment of mixed glia

The previous experiments demonstrated that that Aβ can be used in combination with CFA or CpG to produce Aβ-specific T cells. In order to examine the effect of these T cell subtypes on Aβ-induced microglial activation, it was necessary to first determine the optimal concentration of Aβ required to induce microglial activation. Previous data from this laboratory have shown that treatment of rat mixed glial cells with 10 μg/ml of Aβ for 24 h induced significant production of IL-1β, IL-6, and TNF-α. It was thus decided to treat mixed glial cells prepared from neonatal C57BL/6 animals with 10 - 40 μg/ml of Aβ and to measure production of IL-1β, IL-6, and TNF-α by ELISA. The data show that treatment with Aβ significantly increased production of IL-1β, IL-6, and TNF-α from mixed glial cells (Fig. 3.7). The minimum concentration of Aβ required to significantly increase mixed glial production of IL-1β, IL-6, and TNF-α was found to be 40 μg/ml. This concentration of Aβ was thus used to treat mixed glial cells in all future experiments. To determine the optimum treatment time for detection of proinflammatory cytokine production by mixed glia, mixed glial cells were treated with Aβ (40 μg/ml) for 2-48 h and IL-1β, IL-6, and TNF-α concentrations were determined by ELISA. The data show that concentrations of IL-6 and TNF-α in mixed glial cultures were significantly increased following 24 or 48 h treatment with Aβ (Fig. 3.8). Of note, in this particular experiment Aβ did not induce production of significant concentrations of IL-1β.

3.2.4. LPS synergises with Aβ to induce IL-1β secretion from mixed glia

The data in Figs. 3.7 and 3.8 show that treatment of mixed glia with Aβ induced significant production of IL-6 and TNF-α, but only induced very low levels of IL-1β secretion. This is consistent with previous studies showing a weak inflammatory response following treatment of microglia with Aβ *in vitro*

(Goodwin *et al.* 1995, Ii *et al.* 1996, Meda *et al.* 1995). However, it has been demonstrated that LPS and IFN-γ can synergise with Aβ in inducing proinflammatory cytokine production by microglia (Gasic-Milenkovic *et al.* 2003, Halle *et al.* 2008, Meda *et al.* 1999). To examine the effect of LPS on Aβ-induced proinflammatory cytokine production from glia, mixed glial cells prepared from neonatal C57BL/6 animals were treated with Aβ (10 – 40 μg/ml) in the presence or absence of LPS (10 ng/ml). After 24 h, supernatants were removed and concentrations of IL-1β, IL-6, and TNF-α were measured by ELISA. The data show that treatment with LPS induced glial production of IL-6 and TNF-α (Fig. 3.9). LPS failed to induce IL-1β production from mixed glia, however, it synergised with Aβ in inducing IL-1β, but not TNF-α or IL-6 secretion (Fig. 3.9).

3.2.5. Aβ-specific T cells increase microglial activation *in vitro*

To assess the effect of Aβ-specific T cell subtypes on microglial activation, spleen cells were prepared from mice immunised with Aβ in CFA followed by Aβ in IFA, or Aβ plus CpG followed by Aβ in IFA, with or without PT. Spleen cells were cultured for 10 days, harvested, and the resulting T cells were co-cultured with mixed glia at the ratio of 0.5:1. This was previously determined to be the optimal ratio for co-culture of these cell types. T cell-induced microglial activation was assessed by measurement of the proinflammatory cytokines IL-1β, IL-6, and TNF-α by ELISA, as well as microglial expression of the co-stimulatory molecules MHC class II and CD86 by flow cytometry. The data show that treatment of mixed glia with T cells from any of the immunisation groups increased production of IL-6 and TNF-α, but there was little T cell-induced increase in production of IL-1β (Fig. 3.10). There was no significant difference in cytokine production induced by T cells from the different immunisation groups. It was found that T cells from any of the immunisation groups enhanced microglial expression of MHC class II and CD86 (Fig. 3.11). The greatest enhancement of MHC class II expression on microglia was seen on microglia co-incubated with T

cells from mice immunised with Aβ plus CpG with PT. T cells from mice immunised with Aβ plus CpG induced the largest increase in expression of CD86 on microglia, and this was reduced by immunisation in the presence of PT (Fig. 3.11).

3.2.6. Effect of CT and LPS plus CpG on the induction of Aβ-specific T cells

The data from the previous experiments show that immunisation with the adjuvants CFA and CpG induced primarily a Th1 response. CT has been shown to generate Th2 and Treg responses (Lavelle *et al.* 2004, Plant & Williams 2004), and in certain instances can polarise the immune response towards a Th2 phenotype (Su *et al.* 2004). In an attempt to generate Aβ-specific Th2 cells, mice were injected s.c. into the back with Aβ (100 μg/mouse) and CT (100 ng/mouse). CpG was included as a Th1-inducing control adjuvant. The mice were boosted 21 days later with Aβ in CT or CpG. Seven days after the boost, spleen cells were harvested from immunised mice and restimulated with Aβ for 72 h, at which point cytokine responses were measured by ELISA. Restimulation of spleen cells from mice immunised with Aβ and CT was found to induce substantial production of IFN-γ, with no IL-4, IL-10, or IL-17 (Fig. 3.12). Restimulation of spleen cells from mice immunised with Aβ and CpG induced high levels of IFN-γ, with no IL-4, IL-10, or IL-17.

3.2.7. Aβ-induced increase in microglial activation *in vivo* is unaffected by Aβ-specific T cells

The previous experiments demonstrated that Aβ-specific T cells can alter microglial activation *in vitro*. In parallel with these studies, it was decided to investigate the effect of Aβ-specific T cells in modifying Aβ induced microglial activation *in vivo*. Previous studies have shown that IL-1β, TNF-α, and MHC

class II are increased in the hippocampus of mice injected i.c.v. with Aβ (Lyons *et al.* 2007), and so it was decided to examine the effect of Aβ-specific Th1 and Th2 cells on co-stimulatory molecule expression by microglia as a measure of microglial activation *in vivo*. Aβ-specific Th1 and Th2 cell lines were developed and transferred i.v. to mice that had previously been given an i.c.v. injection of Aβ. Aβ-specific Th1 cell lines were developed from mice that had been immunised with Aβ plus CpG followed by a boost of Aβ in CFA, by restimulating spleen cells *in vitro* with Aβ (25 μg/ml), IL-12 (10 ng/ml), and anti-IL-4 antibody (10 μg/ml). Th2 cell lines were developed from mice immunised with Aβ in alum, by restimulating spleen cells *in vitro* with Aβ, IL-4 (10 ng/ml), and anti-IFN-γ antibody (10 μg/ml).

The data show that in supernatants taken after 4 days of culture, the Th1 cell line supernatants contained large amounts of IFN-γ, intermediate amounts of IL-10, and little or no IL-4 (Fig. 3.13). Th2 cell line supernatants contained large amounts of IL-4, low IL-10, and little or no IFN-γ or IL-17. IL-2 (5 ng/ml) was added to the T cell lines after 4 days of culture, and they were restimulated with antigen and irradiated APCs on day 11. A further 4 days after antigen restimulation, supernatants were analysed for cytokine production by ELISA. It was found that the Th1 cell line produced large amounts of IFN-γ, intermediate amounts of IL-10 and IL-17, and little or no IL-4 (Fig. 3.14). The Th2 cell line produced intermediate amounts of IFN-γ, IL-4, and IL-10, with little or no IL-17. This suggests that after the second round of antigen stimulation, the Th2 cell line was actually a mixed Th1/Th2 cell line, and is referred to as such in following figure legends. On the initiation of the third round of antigen stimulation, T cells were isolated, and injected i.v. at a concentration of 1×10^5 cells/mouse into mice that had been injected i.c.v. with Aβ (5 μg/mouse) 24 hours previously. Mice were sacrificed 24 h after T cell transfer, and the hippocampus of the injected hemisphere of the brain was isolated and processed for FACS analysis of $CD11b^+$ cells co-expressing MHC class II, CD80, CD86, and CD40. It was found that i.c.v. injection of Aβ induced a significant increase in the percentage of $CD11b^+$

cells co-expressing MHC class II and CD80 in the hippocampus (Figs. 3.15 and 3.16). The data also show that i.v. administration of polarised T cell lines had no effect on the percentage of $CD11b^{+}$ cells expressing any of the co-stimulatory molecules studied.

3.3. Discussion

In this chapter, immunisation protocols for the generation of Aβ-specific T cell lines were developed. These immunisation protocols provided a means by which both a Th1/Th17 cell response and an antibody response to Aβ could be produced. Aβ-specific T cell lines were found to enhance Aβ-induced co-stimulatory molecule expression and proinflammatory cytokine production in co-culture with glial cells. This study also showed that i.c.v. injection of Aβ induced microglial activation; however, i.v. transfer of Aβ-specific Th1 and Th1/Th2 cells was unable to modulate these changes.

Immunotherapeutic strategies are a promising avenue of exploration for the treatment of AD. However, while vaccinations with Aβ result in plaque clearance and improved memory in animals models of AD, they were found to induce meningoencephalitis in AD patients, which was associated with T cell infiltration into the brain parenchyma (Weiner & Frenkel 2006). Activated microglia expressing the APC surface markers MHC class II and CD40 have been detected in the vicinity of Aβ plaques in brain sections from patients with AD (Perlmutter *et al.* 1992, Togo *et al.* 2000), suggesting that the interaction of microglia and T cells may be involved in the development of the meningoencephalitis seen in clinical trials. It is therefore important to study the interaction between Aβ-specific T cells and microglia, and to determine if subsets of T cells have differential effects on microglial activation, and on amyloid clearance.

In order to examine the effects of Aβ-specific T cells on microglial activation, it was first necessary to develop polarised T cells specific for Aβ. There are two main ways of achieving this goal. Firstly, T cells can be polarised *ex vivo* by using a combination of polarising cytokines and neutralising antibodies. This protocol has the benefit of generating highly polarised T cell

lines. In order to examine the effects of these T cells *in vivo*, however, they must be transferred into a host animal, which presents technical challenges such as route of administration and number of T cells to administer. T cells can also be polarised *in vivo* by the use of different adjuvants, such as CpG and alum which generate specific Th1 and Th2 cells, respectively, to a co-administered antigen. The major advantage of this technique is that polarised, antigen-specific T cells can be generated in a mouse model of AD without the need for T cell transfer. However, the disadvantage is that the responses are rarely completely polarised to Th1 or Th2. In this study, we initially adopted the second approach, as it was felt that it would be preferable to generate these T cells *in vivo* if possible.

As Aβ is a self-antigen, it is necessary to break immune tolerance to induce T cell responses to Aβ, and so initial experiments focused on immunisation with Aβ in the presence of the Th1 promoting adjuvants, CFA and CpG. PT was also included in the immunisation protocol, as a similar immunisation protocol is used with the MOG antigen to induce EAE in mice, with concomitant production of MOG-specific Th1 and Th17 cells. The data show that a primary immunisation with Aβ in CFA together with i.p. administration of PT, followed by a boost with Aβ in IFA induced a mixed Th1/Th17 response. This is in agreement with data showing that a similar protocol for immunisation with MOG as the antigen induced MOG-specific Th1 and Th17 cells (Sutton *et al.* 2006). This immunisation protocol also promoted the induction of Aβ-specific IgG1. The induction of IgG1 by this immunisation protocol is surprising, given that these antibodies are generally associated with Th2 responses. However, it has previously been shown that injection of CFA can augment MOG-specific IgG1 responses *in vivo* (Kahn *et al.* 2001). In contrast, an initial immunisation with Aβ and CpG together with i.p. administration of PT, followed by a boost with Aβ in CFA induced a robust Th1 response, with some Th17 cells also present. This data is in broad agreement with previously published studies, which show that CpG acts as a Th1 promoting adjuvant (Roman *et al.* 1997, Walker *et al.* 1999). This immunisation protocol did not promote induction of Aβ-specific antibodies.

Analysis of the Aβ-specific proliferative response of spleen cells from immunised animals showed significant cell proliferation by spleen cells from mice immunised with Aβ plus CpG followed by Aβ in CFA. There was no significant Aβ-specific T cell proliferation from cells of mice immunised with Aβ in CFA followed by Aβ in IFA. These immunisation protocols thus provided a means by which either a cell-mediated response or a humoral response to Aβ could be induced.

The effect of the booster immunisation with CFA following immunisation with Aβ and CpG was also studied. It was found that immunisation with CpG followed by a boost with Aβ in IFA generated T cells that did not produce IL-17 in response to stimulation with Aβ. This is in contrast to immunisation with Aβ and CpG, then Aβ in CFA, where significant levels of IL-17 were produced. Therefore, the addition of CFA in the booster immunisation appears to be able to induce a more inflammatory T cell profile than that induced by IFA.

PT is required for the induction of EAE following immunisation with MOG in CFA. It is generally thought that PT facilitates the induction of EAE by increasing the permeability of the BBB, thus allowing the infiltration of T cells into the brain (Kugler *et al.* 2007). However, PT has also been shown to enhance Th1 and Th17 responses (Chen *et al.* 2007b, Hou *et al.* 2003). Indeed, PT has been reported to promote the clonal expansion of neuroantigen-specific Th1 cells, thus promoting the induction of EAE (Hofstetter *et al.* 2002). The data show that addition of PT to the immunisation protocol induced a modest increase in Aβ-specific IL-17 and IFN-γ, but this was not significant.

Given that the immunisation protocol used in clinical trials of active Aβ immunotherapy induced meningoencephalitis, it is noteworthy that no evidence of this disease was seen in immunisation studies using animal models of AD. The meningoencephalitis seen in clinical trials was postulated to be due to a Th1 response, and Aβ restimulation of PBMCs taken from patients enrolled in the clinical trial produced IL-2 and IFN-γ (Pride *et al.* 2008). Furlan and colleagues

found that C57BL/6 mice immunised with Aβ in CFA plus PT developed clinical signs of mild EAE, and had T and B cell infiltration in the CNS (Furlan *et al.* 2003). PT was subsequently found to be required for these neurological impairments. Therefore, in this study mice were examined for signs of any obvious neurological impairment. However, no signs of gait disturbance or limb/tail paralysis were found in this study. This is in agreement with subsequent reports, which have failed to find any evidence of encephalomyelitis in mice immunised with an identical immunisation protocol (Ethell *et al.* 2006, Frenkel *et al.* 2005). As PT appeared to have a minor effect on cytokine production by T cells, it was decided to omit it from the immunisation protocol.

In an examination of the effect of Aβ on microglial activation, Aβ induced production of IL-1β, IL-6, and TNF-α from glial cells. This agrees with other studies showing Aβ-induced proinflammatory cytokine production from mixed glial cultures *in vitro* (Floden & Combs 2006, Ii *et al.* 1996, Murphy *et al.* 1998). However, the data presented here show that treatment with Aβ alone did not induce robust production of IL-1β from mixed glia. This may be due to the relative concentration of oligomers and fibrils present in the particular preparation of Aβ used in each experiment. Each preparation of Aβ is aggregated to preferentially form fibrillar Aβ according to a standard protocol, and tested for the presence of fibrils using a thioflavin T assay (Fig 2.1). However, previous data from this laboratory have shown that these Aβ preparations also contain oligomeric Aβ (Lyons *et al.* 2007), and the relative levels of these species may have subtle effects on the inflammatory response induced by the peptide. Indeed, it has been demonstrated that both oligomeric and fibrillar Aβ stimulate proinflammatory microglial activation, but have different profiles for mitogen-activated protein kinase (MAPK) activation, and subsequent cytokine and chemokine production, with the inference that these species play distinct roles in inducing microglial activation in AD (Sondag *et al.* 2009). Thus, it was deemed important to use a mix of oligomers and fibrils to more accurately mimic the *in vivo* situation.

However, a more likely explanation for the lack of IL-1β production by Aβ-treated mixed glia is that two signals are required for the release of IL-1β from innate immune cells such as microglia. Aβ has recently been demonstrated to activate the NALP3 inflammasome, resulting in the robust activation of caspase-1 (Halle *et al.* 2008). For IL-1β to be secreted by the glial cell, a second signal, such as LPS is required to induce pro-IL-1β production (Mariathasan & Monack 2007). Pro-IL-1β is then cleaved by active caspase-1 to produce mature IL-1β. The results presented here show that treatment of glia with Aβ in combination with LPS induced significantly greater levels of IL-1β release than LPS or Aβ alone, supporting this hypothesis. Additionally, Lorton and colleagues have shown that Aβ stimulated IL-1β release from LPS-activated monocytes (Lorton *et al.* 1996). The results of this study show that LPS did not synergise with Aβ for microglial production of IL-6 or TNF-α, as these cytokines do not require activation of the inflammasome for their production. Despite its synergistic effects on Aβ-induced IL-1β production, LPS was not used in subsequent experiments assessing the effect of T cells on Aβ-induced microglial activation. This is due to the fact that LPS induced high levels of IL-6 and TNF-α production from mixed glia, and it was thought that this would interfere with the ability to detect any modulatory activity of Aβ-specific T cells on Aβ-induced microglial activation.

To investigate the effect of these Aβ-specific T cells in influencing microglial activation by Aβ, T cells from mice immunised with Aβ and CpG and CFA were cultured *ex vivo*, and then restimulated with Aβ in the presence of microglia as an APC. Treatment of glia with T cells from any of the immunisation groups was found to increase production of IL-6 and TNF-α, but there was little or no increase in production of IL-1β. Chabot and colleagues have shown that human microglia shift from a resting ramified phenotype to an activated amoeboid phenotype in response to activated T cells, and that this induced production of TNF-α from the microglia (Chabot *et al.* 1997). In addition, it has been shown that microglia produce IL-1β, TNF-α, and IL-6 in response to MBP-specific T cells

(Dasgupta *et al.* 2005). The T cell-induced increases in cytokine production appear to be unrelated to the immunisation protocol, as there was little difference in cytokine production induced by T cells from the different immunisation groups. However, the cytokine profile showed that the immunisation protocols explored induced similar populations of T cells. An examination of the effect of T cells on expression of co-stimulatory molecules on microglia showed that treatment of glia with Aβ-specific T cells enhanced the expression of the co-stimulatory molecules MHC class II and CD86 on the surface of microglia. This is consistent with data from Aloisi and colleagues, who have shown that interaction of microglia and Th1 cells induces the expression of MHC class II and CD40 on microglia (Aloisi *et al.* 2000a). The data presented here show that Aβ-specific T cells are capable of activating microglia, and perhaps bias their activation towards an adaptive immune response.

Previous work has shown that Th2 cells can restore working memory in APPswe/PS1dE9 mice, and that this is associated with a decrease in the number of plaque-associated microglia (Cao *et al.* 2009). CT was used as an adjuvant to induce Aβ-specific Th2 cells, as it has previously been shown to promote strong Th2 (Xu-Amano *et al.* 1993, Su *et al.* 2004, Yamamoto *et al.* 1997) and Treg (Lavelle *et al.* 2004) responses to co-administered antigens. The results demonstrate that CT induced production of Aβ-specific Th1, but not Th2 or Treg cells. The discrepancy between these results may be due to the fact that CT is normally delivered orally or intranasally, and in this study it was injected s.c. Alternatively, it may be due to a unique feature of the Aβ antigen, as intranasal administration of Aβ in combination with the cholera toxin B subunit was found to induce very little IgG1 antibody production, which would be indicative of a Th2 response (Maier *et al.* 2005).

As CT adjuvant failed to induce a Th2 response to Aβ, the efficacy of an alternative adjuvant, alum, in generating Th2 cells was investigated. Aluminium adjuvants are generally recognised as safe and are known to be stimulators of Th2

responses (Lindblad 2004, Mahon *et al.* 1996). Immunisation of mice with Aβ adsorbed to alum was shown to induce antibodies predominantly of the IgG1 subtype, indicating a Th2-type response (Ghochikyan *et al.* 2006). In the present study, a Th1 cell line was also generated by immunising with Aβ with CpG, then Aβ in CFA. Polarising cytokines were added to these cell lines at the time of Aβ restimulation to further skew the immune response towards Th1 or Th2. The Th1 cell line was shown to produce high levels of IFN-γ, with low levels of IL-4, IL-10, and IL-17, indicating a highly polarised Th1 cell line. The Th2 cell line was found to secrete moderate levels of IL-4 and IFN-γ, with low levels of IL-10 and IL-17, indicative of a mixed Th1/Th2 cell population. This lack of robust production of IL-4 was surprising, given that alum is a Th2 inducing adjuvant. However, the lack of a potent immune response may be due to that fact that Aβ is an autoantigen and thus requires a strong adjuvant to break tolerance. Alum is much less potent than Th1 promoting adjuvants, sometimes up to 200-fold (Ghochikyan *et al.* 2006), and may not have the potency to fully break tolerance.

To study the effect of these T cell lines on microglial activation, an *in vivo* mouse model was used to mimic the microglial activation seen in AD. Intracerebroventricular injection of Aβ induced an inflammatory change in the hippocampus, characterised by an increase in microglial expression of MHC class II and CD80. This agrees with previous data from this laboratory showing increased hippocampal expression of MHC class II following i.c.v. administration of Aβ (Lyons *et al.* 2007). However, i.v. injection of Th1 and Th1/Th2 cell lines had no modulatory effect on Aβ-induced upregulation of co-stimulatory molecules. This may be due to the relatively small number of cells (1×10^5 cells/mouse) injected, and indeed other laboratories have used much higher numbers of T cells in the induction of passive EAE, where as many as 25×10^6 cells have been transferred, although lower numbers have also been used (Kroenke *et al.* 2008, Lees *et al.* 2008). It is also possible that as the T cells were administered 24 hours after Aβ administration, a great deal of the injected amyloid had been phagocytosed by this time. This seems unlikely however, given

that previous work has shown that fibrillar Aβ injected into the CNS persists for at least 30 days (Frautschy *et al.* 1992, Weldon *et al.* 1998). This model of AD is advantageous in that injection of amyloid has been reported to cause neuronal death, which is not usually seen in transgenic models of disease (Frautschy *et al.* 1991). However, the relative lack of Aβ in the CNS and periphery when compared with APP-transgenic mice means that this model is less well suited for studies of the effects of Aβ-specific T cells on microglial activation, and particularly, on plaque clearance.

The data presented in this study show that specific immunisation protocols can be used to produce Aβ-specific Th1 and Th17 cells *in vivo*, as well as an antibody response to Aβ. These T cells were demonstrated to promote proinflammatory cytokine production and co-stimulatory molecule expression following co-culture with Aβ and microglia. None of the adjuvants tested were able to produce a satisfactory Th2 cell line, and it is possible that Th2-promoting adjuvants are not strong enough to generate significant cellular responses to Aβ. Intracerebroventricular injection of Aβ was found to induce adaptive activation of microglia; however Aβ-specific Th1 and Th1/Th2 cells were not able to alter this response.

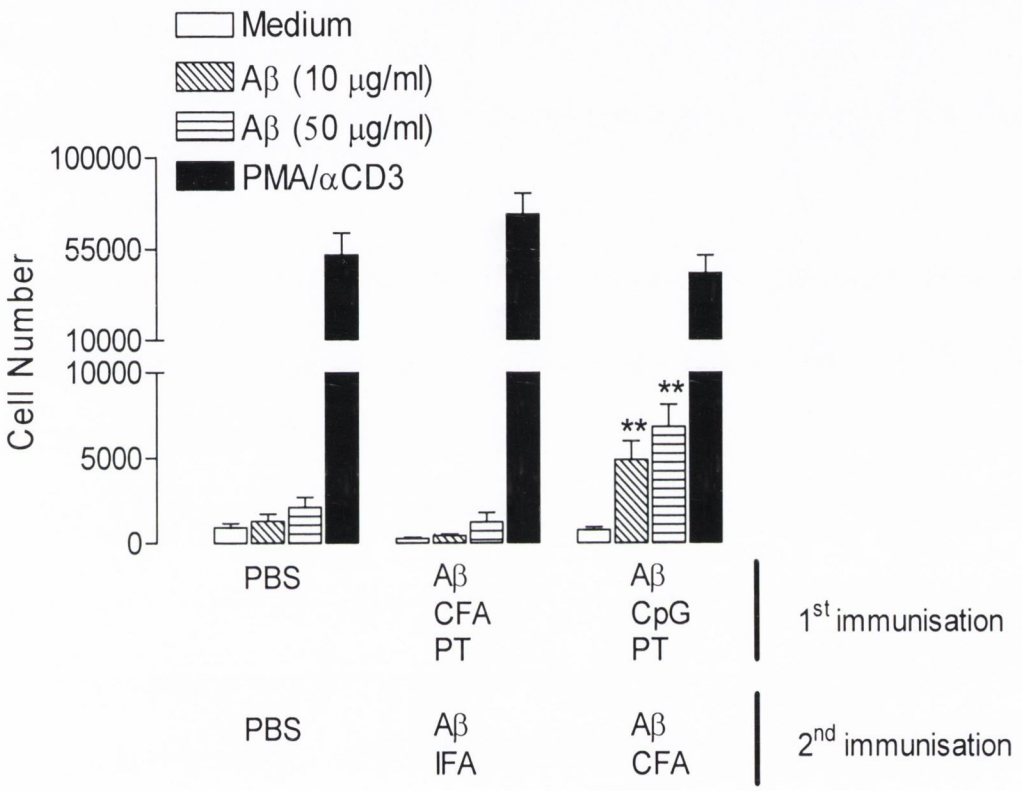


Figure 3.1. Aβ-specific proliferation response in mice immunised with Aβ with CFA/IFA or Aβ with CpG/CFA

C57BL/6 mice were injected subcutaneously with PBS, Aβ (100 μg/mouse) and CFA, or Aβ and CpG (25 μg/mouse). The Aβ-treated groups were also injected intraperitoneally with pertussis toxin (PT, 150 ng/mouse). After 21 days, mice were injected with PBS, Aβ and IFA, or Aβ and CFA respectively. After a further 7 days, mice were sacrificed, spleens were harvested and cultured in medium only, Aβ (10 or 50 μg/ml), or PMA and anti-CD3 for 72 hours. The cells were pulsed with 0.5 μCi of [^{3}H]-thymidine for 18 hours in culture. [^{3}H]-thymidine incorporation was assessed using a Betaplate scintillation counter. ** $p<0.01$ vs medium only (one-way ANOVA). Values are expressed as means ± SEM (n=5).

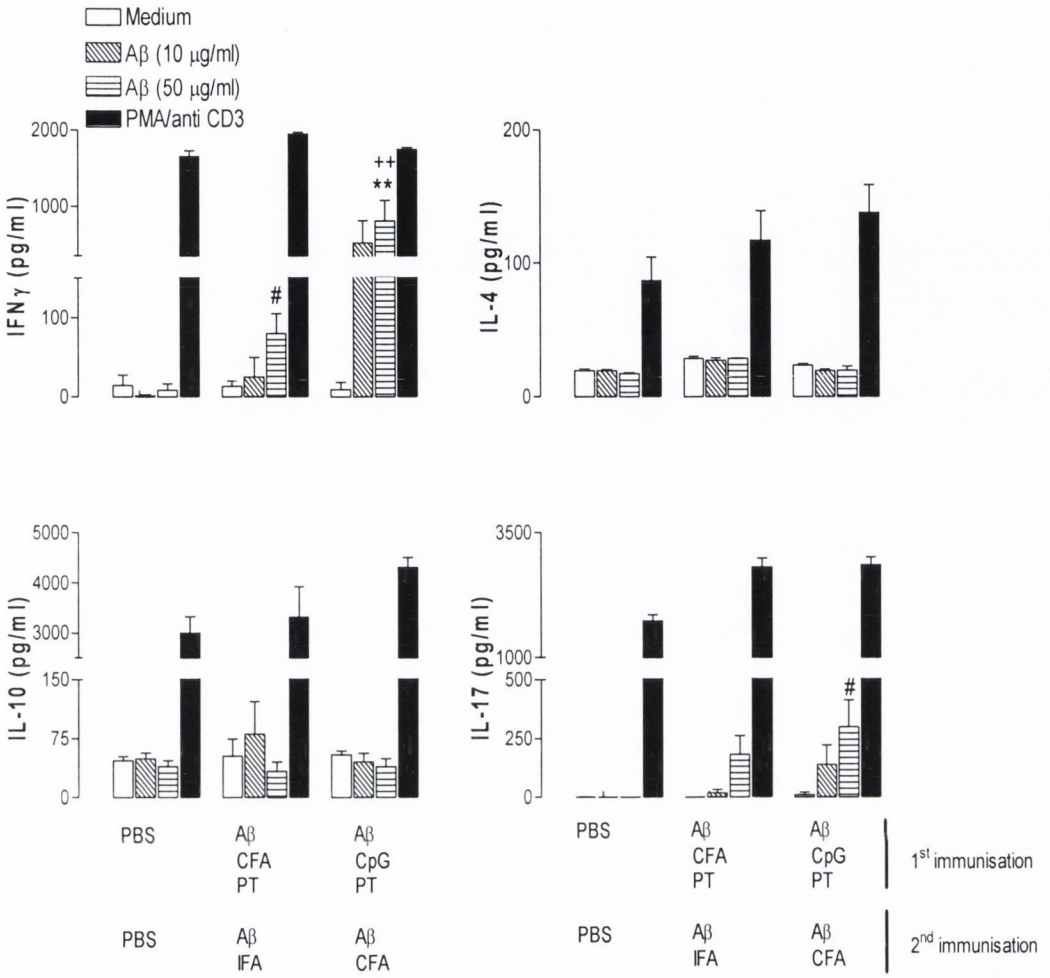


Figure 3.2. Immunisation with Aβ in CpG and PT, followed by a boost with Aβ in IFA induces strong Aβ-specific Th1 and Th17 responses.

C57BL/6 mice were immunised as described in Figure 3.1. Seven days after the second immunisation, mice were sacrificed, spleens were harvested and cultured in medium only, Aβ (10 or 50 μg/ml), or PMA and anti-CD3. After 72 hours IFN-γ, IL-4, IL-10, and IL-17 concentrations in supernatants were quantified by ELISA. ** $p<0.01$ versus medium only; $^{++}$ $p<0.01$ versus Aβ-stimulated (50 μg/ml) spleen cells from mice immunised with Aβ in CFA (one-way ANOVA). $^{\#}$ $p<0.05$ versus medium only (unpaired Student's t-test). Values are expressed as means ± SEM (n=5).

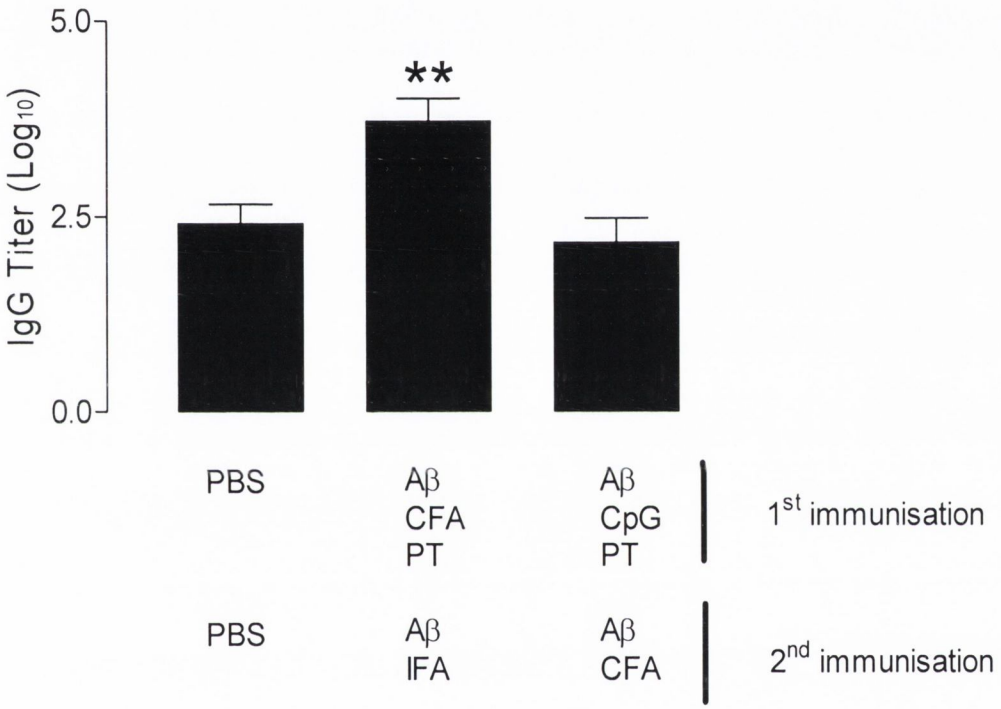


Figure 3.3. Immunisation with Aβ in combination with CFA and PT, followed by boosting with Aβ in IFA, induces IgG in C57BL/6 mice.

C57BL/6 mice were immunised as described in Figure 3.1. Seven days after the second immunisation, mice were sacrificed and serum was taken and analysed for Aβ-specific IgG concentration by ELISA. ** $p<0.01$ versus PBS-treated mice (one-way ANOVA). Values are expressed as means ± SEM (n=5).

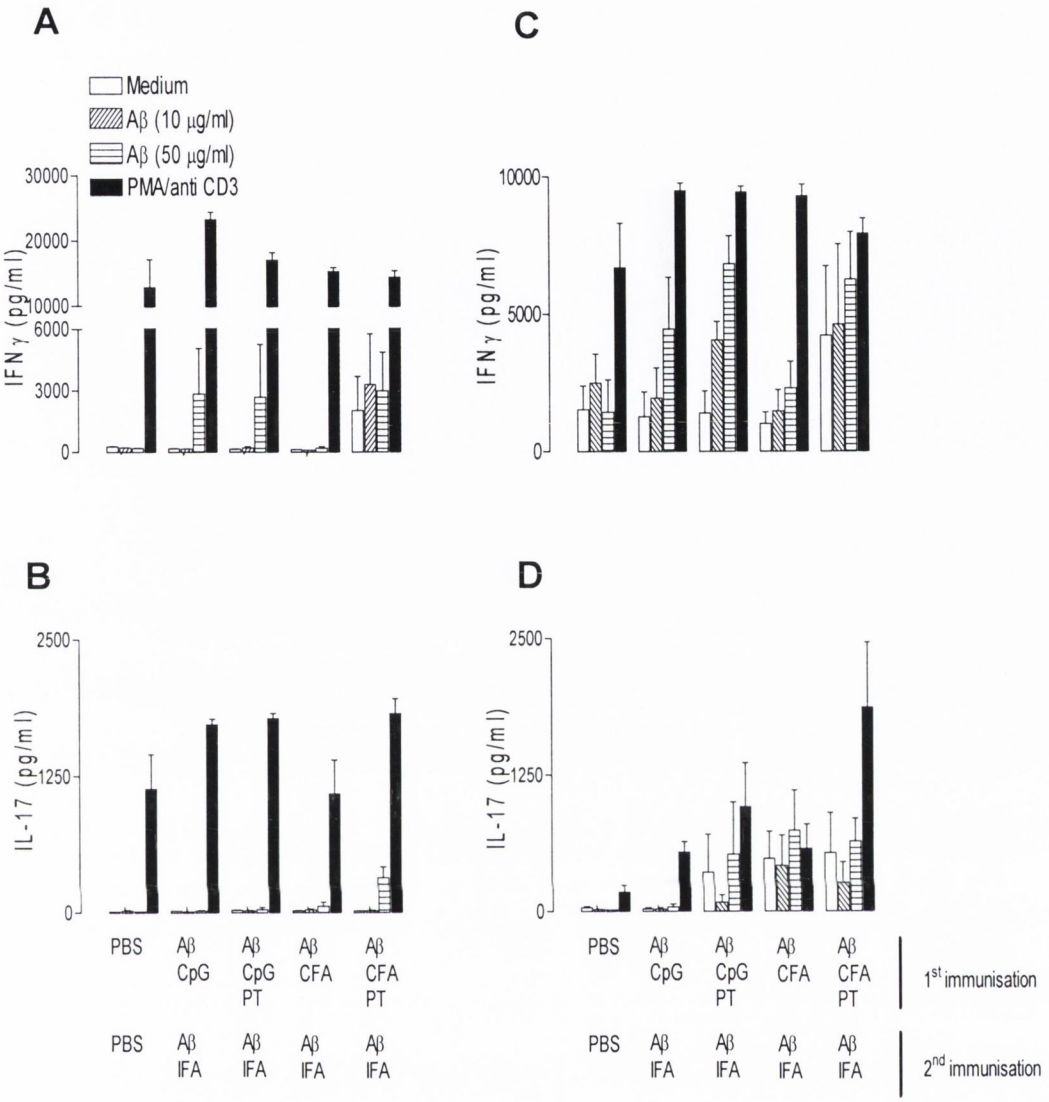


Figure 3.4. Comparison of CpG and CFA with or without pertussis toxin for promoting Aβ-specific Th1 and Th17 responses.

C57BL/6 mice were injected subcutaneously with PBS, Aβ (100 μg/mouse) and CFA, or Aβ and CpG (25 μg/mouse). The Aβ-treated groups were injected intraperitoneally with either PBS or PT (150 ng/mouse). After 21 days, mice were injected with PBS alone (control group) or Aβ and IFA. After a further 7 days, mice were sacrificed and spleens were harvested and cultured in the presence of medium only, Aβ (10 or 50 μg/ml) or PMA and anti-CD3. After 72 hours, IFN-γ (A), and IL-17 (B) were quantified by ELISA. 5 ng/ml of IL-2 was added to the cultures 3, 6, and 9 days later. On day 12, the supernatants were removed and IFN-γ (C), and IL-17 (D) were quantified by ELISA . Values are expressed as means ± SEM (n=5).

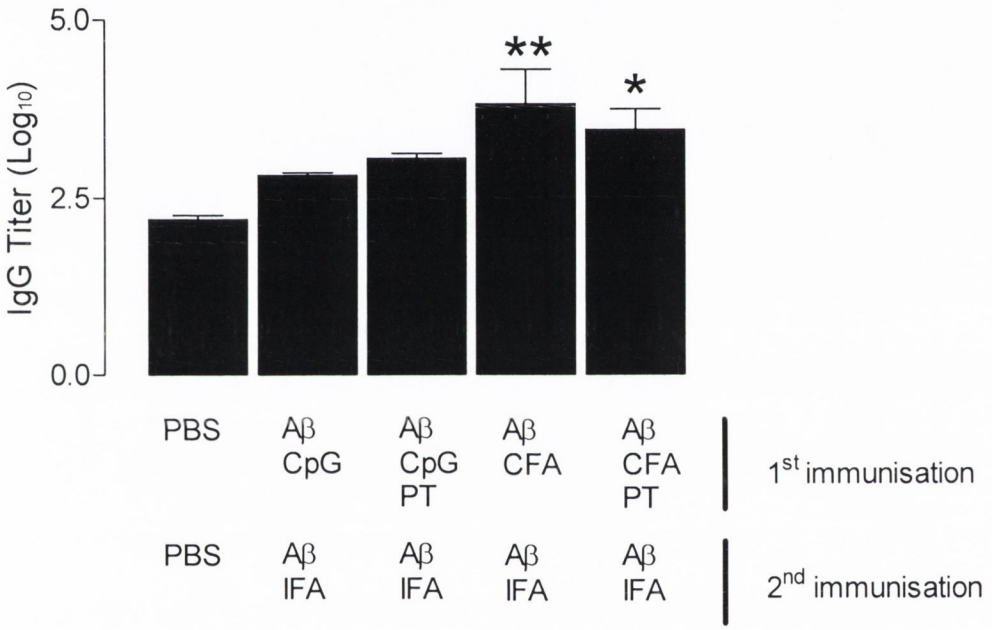


Figure 3.5. Pertussis toxin injection has no effect on IgG responses to Aβ.

C57BL/6 mice were immunised as described in Figure 3.4. Seven days after the second immunisation, mice were sacrificed and serum was taken and analysed for Aβ-specific total IgG concentration by ELISA. * $p<0.05$, ** $p<0.01$ versus PBS-treated animals (one-way ANOVA). Values are expressed as means ± SEM (n=5).

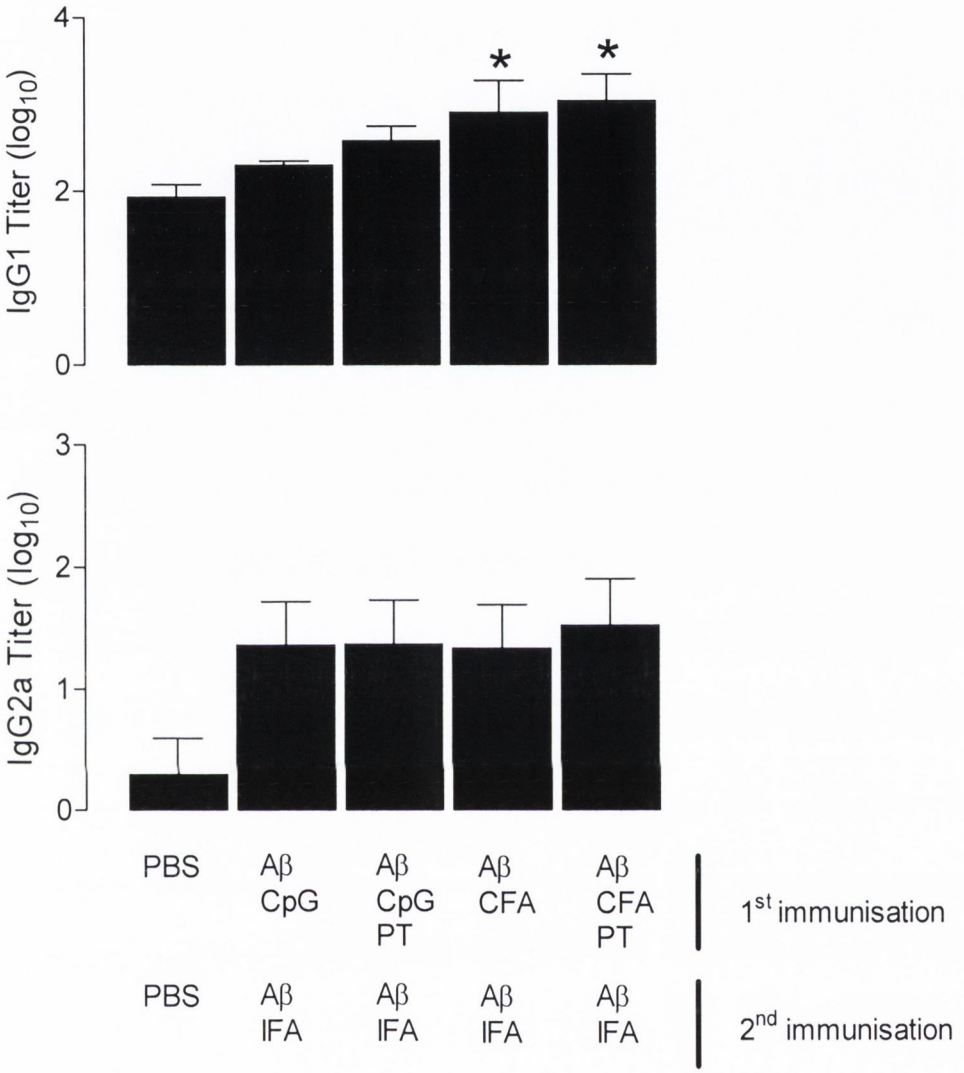


Figure 3.6. Pertussis toxin has no effect on IgG1 or IgG2a responses to Aβ.

C57BL/6 mice were immunised as described in Figure 3.4. Seven days after the second immunisation, mice were sacrificed and serum was taken and analysed for Aβ-specific IgG1 and IgG2a concentration by ELISA. * $p<0.05$ versus PBS-treated animals (one-way ANOVA). Values are expressed as means ± SEM (n=5).

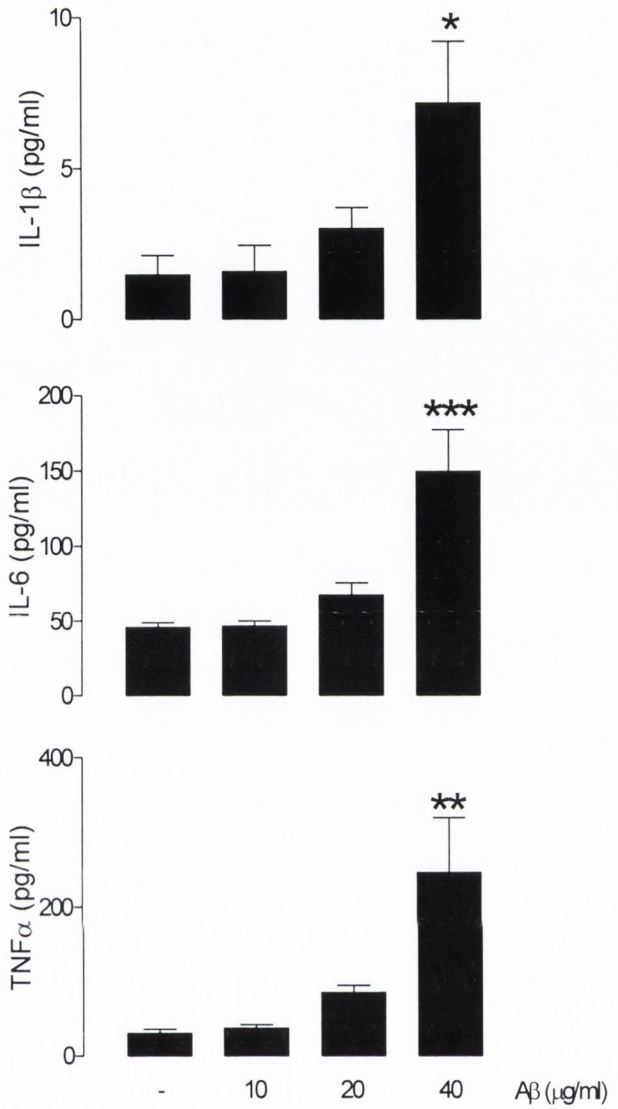


Figure 3.7. Effect of Aβ concentration on induction of proinflammatory cytokine production by mixed glia.

Mixed glia prepared from 0-1 day old C57BL/6 mice were treated with increasing concentrations of Aβ (10, 20, and 40 μg/ml), or medium only. After 24 h of culture, cytokine concentrations of IL-1β, IL-6, and TNF-α in supernatants were measured by ELISA. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ versus medium-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=6).

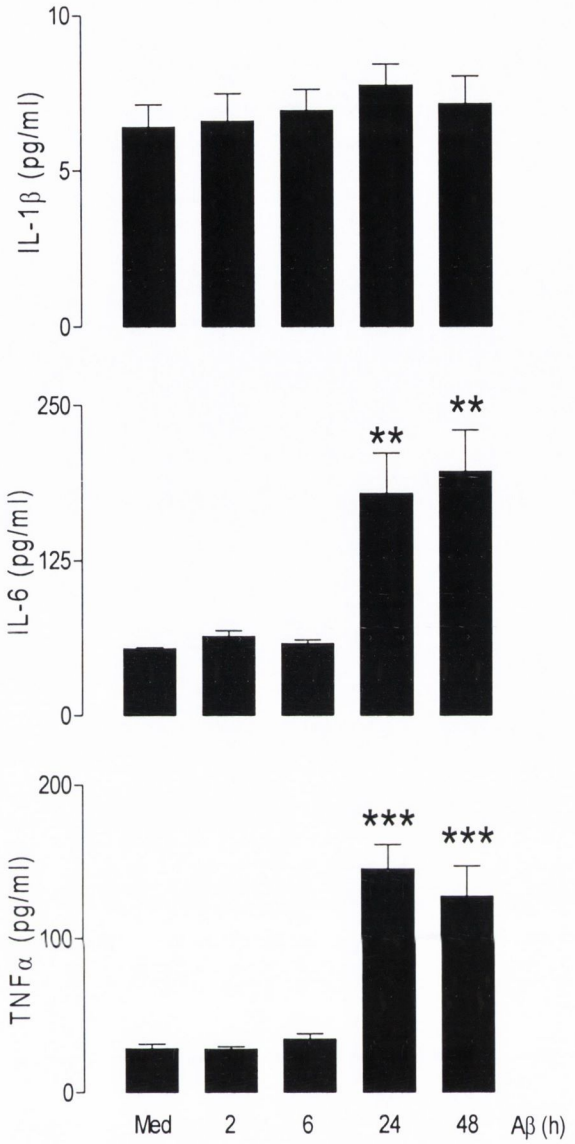


Figure 3.8. Time course of proinflammatory cytokine production by Aβ-stimulated mixed glia.

Mixed glia prepared from 0-1 day old C57BL/6 mice were cultured for between 2 and 48 hours with Aβ (40 μg/ml). Mixed glia cultured for 24 h with medium (med) served as a control. Concentrations of IL-1β, IL-6, and TNF-α in supernatant were measured by ELISA. ** $p<0.01$, *** $p<0.001$ versus medium-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=6).

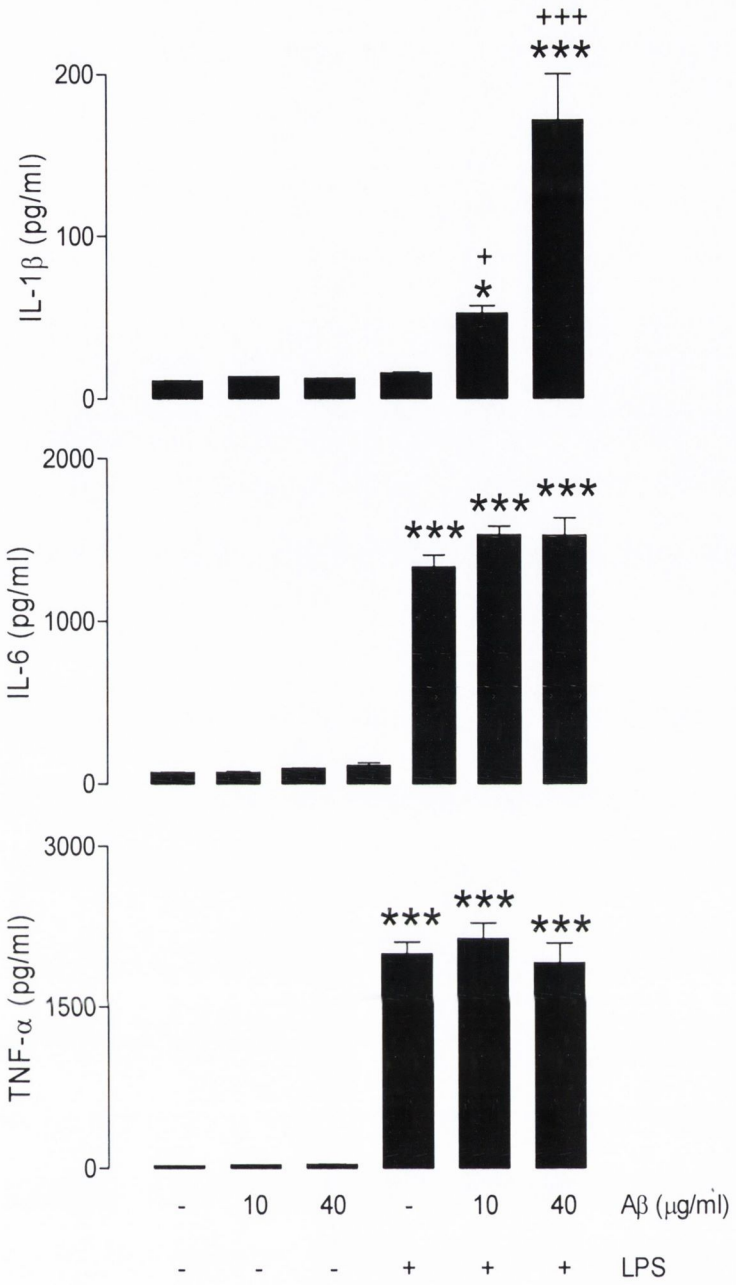


Figure 3.9. Synergistic effect of LPS and Aβ in producing IL-1β from mixed glia.

Mixed glia prepared from 0-1 day old C57BL/6 mice were treated with Aβ (10 - 40 μg/ml), in the presence or absence of LPS (10 ng/ml) for 24h. Cytokine concentrations of IL-1β, IL-6, and TNF-α in supernatants were measured by ELISA. * $p<0.05$, *** $p<0.001$ versus medium-treated mixed glia; $^{+}$ $p<0.05$, $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=5).

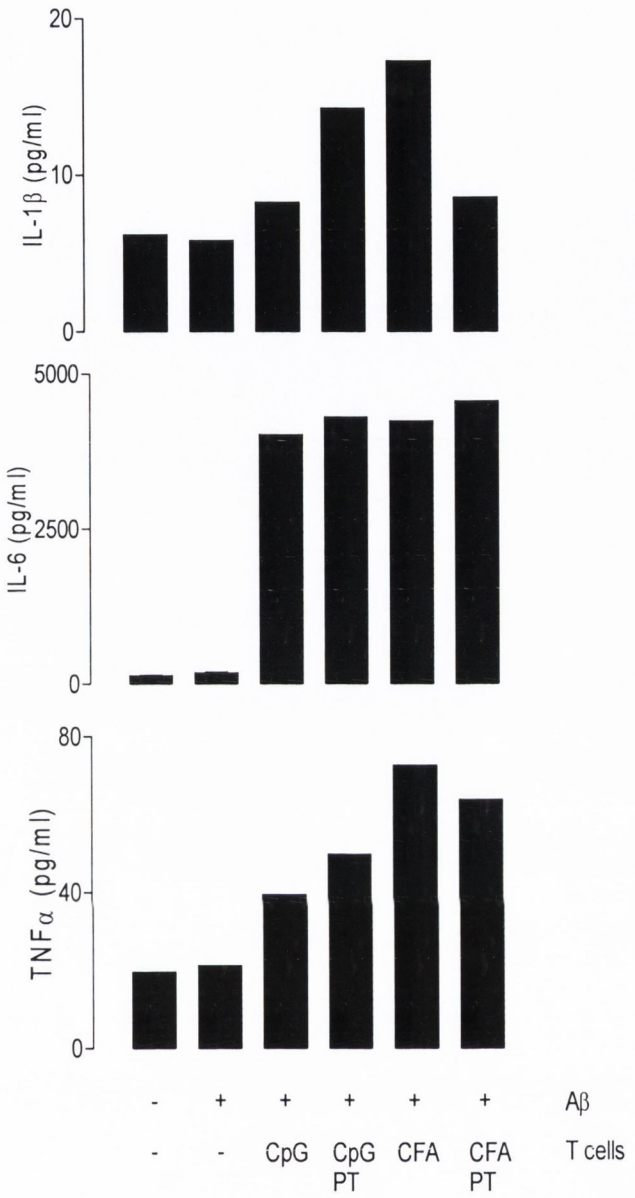


Figure 3.10. Treatment of mixed glial cultures with Aβ-specific T cells enhances proinflammatory cytokine production.

Mice were immunised and T cells prepared as described in Figure 3.4. On day 12, mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ and T cells for 24 h. T cells were added at a ratio of 0.5:1 (T cells:glia). Concentrations of IL-1β, IL-6, and TNF-α in supernatants were measured in triplicate by ELISA. This figure represents data taken from a pilot study, with n=1.

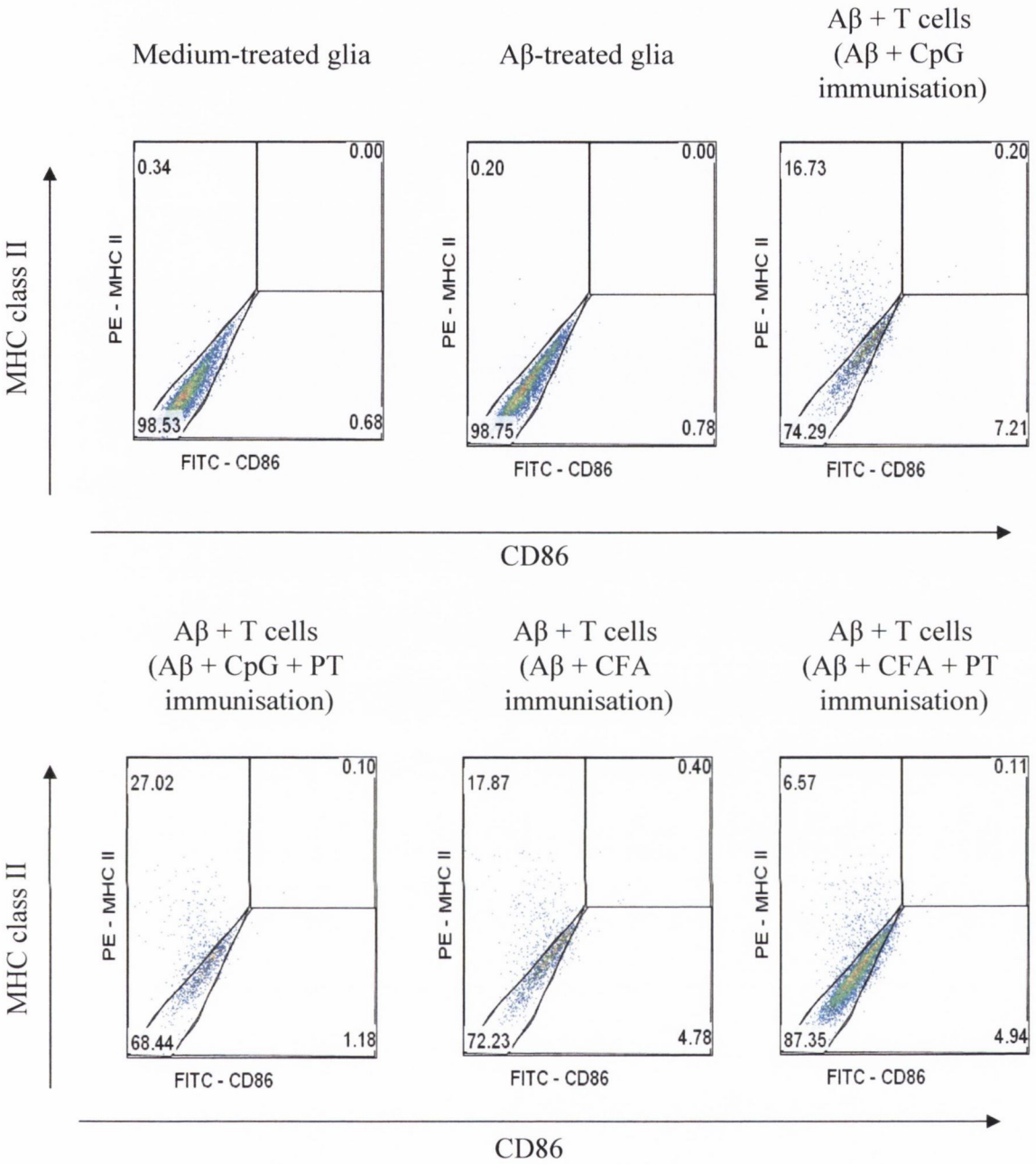


Figure 3.11. Aβ-specific T cells from mice immunised with either CFA or CpG promote MHC class II and CD86 expression on microglial cells *in vitro.*

C57BL/6 mice were immunised as described in Figure 3.4. On day 12, mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ and T cells for 24 h. T cells were added at a ratio of 0.5:1 (T cells:glia). After 24 h, expression of MHC class II and CD86 on $CD11b^+$ cells was assessed by flow cytometry. Numbers represent the percentage of cells in each gate. This figure represents data taken from a pilot study, with n=1.

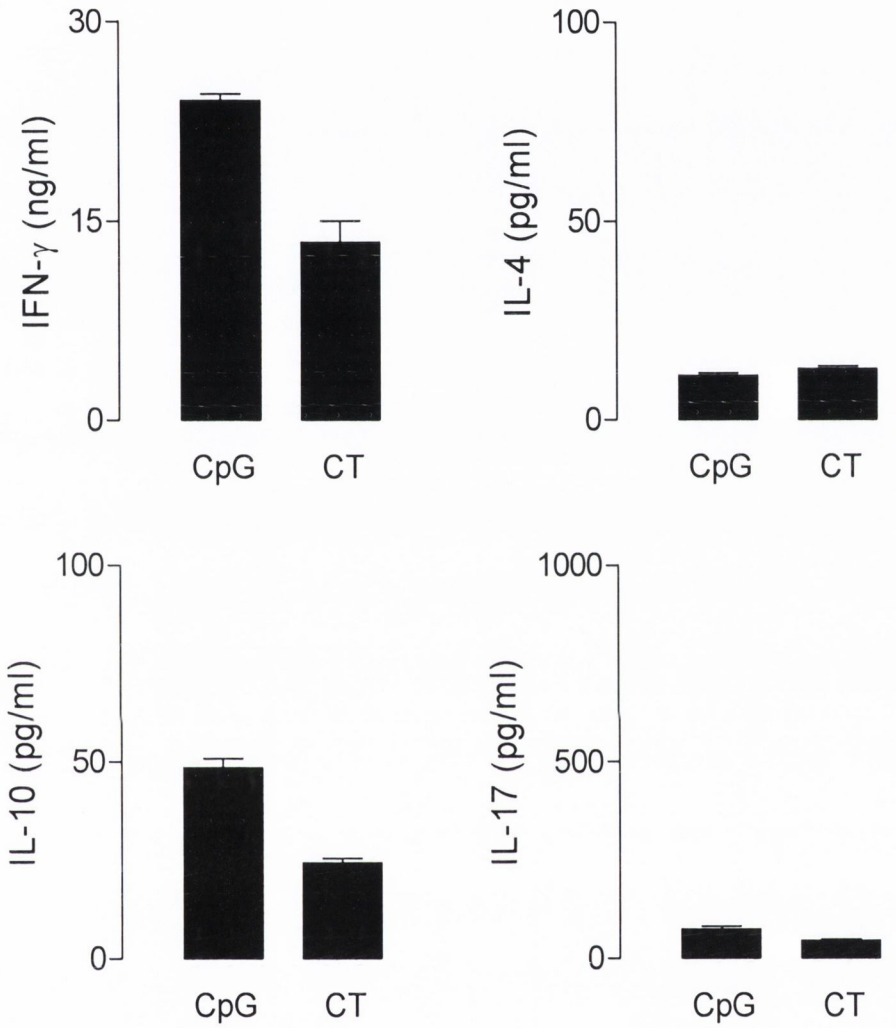


Figure 3.12. Immunising with CT induces a polarised Th1 response.

C57BL/6 mice were injected subcutaneously with Aβ (100 μg/mouse) in combination with CpG (25 μg/mouse) or CT (100 ng/mouse), and boosted 21 days later. Mice were sacrificed 7 days after the boost, spleens were harvested, and T cells were stimulated with Aβ (25 μg/ml). Four days after stimulation, the supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=6).

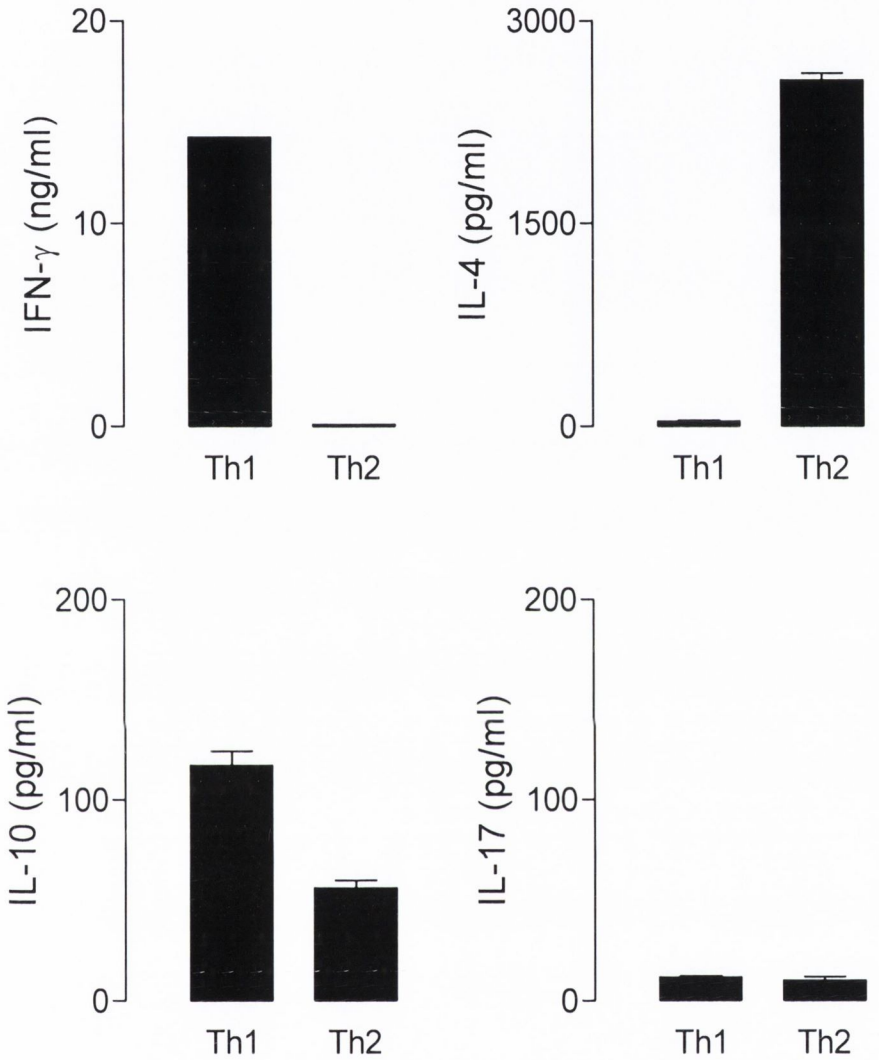


Figure 3.13. Cytokine profile of polarised Aβ-specific Th1 and Th2 cells.

C57BL/6 mice were injected subcutaneously with Aβ (100 μg/mouse) and CpG to generate Th1 cells, or Aβ and alum to generate Th2 cells. Twenty one days later, mice were injected with Aβ and CFA (Th1 group), or Aβ and alum (Th2 group). After a further 7 days, mice were sacrificed, spleens were harvested, and T cells were stimulated with Aβ (25 μg/ml) and Th1 polarising cytokines IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml), or Th2 polarising cytokines IL-4 (10 ng/ml) and anti-IFNγ(10 μg/ml). Four days after polarisation, the supernatants were removed for analysis of IFN-γ, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=3).

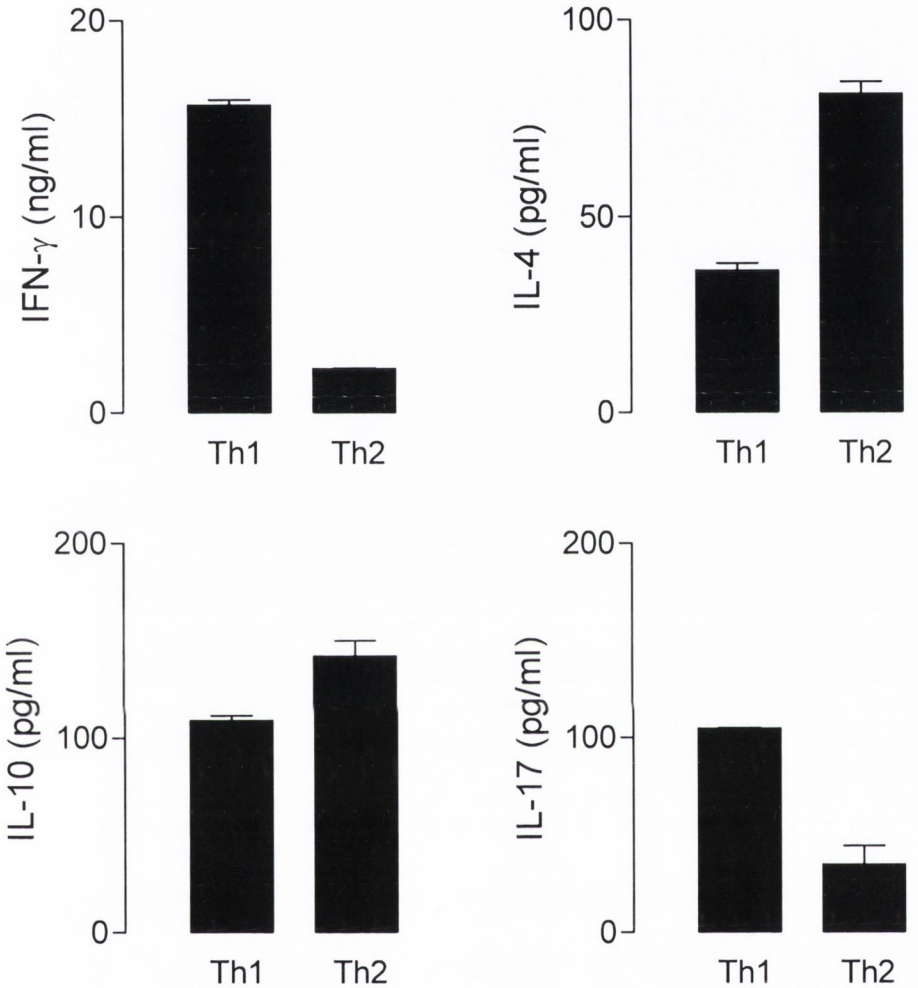


Figure 3.14. Cytokine profile of polarised Aβ-specific Th1 and Th2 cell lines.

C57BL/6 mice were immunised and polarised as in Figure 3.13. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs. Four days after restimulation, supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=3).

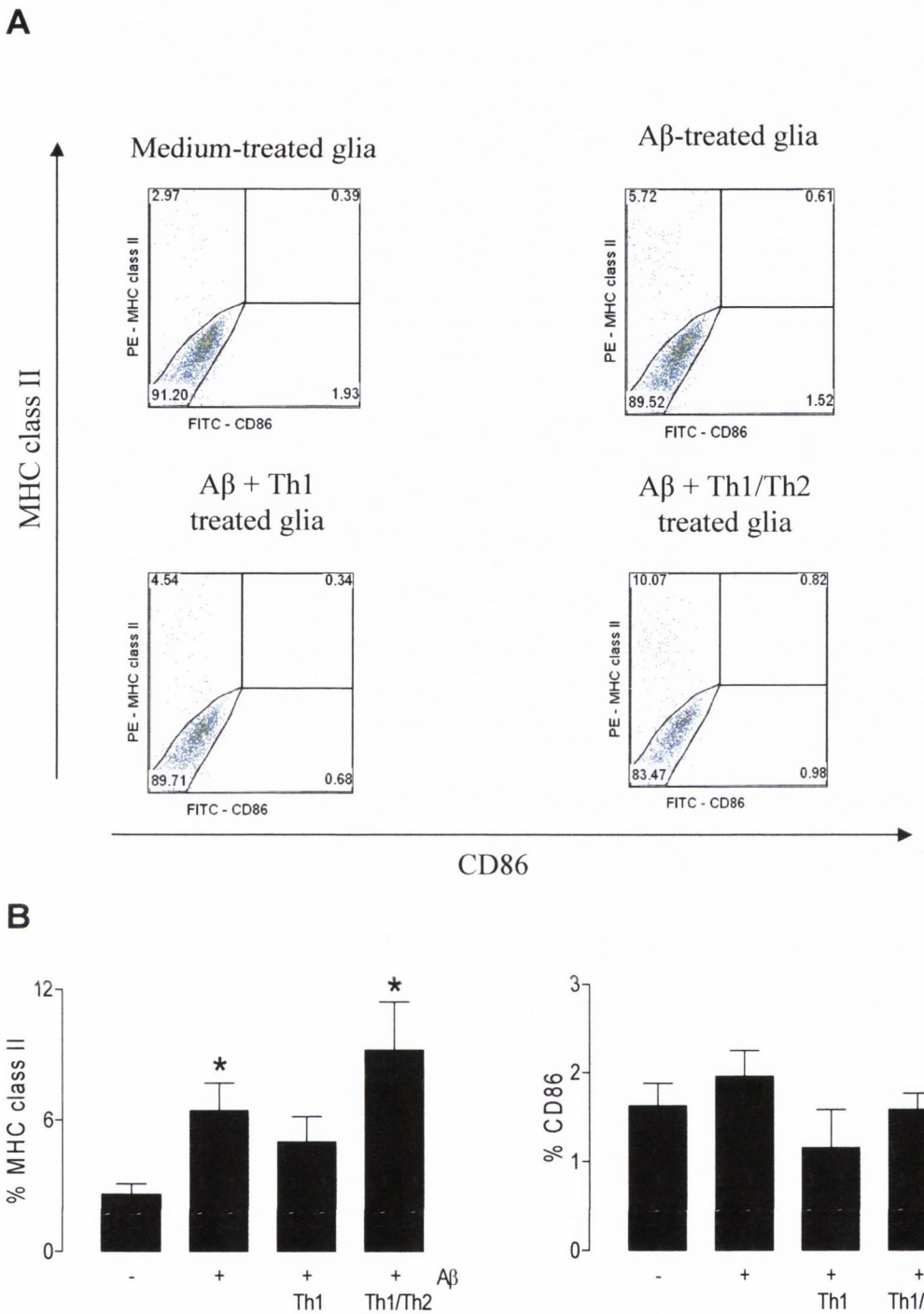


Figure 3.15. Effect of Aβ-specific Th1 or Th1/Th2 cells on Aβ-induced microglial activation.

C57BL/6 mice were immunised and polarised as in Figure 3.13. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs. Ten days after the second round of stimulation, C57BL/6 mice were injected intracerebroventricularly with Aβ (5μg/mouse). Th1 or Th1/Th2 mixed T cells (1 x 10^5) were injected intravenously into these mice 24 h later. After a further 24 hours the mice were sacrificed and the hippocampus was removed. (A) Representative dotplots are shown from each treatment group. (B) Hippocampal $CD11b^+$ cells expressing MHC class II or CD86 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b. * $p<0.05$ versus medium-treated mixed glia (unpaired Students t-test) . Values are expressed as means ± SEM (n=4).

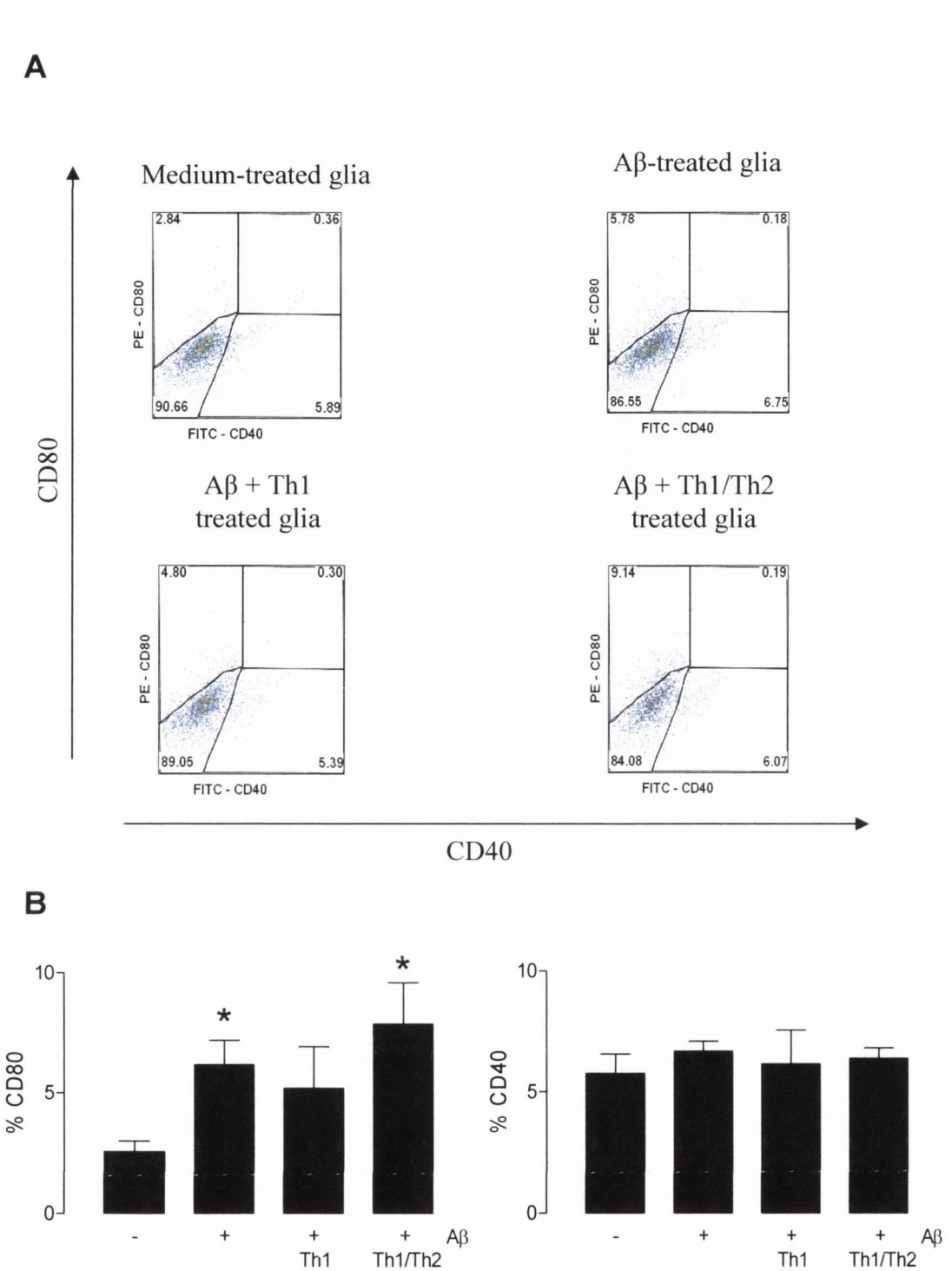


Figure 3.16. Aβ-induced microglial activation was unaffected by the addition of polarised Th1 or mixed Th1/Th2 cells.

Mixed glia from C57BL/6 mice were treated with T cells as described in Figure 3.15. (A) Representative dotplots are shown from each treatment group. (B) Hippocampal $CD11b^+$ cells expressing CD80 or CD40 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b. * $p<0.05$ versus medium-treated mixed glia (unpaired Students t-test). Values are expressed as means ± SEM (n=4).

Chapter 4

Role of distinct T cell subsets in glial activation

4.1. Introduction

Microglia are the innate immune cells of the CNS. In the normal adult brain, microglia exist in a quiescent state and are known as ramified microglia. In this state, most immune functions of microglia are down-regulated, and they act as supportive glial cells (Fetler & Amigorena 2005, Aloisi 2001). They use their long processes to sample the surrounding microenvironment and it is thought that this allows microglia to clear the brain of accumulated metabolic components and cell debris, as well as allowing them to respond rapidly to injury (Nimmerjahn *et al.* 2005). Upon infection or injury, ramified microglia undergo a morphological change. Their processes shorten and the main cell body enlarges, giving rise to an amoeboid phenotype (Vilhardt 2005). It is thought that, once activated, microglia can exist in at least two functionally discernable states: an "innate" phagocytic phenotype, and an "adaptive" antigen-presenting phenotype (Town *et al.* 2005). Furthermore, each of these activation states may be accompanied by production of inflammatory mediators, such as proinflammatory cytokines (Hanisch 2002), chemokines (Aloisi 2001), nitric oxide (Ii *et al.* 1996), and reactive oxygen species (Si *et al.* 1997).

It has become increasingly clear that microglial activation is a fundamental feature of neurodegenerative diseases, such as AD. Compared with control brain tissue, patients with AD have increased expression of proinflammatory cytokines, such as IL-1β and IL-6, in the CNS (Griffin *et al.* 1989, Wood *et al.* 1993). The neuritic plaques found in AD patients have been demonstrated to be associated

with activated microglia, and expression of MHC class II was dramatically upregulated on these microglia (Luber-Narod & Rogers 1988, Rogers *et al.* 1988). Furthermore, Lue and colleagues have reported that microglia isolated from post-mortem samples taken from patients with AD show increased expression of IL-1β, IL-6, and TNF-α following treatment with Aβ (Lue *et al.* 2001a).

While microglial activation is widely accepted to be associated with AD, whether this activation represents a pathogenic or protective response to the disease is currently the subject of much debate. Although microglial activation appears to be neuroprotective in certain models of CNS injury (Nimmerjahn *et al.* 2005, Butovsky *et al.* 2005a), a number of studies have suggested that the chronic activation of microglia contributes to the pathology seen in AD. Treatment of microglia with Aβ induced production of factors which induce neuronal death *in vitro* (Giulian *et al.* 1996). Treatment of microglia with Aβ, especially in the presence of IFN-γ or LPS, results in enhanced production of IL-1β, IL-6, and TNF-α (Gasic-Milenkovic *et al.* 2003, Meda *et al.* 1999). It is thought that the increase in proinflammatory cytokine production by microglia in response to Aβ is involved in the pathogenesis of AD. Polymorphisms in the genes for IL-1β, IL-6, and TNF-α have all been linked with an increased risk of developing AD (McGeer & McGeer 2001, Perry *et al.* 2001). Yamamoto and colleagues demonstrated that TNF-α enhanced Aβ production from APP expressing astrocytes, and reduced degradation of Aβ in APP-transgenic mice (Yamamoto *et al.* 2007). Activated microglia expressing IL-1β and TNF-α have been shown to be associated with Aβ plaques in sections taken from Tg2576 transgenic mice (Benzing *et al.* 1999). Additionally, overexpression of IL-6 in the CNS under an astrocyte promoter gave rise to neurodegeneration and subsequent cognitive decline (Campbell *et al.* 1997).

Microglia have also been reported to phagocytose amyloid aggregates and plaques *in vitro*, supporting a beneficial role of these cells in AD (Paresce *et al.* 1996, Ard *et al.* 1996, DeWitt *et al.* 1998a). In addition, Aβ cores isolated from

AD patients were rapidly phagocytosed following their injection into the hippocampus of rats (Frautschy *et al.* 1992). Additional evidence supporting a beneficial role of microglia in AD comes from immunotherapeutic studies showing that administration of Aβ-specific antibodies induced plaque clearance in a transgenic mouse model of AD, and that this removal was due to Fc-mediated phagocytosis by microglia (Bard *et al.* 2000). Down-regulation of microglial activation using the anti-inflammatory drug, dexamethasone, inhibited the clearance of Aβ (Wilcock *et al.* 2004a). It should be noted however, that microglia in AD patients appear to be unable to effectively phagocytose Aβ plaques *in vivo*. The reason for this discrepancy is unknown, but may be due to the fact that microglia become dysfunctional with aging (Streit 2004). It seems likely that microglia are capable of becoming either neurotoxic or neuroprotective in response to Aβ, and this decision may depend on the cytokine environment present at the time of stimulation.

As well as producing proinflammatory cytokines, microglia can also respond to cytokine signals, and cytokines produced by infiltrating T cells can have a profound effect on microglial activation. IFN-γ is a proinflammatory cytokine produced by Th1 cells, and it has been shown to induce co-stimulatory molecule expression and proinflammatory cytokine secretion by microglia (Aloisi 2001, Nguyen & Benveniste 2000b, O'Keefe *et al.* 1999, O'Keefe *et al.* 2002). In addition, Li and colleagues have demonstrated that priming of microglia with IFN-γ is required for Aβ-induced neuronal toxicity *in vitro* (Li *et al.* 2004), and IFN-γ has been reported to inhibit the phagocytosis of Aβ (Town *et al.* 2005, Yamamoto *et al.* 2008). Treatment of microglia with IL-17 was found to upregulate IL-6 and ICAM-1 expression, as well as inducing the production of nitric oxide (Kawanokuchi *et al.* 2008). In contrast, the Th2-type cytokines, IL-4 and IL-10 appear to down-regulate proinflammatory glial activation, promoting a phagocytic phenotype. IL-4 and IL-10 have also been found to attenuate Aβ-induced proinflammatory cytokine production in microglia (Szczepanik *et al.*

2001), and both of these cytokines reverse an IFN-γ-induced impairment in microglial phagocytosis (Koenigsknecht-Talboo & Landreth 2005).

Activation of microglia is often accompanied by an increase in the expression of MHC class II, CD80, CD86, and CD40 (Aloisi *et al.* 2000b, Issazadeh 1998). Upregulation of the expression of these molecules on the surface of microglia is required for effective presentation of antigen to T cells (Aloisi *et al.* 2000b). Aloisi and colleagues have demonstrated that microglia isolated from the CNS were capable of presenting antigen to Th1 cells, inducing them to produce IFN-γ and IL-2 (Aloisi *et al.* 1998). The data presented in chapter 3 showed that different adjuvants could be used to generate Aβ-specific Th1, Th2 and Th17 cells *in vivo*, and that co-culture of these cells with microglia induced proinflammatory cytokine production and co-stimulatory molecule expression on microglia. However, this protocol failed to generate highly polarised populations of Th2 or Th17 cells, despite the use of a number of different adjuvants, and thus it was not possible to conclusively demonstrate that different Aβ-specific T cell subsets have distinct effects on microglial activation. T cells can also be polarised from immunised mice *ex vivo* using polarising cytokines and neutralising antibodies (Abbas *et al.* 1996). IL-12 is known to be a key determining factor in the development of Th1 cells, and $CD4^+$ cells initially stimulated *in vitro* in the presence of IL-12 and IFN-γ develop into Th1 cells (Gately *et al.* 1998, Rao & Avni 2000). T cells activated with antigen in the presence of IL-4 differentiate into Th2 cells (Rao & Avni 2000), while IL-1 appears to be involved in the induction of Th17 cells (Sutton *et al.* 2006).

The role of T cells in the pathogenesis of AD is uncertain. Though there is no clear T cell response in the brains of AD patients, immunisation of these patients with Aβ in adjuvant led to the development of meningoencephalitis, which is thought to be mediated by Th1 cells specific for Aβ (Boche & Nicoll 2008, Holmes *et al.* 2008). Despite being linked with CNS inflammation, Th1 cells may also have a role in the clearance of toxic Aβ from the brain, as the

majority of AD patients immunised with Aβ in clinical trials had reduced plaque burden (Holmes *et al.* 2008). A recent study by Monsonego and colleagues looked at the effect of immunising mice overexpressing IFN-γ in the CNS with Aβ in CFA (Monsonego *et al.* 2006). It was found that while this immunisation protocol induced encephalitis, it also resulted in clearance of Aβ plaques. A study from the Schwartz laboratory has suggested that an initial Th1 response in the CNS may be beneficial as long as it is followed by an appropriate anti-inflammatory response (Frenkel *et al.* 2005, Butovsky *et al.* 2006). There are currently no published data on the role, if any, of Th17 cells in AD. Nonetheless, Th17 cells are involved in the pathogenesis of a number of autoimmune diseases, including MS and EAE, and therefore might also be involved in the development of AD.

While short-term inflammatory responses may have a beneficial effect, the chronic inflammation seen in the CNS of AD patients is thought to be involved in the pathogenesis of the disease. The cytokines produced by Th2 cells, in particular IL-4, IL-10, and IL-13, have been shown to down-regulate microglial activation and induce phagocytosis (Koenigsknecht-Talboo & Landreth 2005, Szczepanik *et al.* 2001), suggesting that Th2 cells may be beneficial in increasing phagocytosis of Aβ plaques. In support of this hypothesis, immunisation of APP-transgenic mice with a synthetic construct of Aβ in the Th2-promoting adjuvant alum was shown to reduce Aβ plaque burden (Asuni *et al.* 2006). Additionally, Th2 cells induce B cell differentiation and antibody production. As Aβ-specific antibodies have been shown to reduce Aβ deposits in transgenic models of AD (Bacskai *et al.* 2002, Bard *et al.* 2000), this suggests that Th2 cells may increase plaque clearance in AD.

The objectives of this chapter were to:

- Develop polarised, Aβ-specific Th1, Th2, and Th17 cell lines using *ex vivo* polarisation protocols.
- Assess the effect of *ex vivo* polarised, Aβ-specific Th1, Th2, and Th17 cells on proinflammatory cytokine production and co-stimulatory molecule expression by microglia.
- Investigate the ability of Aβ-specific Th2 cells to attenuate microglial activation induced by Th1 or Th17 cells.
- Determine the role of Th1, Th2, and Th17-type cytokines in modulating Aβ-induced cytokine secretion and co-stimulatory molecule expression on microglia.
- Assess the attenuating effects of the Th2-type cytokines, IL-4 and IL-10, on Aβ and IFN-γ-induced microglial activation

4.2. Results

4.2.1. Generation of polarised Aβ-specific Th1 and Th2 cell lines

In an effort to produce a more polarised Aβ-specific Th2 cell line than that induced by adjuvant alone, glucocorticoids were used to polarise T cell responses *in vitro*. Glucocorticoids are powerful immunosuppressive agents and are widely used in the treatment of a number of inflammatory disorders, such as rheumatoid arthritis (Verhoef *et al.* 1999). The immunosuppressive effects of glucocorticoids were initially attributed to their inhibitory effects on cell proliferation and IL-2 production by T cells (Brunetti *et al.* 1998, Daynes & Araneo 1989). In addition to suppressing proinflammatory responses, however, glucocorticoids have been reported to stimulate anti-inflammatory responses, and it has been shown that glucocorticoid treatment suppresses IFN-γ production by $CD4^+$ T cells, while concomitantly enhancing production of IL-4 (Li *et al.* 2007, Ramirez *et al.* 1996). A combination of the synthetic glucocorticoid, dexamethasone, and IL-4 dramatically increased IL-4 production from T cells, while suppressing IFN-γ (Ramirez & Mason 2000), and this is thought to be due to IL-4 selectively rescuing Th2 cells from glucocorticoid-mediated apoptosis (Zubiaga *et al.* 1992). Indeed, Ramirez and colleagues have shown that MBP-specific T cell lines generated in the presence of IL-4 and dexamethasone promote resistance to active EAE (Ramirez & Mason 2000).

In order to assess the efficacy of dexamethasone and IL-4 in promoting Aβ-specific Th2 cells, C57BL/6 mice were injected s.c. into the footpad with Aβ (75 μg/mouse) and CpG (25 μg/mouse). Ten days later, draining lymph nodes were removed, and lymph node cells were stimulated with antigen under Th1-polarising conditions with IL-12 (10 ng/ml), or under Th2-polarising conditions with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ. Cells polarised under Th1-inducing conditions were found to secrete high levels of IFN-γ, with

low levels of IL-4, IL-10, and IL-17 (Fig. 4.1). Th2 polarised cells produced low levels of IL-10, IFN-γ, and IL-17. IL-4 could not be accurately measured in supernatants from Th2-polarised cells at this point, due to the addition of exogenous IL-4.

To determine whether this polarisation protocol induced stably polarised Th1 and Th2 cell lines, Th1 and Th2 cells were restimulated with Aβ (25 μg/ml) and irradiated APC in the absence of polarising cytokines. The Th1 cell line produced high concentrations of IFN-γ, intermediate concentrations of IL-17, and low concentrations of IL-4 and IL-10 (Fig. 4.2). The Th2 cell line secreted high levels of IL-4, with low levels of IFN-γ, IL-10, and IL-17. This suggests that this method of polarisation induced homogenous, Aβ-specific Th1 and Th2 cell lines.

4.2.2. Aβ-specific Th1 cells are more potent activators of glial proinflammatory cytokine production and APC function than Aβ-specific Th2 cells

The data shown in Figs. 4.1 and 4.2 suggest that polarised, Aβ-specific Th1 and Th2 cell lines can be generated *ex vivo*. Previous studies have shown that the interaction of microglial cells and antigen-specific T cells results in the upregulation of co-stimulatory molecules and production of proinflammatory cytokines (Aloisi *et al.* 2000a, Chabot *et al.* 1997, Dasgupta *et al.* 2005). To assess the comparative effect of Aβ-specific Th1 and Th2 cells on microglial activation, polarised Th1 and Th2 cell lines (11 days after their second round of antigen stimulation) were co-cultured with Aβ-treated mixed glia at a ratio of 0.5:1. Consistent with data shown in Fig. 3.7, Aβ induced significant production of IL-6 and TNF-α from mixed glia. Treatment of glia with Th1 cells enhanced Aβ-induced production of IL-1β and IL-6 (Fig. 4.3A). In contrast, Th2 cells were found to have no effect on proinflammatory cytokine production. T cells cultured in the absence of microglia and Aβ produced low levels of IL-1β, IL-6, and TNF-

α (Fig. 4.3B). Th1 cells alone secreted substantially less IL-6 than co-culture of Th1 cells and glia, suggesting that IL-6 is produced by the interaction of Th1 cells and microglia. The concentration of IL-1β produced by Th1 cells alone was comparable to that found in co-culture of glia and T cells, indicating that IL-1β is primarily produced by the T cells. Analysis of co-stimulatory molecule expression revealed that both Th1 and Th2 cells induced MHC class II and CD40 expression on the surface of microglia (Fig. 4.5). Neither T cell subset enhanced expression of CD80 or CD86 on microglia (Fig. 4.4).

4.2.3. Stimulation of T cells from mice immunised with Aβ and CpG with IL-1 and IL-23 promotes IL-17 production

It has been demonstrated that Th17 cells are involved in the pathogenesis of a number of diseases, including rheumatoid arthritis (Chabaud *et al.* 1999), psoriasis (Kryczek *et al.* 2008), and MS (Langrish *et al.* 2005). There are currently no published data on the involvement of Th17 cells in the pathogenesis of AD. Th17 cells were generated by culture with Aβ in the presence of IL-1β and IL-23. IL-23 has been shown to drive a population of IL-17 producing T cells (Langrish *et al.* 2005), and recent studies from this laboratory have implicated IL-1 as an important factor in promotion of MOG-specific Th17 responses (Sutton *et al.* 2006). Mice were immunised and boosted with Aβ and CpG, and the harvested spleen cells were stimulated with antigen and either IL-1 (10 ng/ml), IL-23 (10 ng/ml) and anti-IFN-γ (10 μg/ml), or IL-23 and anti-IFN-γ. Additionally, two routes of immunisation were used, s.c. into the footpad and i.p, to determine the effect of the route of immunisation on the induction of Th17 cells. It was found that the route of immunisation had little effect on the cytokine profile of the induced T cells. After the first round of antigen stimulation *in vitro* (Fig. 4.6), T cells polarised with IL-23 alone produced low levels of IL-17 and no IL-5, IL-10, IL-4, or IFN-γ. In contrast, T cells polarised with a combination of IL-1 and IL-23 secreted high levels of IL-17 and low levels of IL-5.

Restimulation of Th17 cells through their TCR has previously been shown to induce their conversion to a Th1 phenotype by increasing T-bet expression (Mathur *et al.* 2006). To examine the effect of antigen restimulation on Aβ-specific Th17 cells, these cells were restimulated with Aβ and irradiated APC in the absence of polarising cytokines. Antigen restimulation was found to produce four dramatically different cytokine profiles (Fig. 4.7). T cells from mice immunised i.p. with Aβ, and polarised with IL-23, expressed high levels of IL-5, IL-10, and IL-17, with low levels of IL-4 and no IFN-γ (Treg/Th17 cells; Fig. 4.7 A). Addition of IL-1 and IL-23 to spleen cells from i.p. immunised mice generated T cells that secreted high levels of IFN-γ and IL-17, with no IL-4, IL-5, or IL-10 (Th1/Th17 cells; Fig. 4.7A). Antigen restimulation of T cells from mice immunised in the footpad and polarised with IL-23 *in vitro* did not produce significant levels of any of the cytokines measured (Fig. 4.7B). The only highly polarised Th17 cell line was generated from mice immunised s.c. in the footpad and polarised *in vitro* with Aβ in the presence of IL-1 and IL-23. This Th17 cell line produced high levels of IL-17 with no IFN-γ, IL-4, IL-5, or IL-10 (Fig. 4.7B). This immunisation protocol was therefore adopted for subsequent generation of Th17 cell lines.

In order to confirm the induction of Th17 cells, intracellular cytokine staining was performed on $CD4^+$ cells from these T cell lines. Intraperitoneal immunisation of C57BL/6 mice with Aβ and subsequent polarisation with IL-23 was found to generate T cells which secreted IL-17 and IL-10, while polarisation with IL-1 and IL-23 resulted in Aβ-specific T cells producing IFN-γ and IL-17 (Fig. 4.7A). Previous studies have demonstrated the existence of a Th17 cell population co-expressing IFN-γ (Annunziato *et al.* 2007), as well as a regulatory population of Th17 cells that secrete both IL-17 and IL-10 (McGeachy *et al.* 2007). To determine if Aβ-specific Th17 cells generated by i.p. immunisation co-express either IFN-γ or IL-10, Th17 cells were restimulated with a cocktail of PMA (10 ng/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/ml) for 5 h. T cell

lines were then analysed for intracellular production of IFN-γ, IL-10, and IL-17 by flow cytometry. The data show that the Th17 cell line polarised with IL-1 and IL-23 (Th1/Th17) contained cells producing IFN-γ or IL-17 alone, as well as a significant population of T cells that co-produced IFN-γ and IL-17 (Fig. 4.8A). T cells polarised with IL-23 alone (Treg/Th17) were found to be comprised of a large number of IL-17-producing cells, a smaller population of IL-10-expressing cells, as well as a population of cells that co-produced IL-10 and IL-17 (Fig. 4.8B). These data show that different populations of IL-17 producing T cells exist and that the polarising cytokines used have important effects on the cytokine profile of Th17 cells.

4.2.4. Generation of Aβ-specific Th2 cells by restimulation of spleen cells with dexamethasone and IL-4

The experiments outlined above show that the route of immunisation has a significant impact on the subsequent cytokine profile of T cell lines. To assess the effect of the site of immunisation on the polarisation of Th1 and Th2 cell lines, mice were immunised either i.p. or s.c. in the footpad with Aβ and CpG. Spleen cells were polarised with IL-12 to generate a Th1 cell line, or with dexamethasone and IL-4 to generate a Th2 cell line. It was found that Th2 cell lines generated from mice immunised i.p. with Aβ secreted high levels of IFN-γ, as well as IL-4, IL-10, and IL-5, after 2 rounds of antigen stimulation (Fig. 4.7A). These cells were thus termed Th1/Th2 cells. Th2 cell lines polarised from spleen cells taken from mice immunised s.c. in the footpad produced high levels of IL-4, IL-10, and IL-5, with low levels of IFN-γ and no IL-17 (Fig. 4.7B). Immunisation in the footpad thus generated cells that were readily polarised to a Th2 phenotype. The route of administration of Aβ was found to have no effect on the cytokine profile of Th1 cell lines (Figs. 4.6 and 4.7).

4.2.5. Aβ-specific Th17 cells are stronger activators of glial proinflammatory cytokine production and co-stimulatory molecule expression than Aβ-specific Th2 cells

The results shown in Figs. 4.3 to 4.5 show that Th1 and Th2 cell lines can be generated from T cells of mice immunised with Aβ and CpG, and that these cells have distinct modulatory effects on glial cell activation. The ability of Aβ-specific Th17 cells to modulate glial proinflammatory cytokine production and co-stimulatory molecule expression was examined and compared with that of Th1 and Th2 cells. Mice were immunised s.c. in the footpad with Aβ and CpG and boosted 21 days later. Spleens were removed and cells were stimulated with Aβ in the presence of IL-12 for Th1 cell polarisation, dexamethasone and IL-4 to generate Th2 cells, or IL-1 and IL-23 to induce Th17 cells. After 4 days, supernatants were removed, and IFN-γ, IL-4, IL-10, IL-17, and IL-5 production was measured by ELISA. Th1 cells produced high levels of IFN-γ, low levels of IL-5 and IL-10, and no IL-4 or IL-17 (Fig. 4.9). Th2 cells secreted high levels of IL-4, low levels of IL-5 and IL-10, and no IL-17 or IFN-γ. Th17 cells were found to produce high levels of IL-17, low levels of IL-5 and IL-10, and no IL-4 or IFN-γ.

Eleven days after the second round of antigen stimulation, Th1, Th2, and Th17 cells were co-cultured with Aβ-treated mixed glia at a ratio of 0.5:1. Addition of Aβ-specific Th17 cell lines enhanced IL-1β, IL-6, and TNF-α production from mixed glia (Fig. 4.10). Th17 cells were also found to upregulate the expression of MHC class II, CD80, and CD86 on microglia (Figs. 4.11 and 4.12). Comparison of the effects of the different T cell subsets on microglial activation revealed that Aβ-specific Th17 cells induced greater IL-1β, IL-6, and TNF-α production, as well as CD80 and CD86 expression, than Aβ-specific Th2 cells (Figs. 4.10 to 4.12). This indicates that Th17 cells are more potent activators of glia than Th2 cells. In addition, supernatants from co-cultures of glia and Th17

cells had significantly higher IL-1β and IL-6 levels than glia co-cultured with Th1 cells (Fig. 4.10).

4.2.6. Aβ-specific Th2 cells attenuate proinflammatory activation of microglia by Th17 cells, but not by Th1 cells

Th2-type cytokines are reported to be capable of inhibiting differentiation of Th1 and Th17 cells (Moser & Murphy 2000, Harrington *et al.* 2005). In addition, Th2 cells have recently been shown to reduce the number of plaque-associated microglia in a transgenic mouse model of AD (Cao *et al.* 2009). Thus, Th2 cells may be capable of suppressing cytokine production and co-stimulatory molecule expression induced by co-culture of mixed glia and Th1 or Th17 cells. Analysis of cytokine concentrations from Th1, Th2, and Th17 cell lines confirmed that these cells were effectively polarised to the relevant subtype (Fig. 4.13). Co-culture of glia and Th17 cells induced significant increases in production of IL-1β and IL-6, while co-culture of glia and Th1 cells induced significant increases in IL-6 (Figs. 4.14 and 4.15). Addition of Th2 cells to co-cultures of glia and Th17 cells significantly inhibited the production of IL-1β and IL-6 (Figs. 4.14 and 4.15). Addition of Th2 cells did not reduce production of IL-6 induced by co-culture of glia and Th1 cells. Th2 cells did not attenuate increases in TNF-α production induced by co-culture of glia and Th1 or Th17 cells (data not shown).

Increases in cytokine production seen following co-culture of glia and Aβ-specific T cells may be due to the production of these cytokines by either the glia or the T cells. To determine the contribution of T cells to the proinflammatory cytokine production seen following co-culture, cytokine production by T cells was assessed by intracellular cytokine staining. Brefeldin A (5 μg/ml) was added 8 h after the initiation of co-culture to block cytokine secretion. Twelve hours after the addition of brefeldin A, $CD4^+$ T cells were analysed for their expression of IL-6 and TNF-α by flow cytometry. It was found that only a small percentage of T

cells produced IL-6 and TNF-α in response to co-culture with Aβ-stimulated microglia, indicating that they are unlikely to contribute significantly to the proinflammatory cytokine production seen in co-culture of T cells with mixed glia (Fig. 4.16).

The effect of Th2 cells on the expression of co-stimulatory molecules on the surface of microglia was examined by flow cytometry. Co-culture of glia and Th1 or Th17 cells enhanced Aβ-induced MHC class II, CD80, CD86, and CD40 expression on microglia (Figs. 4.17 and 4.18). Addition of Th2 cells attenuated Th1 cell-induced CD86 and CD40 expression on microglia, while enhancing Th1 cell-induced CD80 and MHC class II expression. Th17 cell-induced CD80 and MHC class II expression on microglia was enhanced by addition of Th2 cells, while CD86 expression on microglia was attenuated by Th2 cells (Figs. 4.17 and 4.18). The data show that Aβ-specific Th2 cells can inhibit proinflammatory cytokine production induced by co-culture of microglia and Th17, but not Th1 cells. In addition, Th2 cells have distinct modulatory effects on Th1 and Th17 cell-induced co-stimulatory molecule expression on microglia.

4.2.7. Aβ-specific Th2 cells inhibit IFN-γ and IL-17 produced by co-cultures of glia Th1 and Th17 cells respectively

Previous studies have shown that microglia are capable of acting as a APCs to induce T cell proliferation and cytokine production *in vitro* (Aloisi *et al.* 2000a, Becher *et al.* 2000). To determine the ability of glia to induce cytokine production from Aβ-specific T cell lines, Aβ-specific Th1, Th2 or Th17 cells were co-cultured with microglia at a ratio of 0.5:1. Eight hours after the initiation of co-culture, brefeldin A was added for 12 hours to block cytokine secretion, and the percentage of $CD4^+$ T cells expressing IFN-γ or IL-17 was analysed by flow cytometry. The data show that co-culture of glia and Th1 cells induced T cell production of IFN-γ, while Th17 cells co-cultured with glia produced significant

levels of IL-17 (Fig. 4.19). Addition of Th2 cells attenuated production of IFN-γ in co-cultures of Th1 cells and glia (red numbers; Fig. 4.19), as well as production of IL-17 in co-cultures of Th17 cells and glia (blue numbers; Fig. 4.19).

4.2.8. IFN-γ enhances Aβ-induced activation of microglia

The data presented above demonstrate that Aβ-specific Th1, Th2, and Th17 cells have distinct modulatory effects on glial activation. These T cell-induced changes may be cell-contact mediated, or may involve the secretion of soluble factors by the T cell. In order to assess the ability of Th1, Th2, and Th17-type cytokines to modulate Aβ-induced glial activation, mixed glia from C57BL/6 mice were treated with Aβ (40 μg/ml) in the presence or absence of the Th1-type cytokine, IFN-γ (20 ng/ml), the Th2-type cytokines, IL-4 (100ng/ml) and IL-10 (100 ng/ml), or the Th17-type cytokine, IL-17 (20 ng/ml). After 24 h, concentrations of IL-6 and TNF-α were measured by ELISA, while expression of MHC class II and CD86 on microglia was determined by flow cytometry. The data show that treatment with IFN-γ enhanced IL-6 and TNF-α production from Aβ-stimulated glia, while treatment with IL-17 induced IL-6 secretion (Fig. 4.20). IL-4 and IL-10 were found to have no effect. Analysis of co-stimulatory molecule expression revealed that treatment of glia with IFN-γ increased microglial expression of MHC class II and CD86 (Fig. 4.21). Treatment with IL-4, IL-10, or IL-17 was found to have little effect on co-stimulatory molecule expression. The data indicate that IFN-γ induced significant activation of Aβ-treated glia, IL-17 moderately activated glia, while IL-4 and IL-10 had little effect on proinflammatory cytokine secretion or co-stimulatory molecule expression.

4.2.9. IL-4 attenuates glial activation induced by Aβ and IFN-γ

The above experiments show that IFN-γ in combination with Aβ greatly enhanced glial proinflammatory cytokine production and co-stimulatory molecule expression. IL-4 is an anti-inflammatory cytokine produced by Th2 cells, and it has been shown that it can inhibit IFN-γ-induced glial activation *in vitro* (Nguyen & Benveniste 2000a, O'Keefe *et al.* 1999). In addition, previous data from this laboratory have indicated that IL-4 down-regulates glial activation, and it has been shown to attenuate Aβ-induced increases in IL-1β production and MHC class II expression *in vivo* (Lyons *et al.* 2007). IL-4 was assessed for its ability to down-regulate activation of microglia induced by Aβ and IFN-γ. Treatment of glia with Aβ in combination with IFN-γ induced production of significantly greater concentrations of IL-6 and TNF-α than either Aβ or IFN-γ alone (Fig. 4.22). The addition of IL-4 significantly attenuated Aβ-induced production in TNF-α, and also repressed the increases in IL-6 and TNF-α induced by the combination of Aβ and IFN-γ (Fig. 4.22).

The effect of IL-4 in regulating Aβ and IFN-γ induced expression of co-stimulatory molecules on microglia was assessed by flow cytometry. The data show that treatment with Aβ enhanced CD86, CD80, and CD40 expression on the surface of microglia, which were further enhanced by co-treatment with IFN-γ (Figs. 4.23 and 4.24). IL-4 attenuated Aβ-induced expression of CD86 on microglia (Fig. 4.23) IL-4 was also found to attenuate an increase in CD40 expression on microglia induced by treatment with Aβ plus IFN-γ (Fig. 4.24). The data suggest that IL-4 can down-regulate microglial activation induced by Aβ and IFN-γ.

4.2.10. IL-10 attenuates glial activation induced by Aβ and IFN-γ

IL-10 is another anti-inflammatory cytokine produced by Th2 cells and has previously been shown to inhibit IFN-γ-induced MHC class II expression on microglia (O'Keefe *et al.* 1999). Thus, the inhibitory effect of IL-10 on Aβ and IFN-γ-induced glial activation was determined. The data show that IL-10 attenuated Aβ-induced production of TNF-α from mixed glia (Fig. 4.25). IL-10 was found to have no effect on IL-6 or TNF-α production induced by the treatment with the combination of Aβ and IFN-γ. Analysis of co-stimulatory molecule expression on microglia by flow cytometry revealed that IL-10 attenuated an increase in CD86 expression induced by Aβ plus IFN-γ (Fig. 4.26). In addition, IL-10 inhibited Aβ-induced increases in CD40 and CD80 expression on microglia (Fig. 4.27). The data indicate that IL-10 is able to inhibit microglial activation induced by Aβ and IFN-γ.

4.3. Discussion

In this chapter, a method for the generation of Aβ-specific Th1, Th2, and Th17 cell lines *ex vivo* was developed. The results demonstrate that co-culturing Aβ-specific Th1 or Th17 cells with Aβ-treated microglia markedly enhanced proinflammatory cytokine production and microglial expression of MHC class II and co-stimulatory molecules. Th2 cells were unable to induce proinflammatory cytokine production from microglia, but upregulated co-stimulatory molecule expression to a lesser extent than Th1 or Th17 cells. In addition, Th2 cells attenuated inflammatory responses induced by co-culture of Aβ-treated microglia and Th17, but not Th1 cells. The prototypic Th1 and Th17 cytokines, IFN-γ and IL-17, were found to synergise with Aβ in triggering microglial activation, while the Th2-type cytokines, IL-4 and IL-10, mitigated the proinflammatory cytokine production and co-stimulatory molecule expression induced by Aβ and IFN-γ.

T cells that have differentiated and migrated from the thymus, but that have not yet encountered their specific antigens, are known as naïve T cells. To participate in an adaptive immune response and become effector Th cells, naïve T cells require two separate signals. They must firstly encounter their cognate antigen in the context of an antigen:MHC complex on the surface of an APC (Glimcher & Murphy 2000). They also require a co-stimulatory signal from the APC, such as binding of CD80 or CD86 on the APC to CD28 on the T cell. However, neither of these 2 signals has a profound effect on the subset of T cell induced. The phenotype of the activated CD4 cell is predominantly determined by the cytokine environment present at the time of stimulation (Mosmann *et al.* 1986, Glimcher & Murphy 2000). T helper cells stimulated with antigen in the presence of IL-12 differentiate into Th1 cells (Gately *et al.* 1998), while the presence of IL-4 at the time of activation of a naïve T cell induces development of Th2 cells (Diehl *et al.* 2002, Rao & Avni 2000). The results of the present study show that Aβ-specific Th2 cells were induced by polarisation with a combination of

dexamethasone and IL-4. This is consistent with a previous study which found that this combination of polarising substances generated MBP-specific Th2 cells (Ramirez & Mason 2000). It has been proposed that this is due to IL-4 rescuing Th2 cells from dexamethasone-mediated apoptosis (Zubiaga *et al.* 1992). A combination of IL-1 and IL-23, but not IL-23 alone, was found to be capable of inducing Th17 cell differentiation. This is supported by a previous study that demonstrated an essential role for IL-1 in the development of Th17 cells (Sutton *et al.* 2006). Kimura and colleagues have reported that TGF-β and IL-6 are required for the differentiation of Th17 cells (Kimura *et al.* 2007). However, Th17 cells generated in this manner have been found to co-express IL-10, and appear to be unable to induce CNS inflammation (McGeachy *et al.* 2007).

In addition to the nature of the adjuvant and the antigen being used, the route of immunisation has an effect on the magnitude and type of immune response induced. The data shown in Chapter 3 demonstrated that s.c. immunisation with Aβ in CpG generated Th1 cells, and these cells have been implicated in the development of meningoencephalitis in clinical trials of an Aβ vaccine for AD (Holmes *et al.* 2008). A number of recent studies have thus focused on intranasal administration of Aβ, which has been shown to generate strong antibody and Th2 responses to the peptide (Weiner *et al.* 2000, Lemere *et al.* 2002). The results shown here have demonstrated that the addition of different polarising cytokines to Aβ-specific T cells from mice immunised i.p. generated polarised Th1 cells, but did not produce stable Th2 or Th17 cell lines. In particular, the data from the Th17 cell line polarising approach was interesting as it showed that it is possible to expand T cells that co-express IFN-γ and IL-17, as well as T cells that co-express IL-10 and IL-17. This is consistent with previous studies showing that Th17 cells can be generated which co-produce either IFN-γ (Annunziato *et al.* 2007, Suryani & Sutton 2007), or IL-10 (McGeachy *et al.* 2007), and suggests that Th17 cells may be phenotypically linked to both Th1 and Treg cells. In contrast to the mixed populations generated by polarisation of T cells from i.p. immunised mice, s.c. immunisation in the footpad was found to

induce T cells that could be polarised *in vitro* into Th1, Th2, or Th17 cells following the addition of the appropriate polarising cytokines.

It has been suggested that Th1 cells are detrimental in AD, and the development of meningoencephalitis in a subset of AD patients following immunisation with Aβ may be due to a Th1 cell response to Aβ (Monsonego & Weiner 2003). T cells have detected in the brains of immunised patients in clinical trials (Ferrer *et al.* 2004, Nicoll *et al.* 2003), and stimulation of peripheral blood mononuclear cells from these individuals with Aβ produced IL-2 and IFN-γ, indicative of a Th1 response (Pride *et al.* 2008). Additionally, immunisation with Aβ has been found to induce encephalitis in APP-transgenic mice overexpressing IFN-γ in the CNS (Monsonego *et al.* 2006). In the present study, co-culture of either Th1 or Th17 cells with glia significantly enhanced Aβ-induced production of IL-1β, IL-6, and TNF-α. This data is in agreement with previous work showing that T cells induced production of proinflammatory cytokines from glia (Dasgupta *et al.* 2005, Chabot *et al.* 1997). A comparison of the effects of Th1 and Th17 cells revealed that Aβ-specific Th17 cells induced greater production of IL-1β and IL-6 than Aβ-specific Th1 cells when cultured with glia. To date, there are no published data on the role of Th17 cells in AD. Analysis of co-stimulatory molecule expression showed that both Aβ-specific Th1 and Th17 cells enhanced microglial expression of MHC class II, CD80, CD86, and CD40. These results are consistent with those reported by Aloisi and colleagues, who showed that co-culture of OVA-specific Th1 cells with glia resulted in increased expression of MHC class II and CD40 by the microglia (Aloisi *et al.* 1998). The results presented here show no significant differences between Th1 and Th17 cells in terms of their induction of the expression of antigen-presenting molecules on microglia.

Binding of CD40 to its ligand (CD154) on T cells appears to be important in the interaction of T cells and microglia. It has been shown to reduce microglial phagocytosis of Aβ (Townsend *et al.* 2005), and APP-transgenic mice deficient in

CD154 showed decreased microglial activation and lower numbers of Aβ plaques (Laporte *et al.* 2006, Tan *et al.* 1999). Furthermore, administration of anti-CD154 antibody to APP/PS1 double transgenic mice decreased microgliosis and astrocytosis while increasing plaque clearance (Tan *et al.* 2002). The data shown here demonstrate that Th1 and Th17 cells can enhance Aβ-induced activation of microglia. This provides indirect evidence of a role for Aβ-specific T cells *in vivo* and perhaps in the pathogenesis of AD. It should be emphasised, however, that despite inducing proinflammatory cytokine production, Th1 and Th17 cell responses may have a beneficial effect in clearance of Aβ plaques. Frenkel and colleagues have shown that induction of EAE in APP-transgenic mice, which induces MOG-specific Th1 and Th17 cells, resulted in the clearance of Aβ plaques (Frenkel *et al.* 2005). In addition, it has been reported that while immunisation of APP-transgenic mice overexpressing IFN-γ in the CNS with Aβ induced encephalitis, it also led to a T cell-dependent clearance of Aβ plaques from the brain (Monsonego *et al.* 2006).

A number of studies have reported that microglia are competent APCs, and can stimulate proliferation and cytokine production by antigen-specific T cells (Juedes & Ruddle 2001, Li *et al.* 2007, Matsumoto *et al.* 1992). In this study, microglia acted as an effective APC for Aβ-specific T cells, inducing IFN-γ and IL-17 production by Th1 and Th17 cells respectively. This is consistent with results from Aloisi and colleagues, who demonstrated that microglia can induce IFN-γ production from OVA-specific Th1 cells (Aloisi *et al.* 1998). Furthermore, treatment with glucocorticoids has been shown to inhibit microglial-induced proliferation and cytokine production by anti-CD3-stimulated T cells (Li *et al.* 2007). However, a number of studies have reported that microglia induce T cell activation only in the presence of IFN-γ (Matsumoto *et al.* 1992, Li *et al.* 2004, Aloisi *et al.* 1998). The discrepancy between these results and those in the present study may be due to the uniqueness of Aβ as an autoantigen. We and others have shown that Aβ can induce the production of co-stimulatory molecules from glia, and it has been suggested that Aβ may act as both antigen and co-stimulator to

promote an immune response (Loewenbrueck *et al.* 2008). In contrast to the results shown here, Townsend and colleagues found that microglia treated with Aβ could only induce cytokine production from Aβ-specific T cells in the presence of CD40L (Townsend *et al.* 2005), however they used a monomeric form of the peptide which did not induce proinflammatory cytokine production, or presumably, co-stimulatory molecule expression on microglia. The preparation of Aβ used in the present study was found to upregulate CD40 expression on the surface of microglia, and this may be required for T cell activation.

Given that Th1 and Th17 cells induced proinflammatory cytokine production by microglia, and that proinflammatory cytokines have been associated with AD (Griffin *et al.* 1995, McGeer & McGeer 2001, Wood *et al.* 1993), it was postulated that Th2 cells may have a beneficial effect in AD. In support of this hypothesis, IFN-γ and IL-12 were found to be upregulated, and IL-4 down-regulated, in the cerebral cortex of APP-transgenic mice (Abbas *et al.* 2002). Recent vaccination studies have thus focused on immunisation with alternate adjuvants to CFA, aimed at biasing the immune response towards a Th2 phenotype (Asuni *et al.* 2006, Cribbs *et al.* 2003). APP-transgenic mice immunised with an amyloid-based peptide in alum showed reductions in amyloid levels in the CNS (Asuni *et al.* 2006). Additionally, Aβ-specific Th2 cells have been demonstrated to reduce plaque-associated microglia and improve cognition in APP/PS1 transgenic mice (Cao *et al.* 2009). Nonetheless, the reductions in Aβ plaques seen in these studies were minor in comparison with the dramatic effect seen with immunisation in the Th1-based adjuvant, CFA. In contrast to the significant proinflammatory cytokine production seen in co-cultures of glia and Th1 or Th17 cells, the present study showed that Th2 cells did not induce IL-1β, IL-6, or TNF-α production when co-cultured with glia. These results are consistent with the anti-inflammatory role of Th2 cells, and indeed, a study by Seguin and colleagues has shown that supernatants from Th2 cells did not induce IL-6 or TNF-α production by microglia (Seguin *et al.* 2003). They also found that supernatants from Th2 cells did not induce co-stimulatory molecule expression on

microglia. This is in contrast to the results shown here, where Aβ-specific Th2 cells were found to induce MHC class II, CD80, CD86, and CD40 expression on microglia, and suggests that this may be cell-contact mediated. Notably, while Th2 cells induced co-stimulatory molecule expression on microglia, they did not do so to the same extent as either Th1 or Th17 cells.

This study has also provided new information on the ability of Th2 cells to modulate Th1 and Th17 cell-induced activation of microglia. Addition of Aβ-specific Th2 cells to co-cultures of glia and Aβ-specific Th17 cells inhibited the production of IL-1β and IL-6. This may in part be mediated by the inhibitory effect of Aβ-specific Th2 cells on IL-17 production by T cells. The Th2-type cytokines, IL-4 and IL-10, have previously been shown to reduce microglial proinflammatory cytokine production induced by LPS and IFN-γ (Chao *et al.* 1993, Ledeboer *et al.* 2002, Sawada *et al.* 1999, Nguyen & Benveniste 2000a). Despite producing large amounts of IL-4 however, Th2 cells were unable to attenuate proinflammatory cytokine production induced by co-culture of glia and Th1 cells. However, Th1 cell-induced IFN-γ production was found to be inhibited by Th2 cells, and this suggests that production of proinflammatory cytokines in co-cultures of glia and Th1 cells is predominantly cell-contact dependent. Th2 cells were also found to modulate co-stimulatory molecule expression on microglia induced by Th1 and Th17 cells. Addition of Th2 cells increased both Th1 and Th17 cell-induced MHC class II and CD80, while reducing Th1 and Th17 cell-induced CD86. Th2 cells were also found to decrease Th1 cell-induced CD40. It has previously been reported that CD40:CD154 interaction is important in the interaction of microglia and T cells, and Nguyen and colleagues have found that IL-4 inhibits IFN-γ-induced CD40 (Nguyen & Benveniste 2000a). However, inhibition of CD40 by Th2 cells did not affect their induction of proinflammatory cytokine production by co-cultures of glia and Th1 cells.

To investigate the role of Th1, Th2, and Th17 derived cytokines in T cell-induced microglial activation, microglia were treated with IFN-γ, IL-4, IL-10, and

IL-17. The data show that treatment of glia with IL-17 enhanced Aβ-induced IL-6 production, but did not increase TNF-α production, or expression of MHC class II or CD86 on microglia. This is in agreement with a previous study showing that IL-17 increased IL-6 production, but not TNF-α production or MHC class II expression on microglia (Kawanokuchi *et al.* 2008). In the present study, Aβ and IFN-γ synergised in triggering glial production of IL-6 and TNF-α, as well as microglial expression of CD86 and CD40. Aβ has previously been reported to synergise with IFN-γ in producing TNF-α and nitric oxide (Meda *et al.* 1995, Goodwin *et al.* 1995). IL-4 and IL-10 both failed to induce cytokine production or co-stimulatory molecule expression on microglia. A comparison of the effects of T cell subsets and their respective cytokines showed that while Th2 and Th17 cells induced significant expression of MHC class II and co-stimulatory molecules on microglia, their prototypical cytokines failed to do so. Thus, it would appear that induction of co-stimulatory molecule expression on microglia is mediated by factors other than these cytokines.

IL-4 and IL-10 have previously been shown to inhibit LPS-induced production of proinflammatory cytokines by microglia *in vitro* (Sawada *et al.* 1999, Szczepanik *et al.* 2001). The data presented here show that IL-4 attenuated Aβ and IFN-γ-induced increases in TNF-α production, and CD86 and CD40 expression on microglia. IL-10 was found to inhibit Aβ-induced TNF-α and CD80, and Aβ plus IFN-γ-induced CD86 expression on microglia. These results demonstrate that IL-4 and IL-10 can down-regulate inflammatory microglial activation induced by Aβ and IFN-γ, and therefore may be beneficial in the treatment of AD. Indeed, immunisation of APP-transgenic mice with a cocktail of Aβ, granulocyte macrophage colony-stimulating factor, and IL-4 produced a Th2-type response and reduced Aβ deposition (DaSilva *et al.* 2006).

It has been hypothesised that, once activated, microglia exist in at least two functionally discernable states: an antigen presenting phenotype termed "adaptive activation", and a phagocytic phenotype termed "innate activation"

(Town *et al.* 2005). In this model, the activation state adopted by the microglial cell following stimulation is determined by the stimulatory environment. Treatment of microglia with Aβ has been shown to stimulate microglia to phagocytose Aβ aggregates (Paresce *et al.* 1996, Ard *et al.* 1996). Addition of CD40L reversed this response and increased production of IL-6 and TNF-α by microglia (Town *et al.* 2005, Townsend *et al.* 2005). Phagocytosis was inhibited by IFN-γ, while IL-4 and IL-10 enhanced engulfment of Aβ. This model proposes that when microglia are activated in a proinflammatory environment, they switch from being "innately activated" to being "adaptively activated", and their phagocytic response to Aβ is reduced. In support of this theory, IL-10, IL-4, and IL-13 have all been shown to suppress Aβ and LPS-induced proinflammatory cytokine production to varying extents (Szczepanik *et al.* 2001). Furthermore, it has been shown that phagocytosis of fibrillar Aβ by microglia can be inhibited by the addition of proinflammatory cytokines (Koenigsknecht & Landreth 2005), and that this is reversed by co-incubation with anti-inflammatory cytokines.

This study provides new insights into the role of Aβ-specific T cells and their associated cytokines in microglial activation. Th1 and Th17 cells, along with their associated cytokines, activated microglia to an "adaptive phenotype", characterised by proinflammatory cytokine production and co-stimulatory molecule expression. In contrast, Th2 cells and their associated cytokines induced less proinflammatory cytokine production and co-stimulatory molecule expression by microglia, and in fact were able to inhibit "adaptive activation" of microglia by Th17 cells. Based on the data shown here, Th2 cells might be expected to induce microglia that are more phagocytic, while Th1 cells may induce less phagocytosis by microglia and more proinflammatory cytokine production. Further experiments are required to determine the ability of microglia to phagocytose Aβ in the presence of Th1, Th2, and Th17 cells.

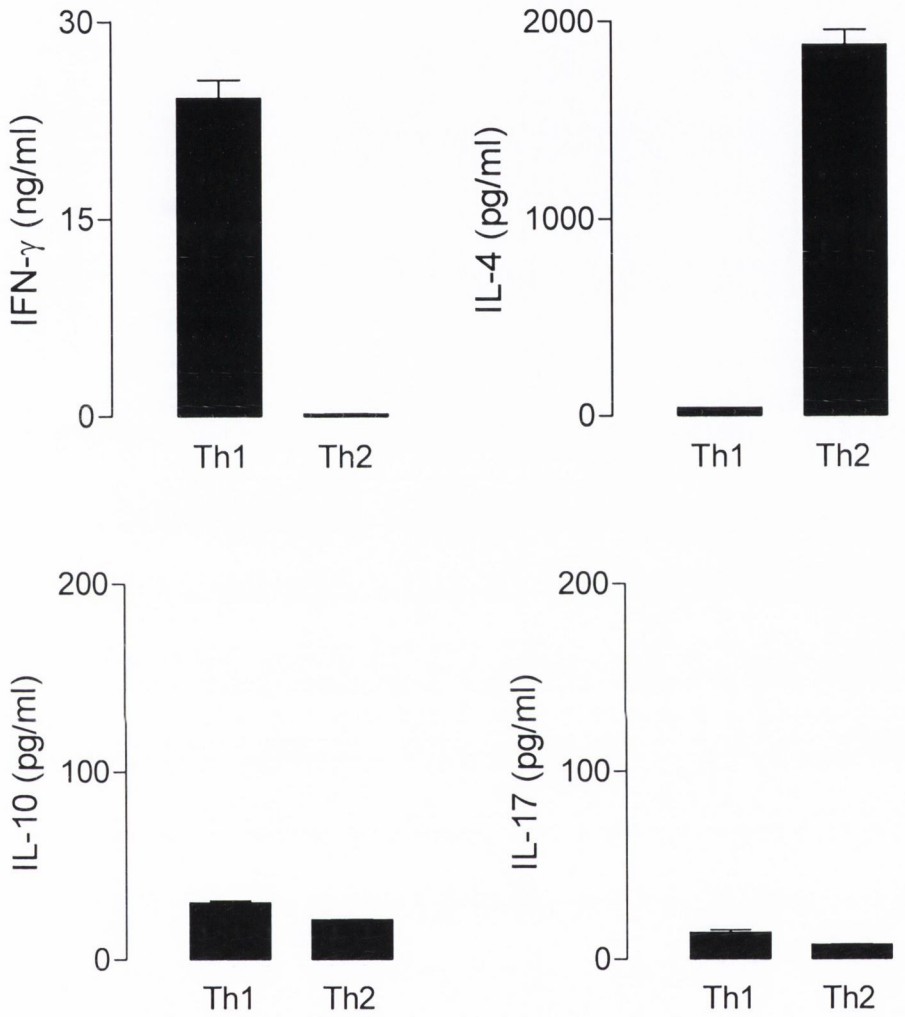


Figure 4.1. ***Ex vivo*** **polarisation of T cells from mice immunised with Aβ and CpG to generate Aβ-specific Th1 and Th2 cell lines – cytokine production after first round of Aβ stimulation.**

C57BL/6 mice were injected subcutaneously in the footpad with Aβ (75 μg/mouse) and CpG (25 μg/mouse). After 10 days, mice were sacrificed, popliteal lymph nodes were harvested, and the cells were stimulated with Aβ (25 μg/ml) under Th1 polarising conditions with IL-12 (10 ng/ml), or under Th2 polarising conditions with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml). On day 4 of culture the supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=10).

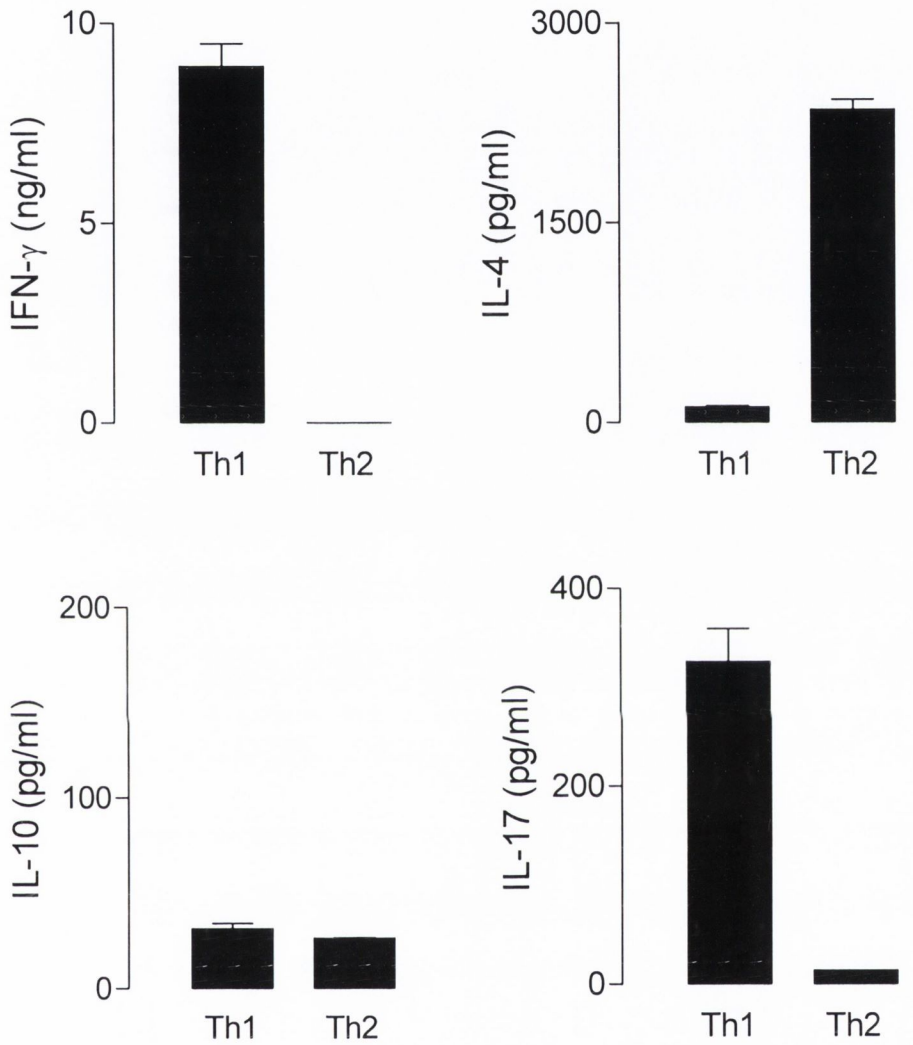


Figure 4.2. Cytokine production from Aβ-specific Th1 and Th2 polarised T cell lines 4 days after second round of antigen stimulation.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.1. Four days after stimulation with Aβ, IL-2 (5 ng/ml) was added. In addition, IL-4 and dexamethasone were added to Th2 polarised cells only. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. On day 4 of this second round of stimulation, the T cell supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=4).

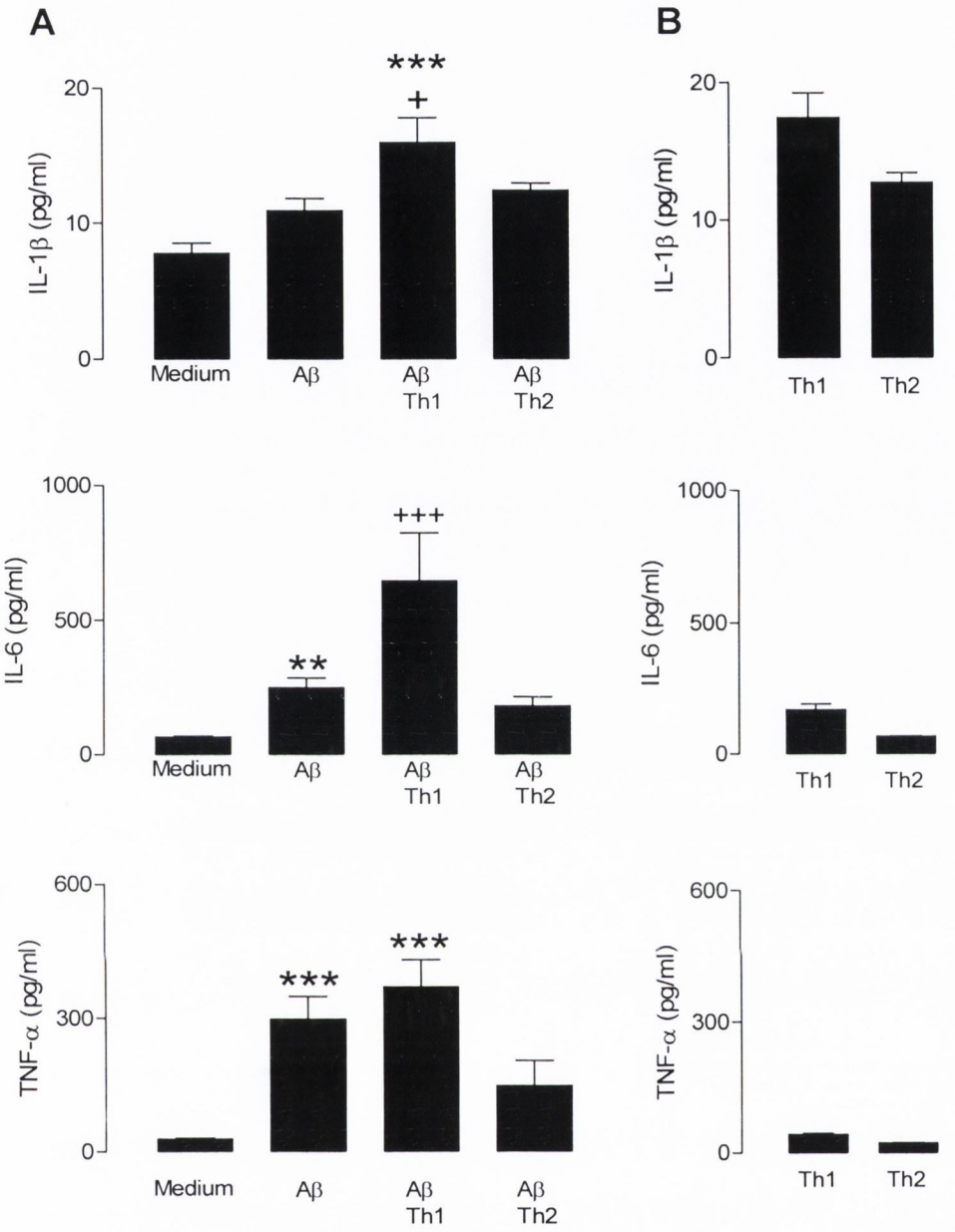


Figure 4.3. Co-culture of mixed glia and Aβ-specific Th1 cells enhances IL-1β and IL-6 production.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.1. Four days after stimulation with Aβ, IL-2 (5 ng/ml) was added. In addition, IL-4 and dexamethasone were added to Th2 polarised cells only. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. Eleven days after this second round of antigen stimulation, mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1 or Th2 cells. Concentrations of IL-1β, IL-6, and TNF-α in supernatants of glia treated with T cells (A), or in T cell supernatants in the absence of glia and Aβ (B), were measured by ELISA. ** $p<0.01$, *** $p<0.001$ versus medium-treated mixed glia; $^{+}$ $p<0.05$, $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=6).

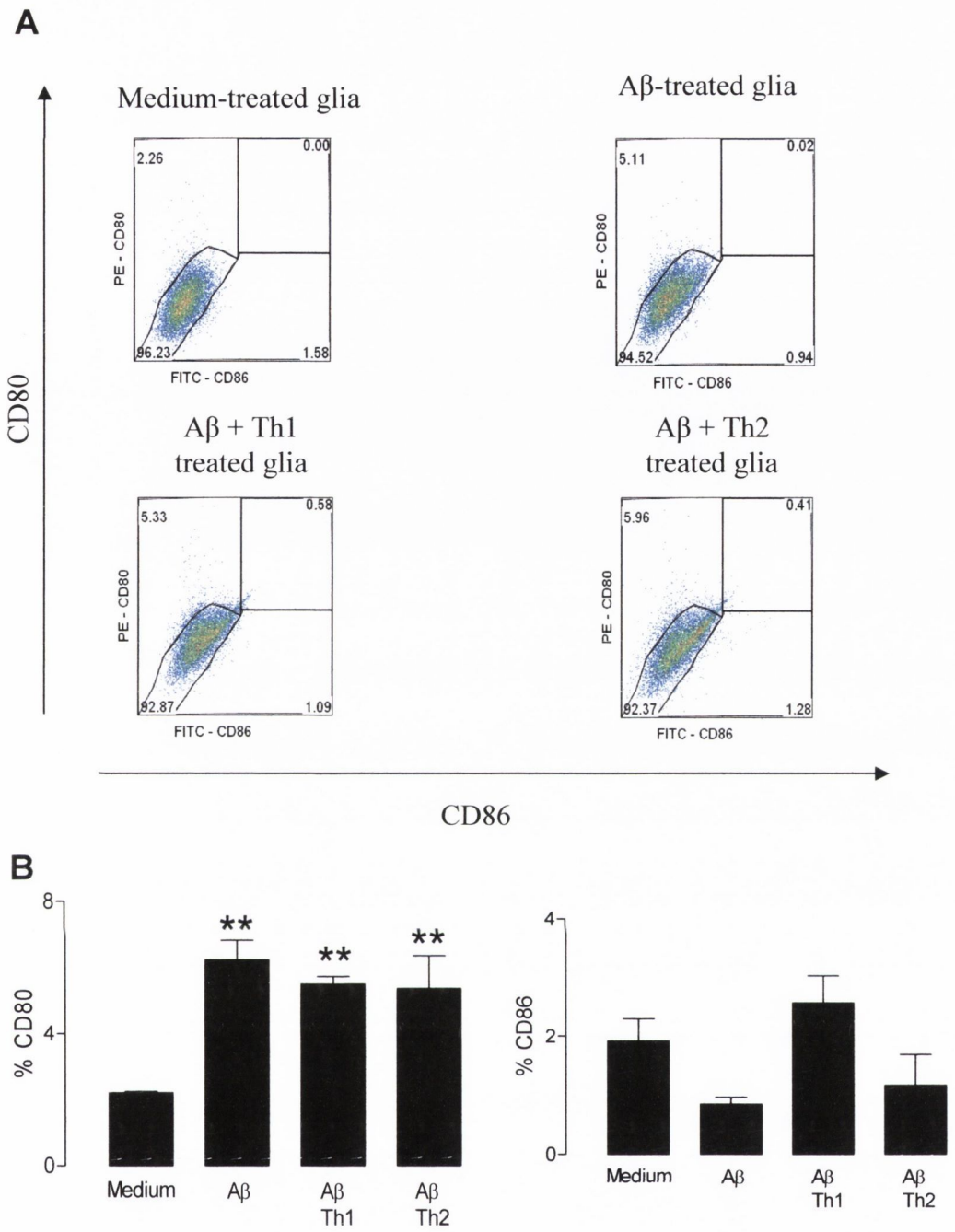


Figure 4.4. Aβ-specific Th1 and Th2 cells have no effect on CD80 or CD86 expression on microglia.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.1. Four days after stimulation with Aβ, IL-2 (5 ng/ml) was added. In addition, IL-4 and dexamethasone were added to Th2 polarised cells only. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. Eleven days after this second round of antigen stimulation, mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1 or Th2 cells for 24 h. (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells expressing CD80 or CD86 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b. ** $p<0.01$ versus medium-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=3).

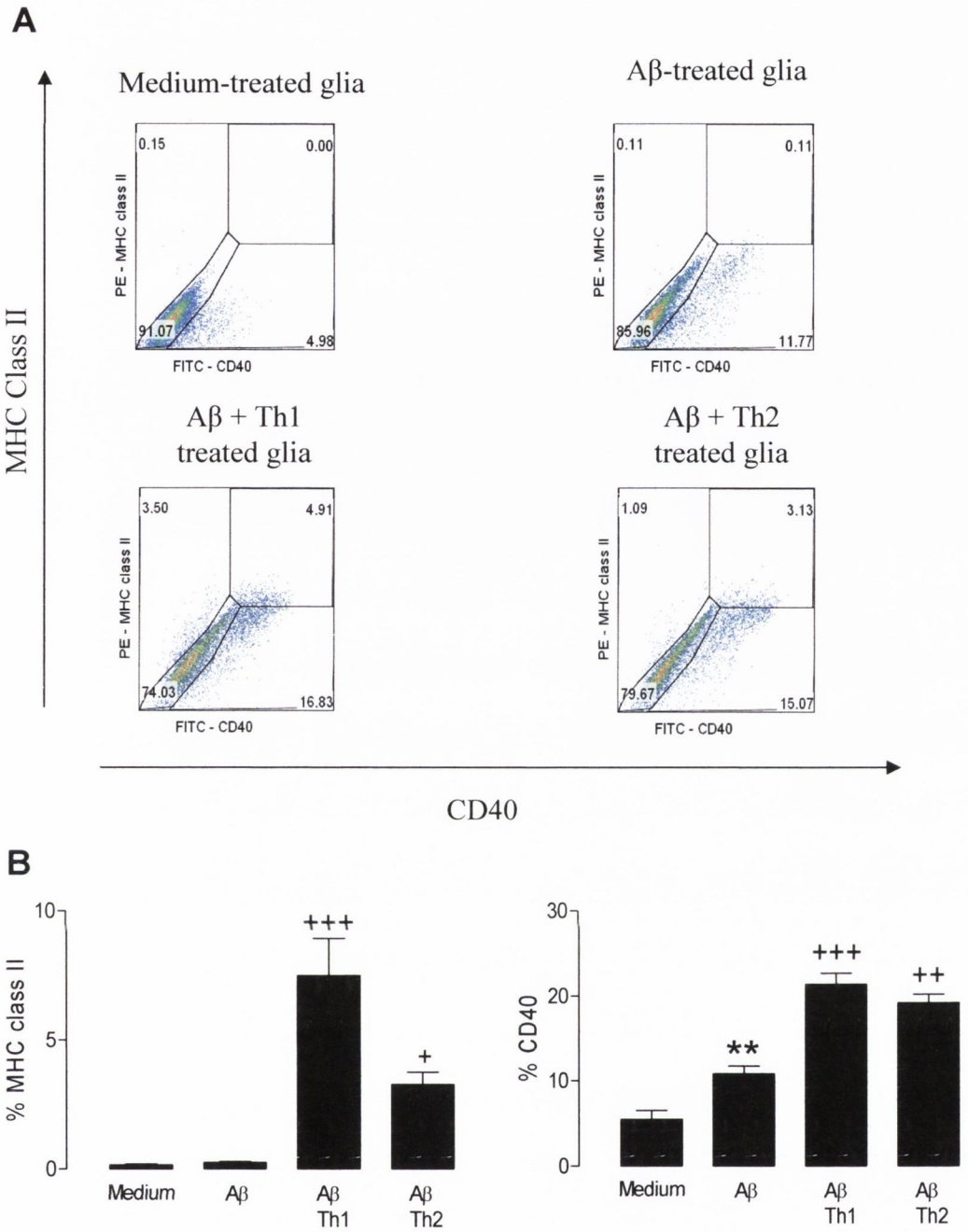


Figure 4.5. MHC class II and CD40 expression on microglia are enhanced by Aβ-specific Th1 or Th2 cells.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.1. Four days after stimulation with Aβ, IL-2 (5 ng/ml) was added. In addition, IL-4 and dexamethasone were added to Th2 polarised cells only. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. Eleven days after this second round of antigen stimulation, mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1 or Th2 cells for 24 h. (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells expressing MHC class II or CD40 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b. ** $p<0.01$ versus medium-treated mixed glia; $^+$ $p<0.05$ $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=3).

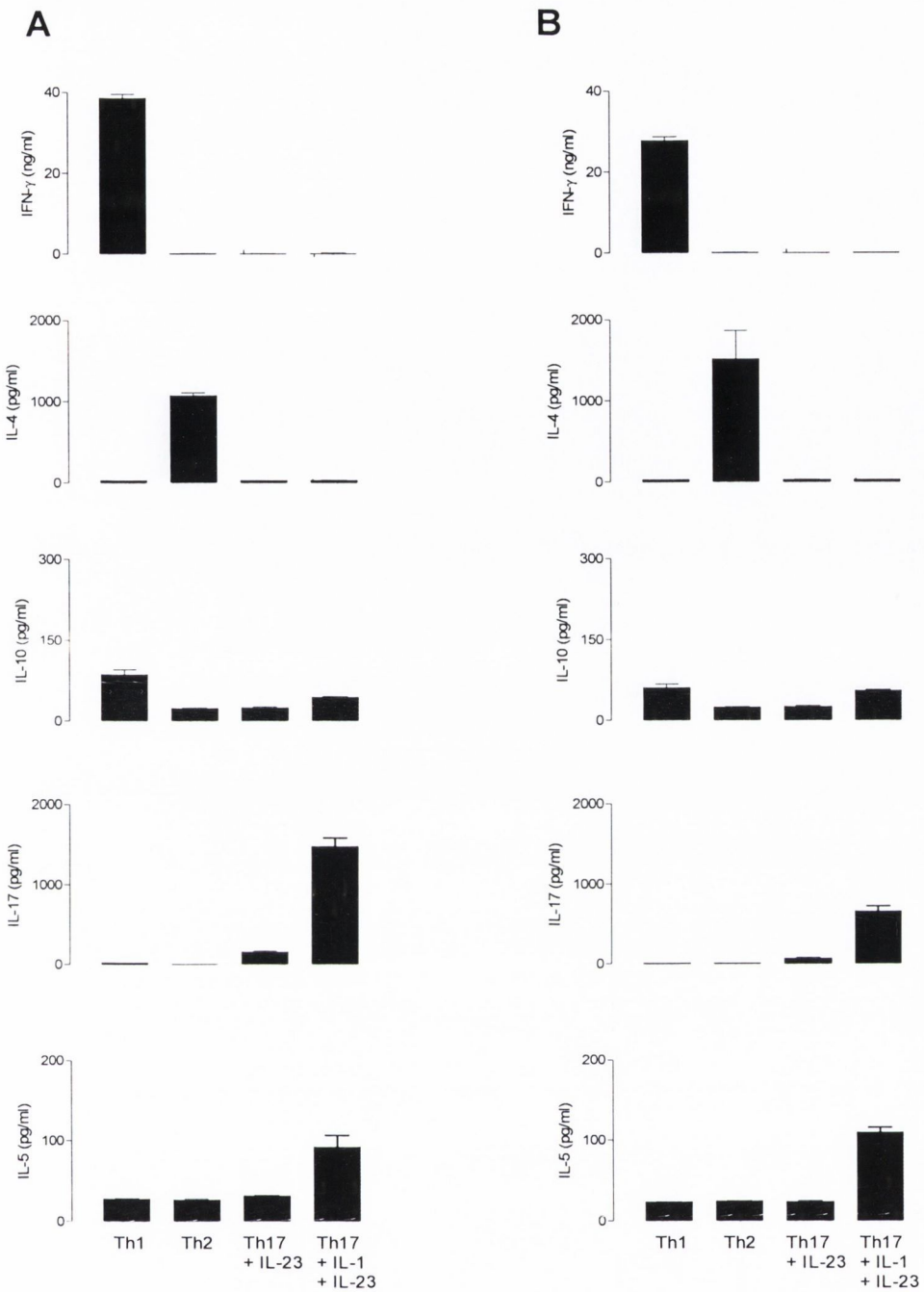


Figure 4.6. Cytokine profile of bulk T cell cultures of Aβ-specific Th1, Th2, and Th17 cells generated from mice immunised sub-cutaneously or intraperitoneally with Aβ and CpG.

C57BL/6 mice were injected intraperitoneally with Aβ (100 μg/mouse) and CpG (25 μg/mouse) (A), or subcutaneously in the footpad with Aβ (75 μg/mouse) and CpG (B). After 21 days, mice were injected again with Aβ and CpG. Seven days later, mice were sacrificed, spleens were harvested, and the cells were stimulated with Aβ (25 μg/ml) under Th1 polarising conditions with IL-12 (10 ng/ml), under Th2 polarising conditions with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml), or under Th17 polarising conditions with either IL-1β (10 ng/ml), IL-23 (10 ng/ml), and anti-IFN-γ (5 μg/ml), or IL-23 and anti-IFN-γ. On day 4 of culture the supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=3).

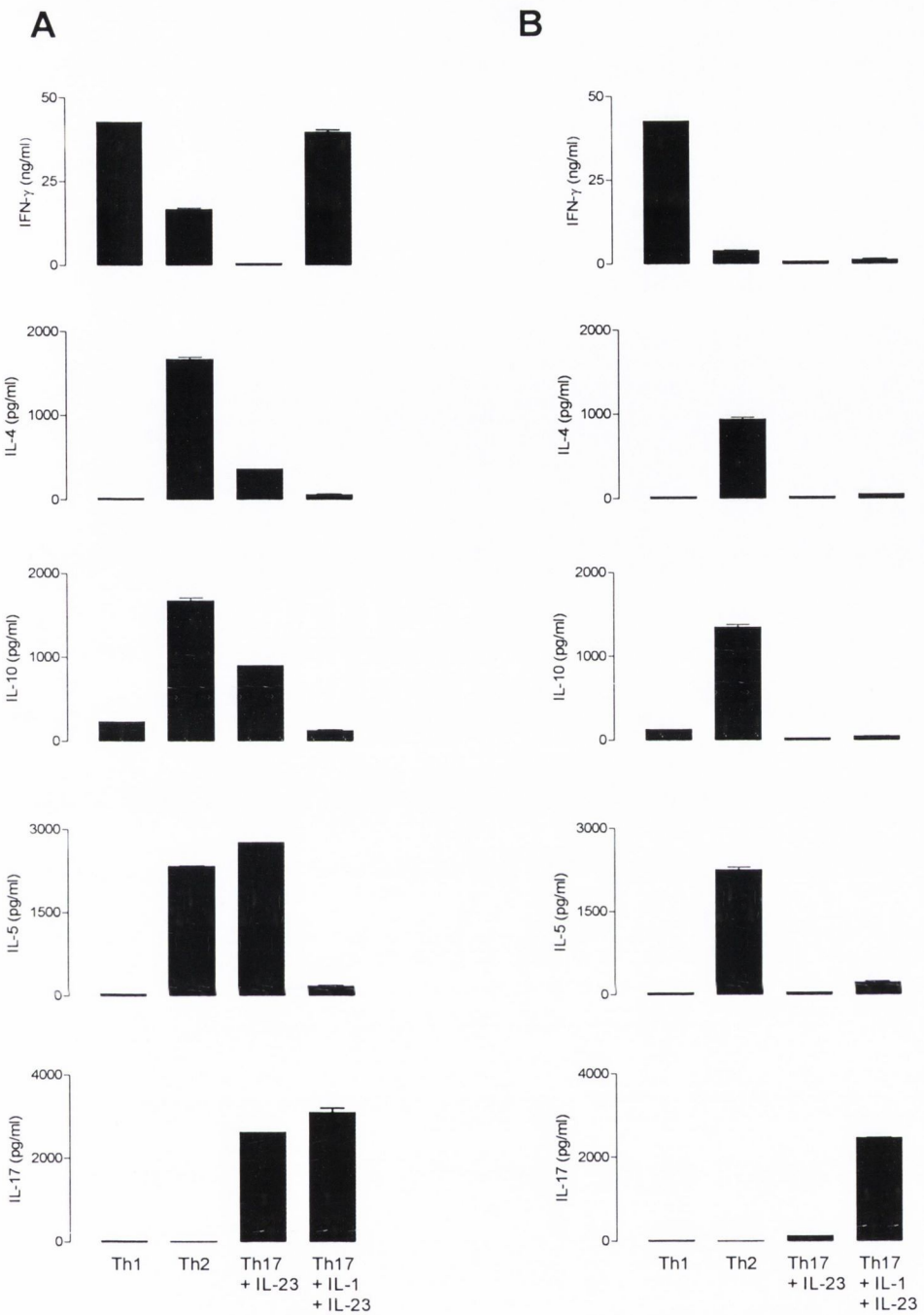


Figure 4.7. Cytokine profile of Aβ-specific T cell lines generated from mice immunised with Aβ + CpG after the second round of antigen stimulation.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.6. On day 4 of culture, Th1 and Th2, but not Th17 cell lines were stimulated with IL-2 (5 ng/ml). IL-4 and dexamethasone were added to Th2 polarised cells only. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. On day 4 of this second round of stimulation, the T cell supernatants were removed for analysis of concentrations of IFN-γ, IL-4, IL-10, and IL-17 in supernatants of mice immunised intraperitoneally (A) or subcutaneously in the footpad (B) were measured in triplicate by ELISA.

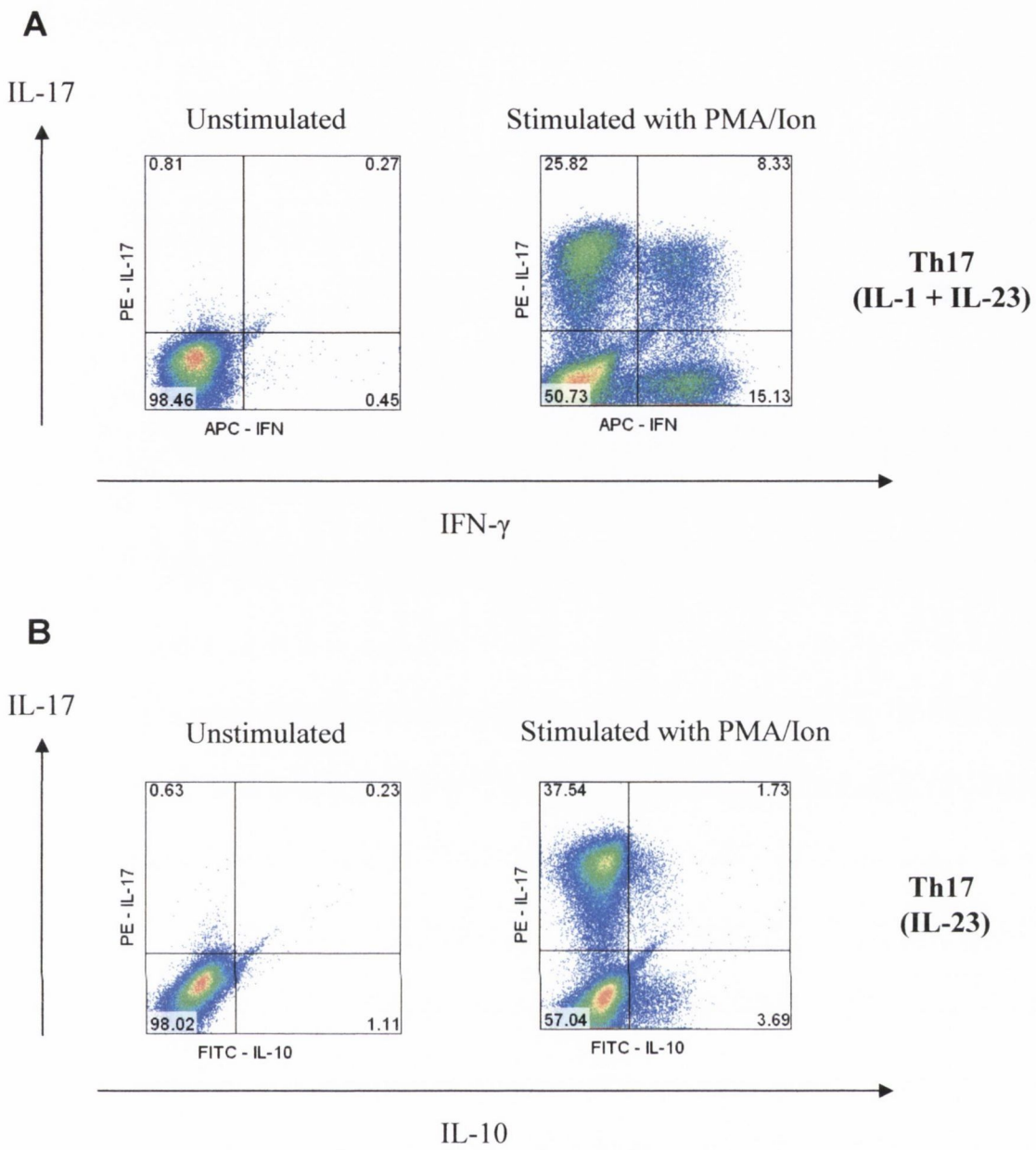


Figure 4.8. Intracellular cytokine staining of Aβ-specific Th17 cell lines.

C57BL/6 mice were injected intraperitoneally with Aβ (100 μg/mouse) and CpG (25 μg/mouse). After 21 days, mice were injected again with Aβ and CpG. A further 7 days later, mice were sacrificed, spleens were harvested, and the cells were stimulated with Aβ (25 μg/ml) under Th17 polarising conditions with either IL-1β (10 ng/ml), IL-23 (10 ng/ml), and anti-IFN-γ (5 μg/ml) (A), or IL-23 and anti-IFN-γ (B). After 11 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. After a further 10 days, T cell lines were stimulated with PMA (10 ng/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/ml) for 5 h. T cell lines were analysed for intracellular expression of IL-17, IFN-γ, and IL-10 on $CD4^+$ cells by flow cytometry.

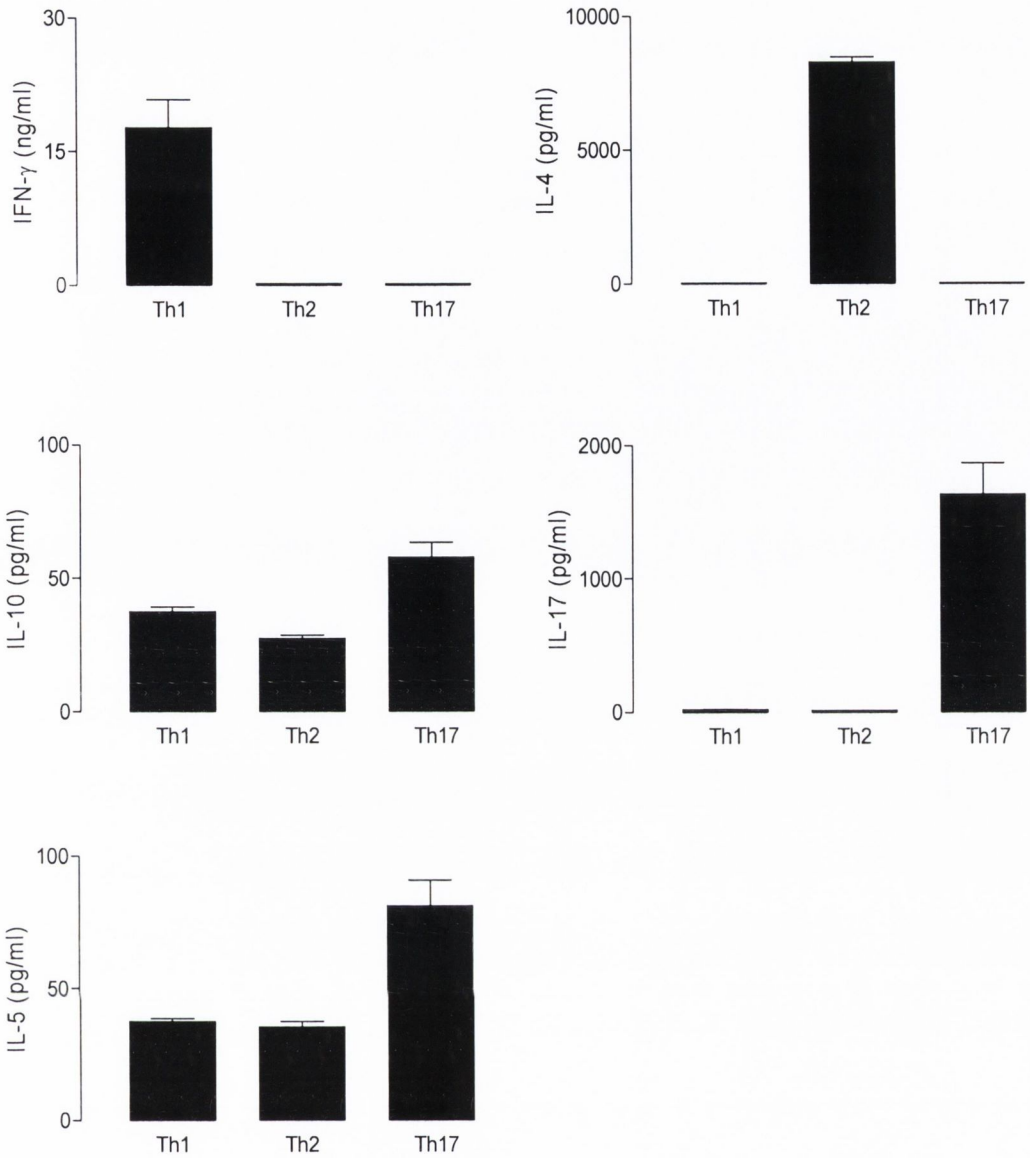


Figure 4.9. Generation of Aβ-specific Th1, Th2, and Th17 T cell lines.

C57BL/6 mice were injected subcutaneously in the footpad with Aβ (75 μg/mouse) and CpG (25 μg/mouse). After 10 days, mice were sacrificed, popliteal lymph nodes were harvested, and the cells were stimulated with Aβ (25 μg/ml) under Th1 polarising conditions with IL-12 (10 ng/ml), under Th2 polarising conditions with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml) or under Th17 polarising conditions with IL-1β, IL-23, and anti-IFN-γ. On day 4 of culture the supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=8).

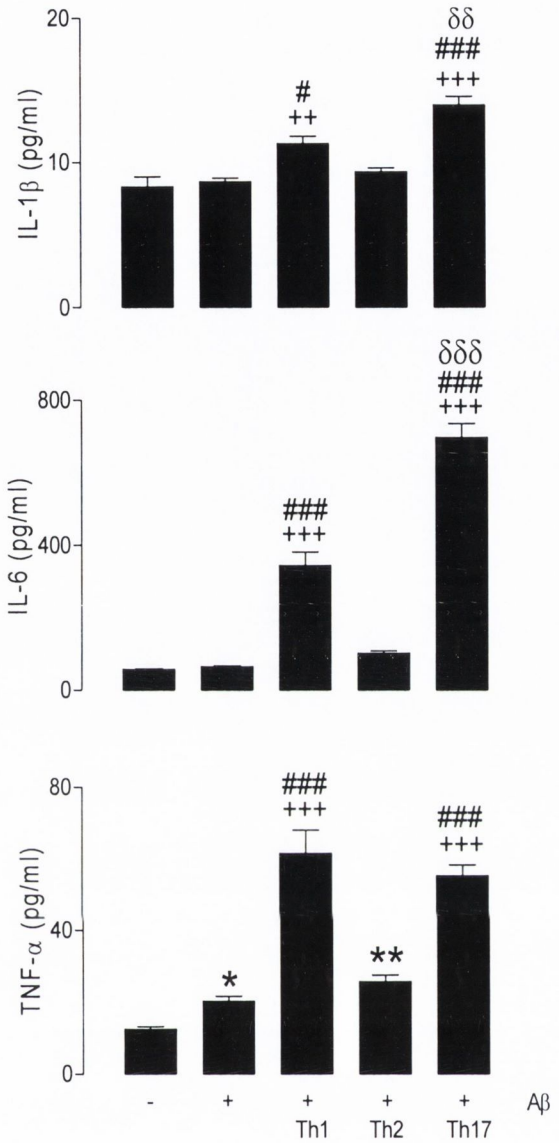


Figure 4.10. Co-culture of mixed glia and Aβ-specific Th17 cell lines enhances IL-1β, IL-6, and TNF-α production.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.9 Mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1, Th2, or Th17 cells. After 24 h, concentrations of IL-1β, IL-6, and TNF-α in supernatants were measured by ELISA. * $p<0.05$, ** $p<0.01$ versus medium-treated mixed glia; $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia; $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ versus Th2-treated mixed glia; $^{\delta\delta}$ $p<0.01$, $^{\delta\delta\delta}$ $p<0.001$ versus Th1-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=4).

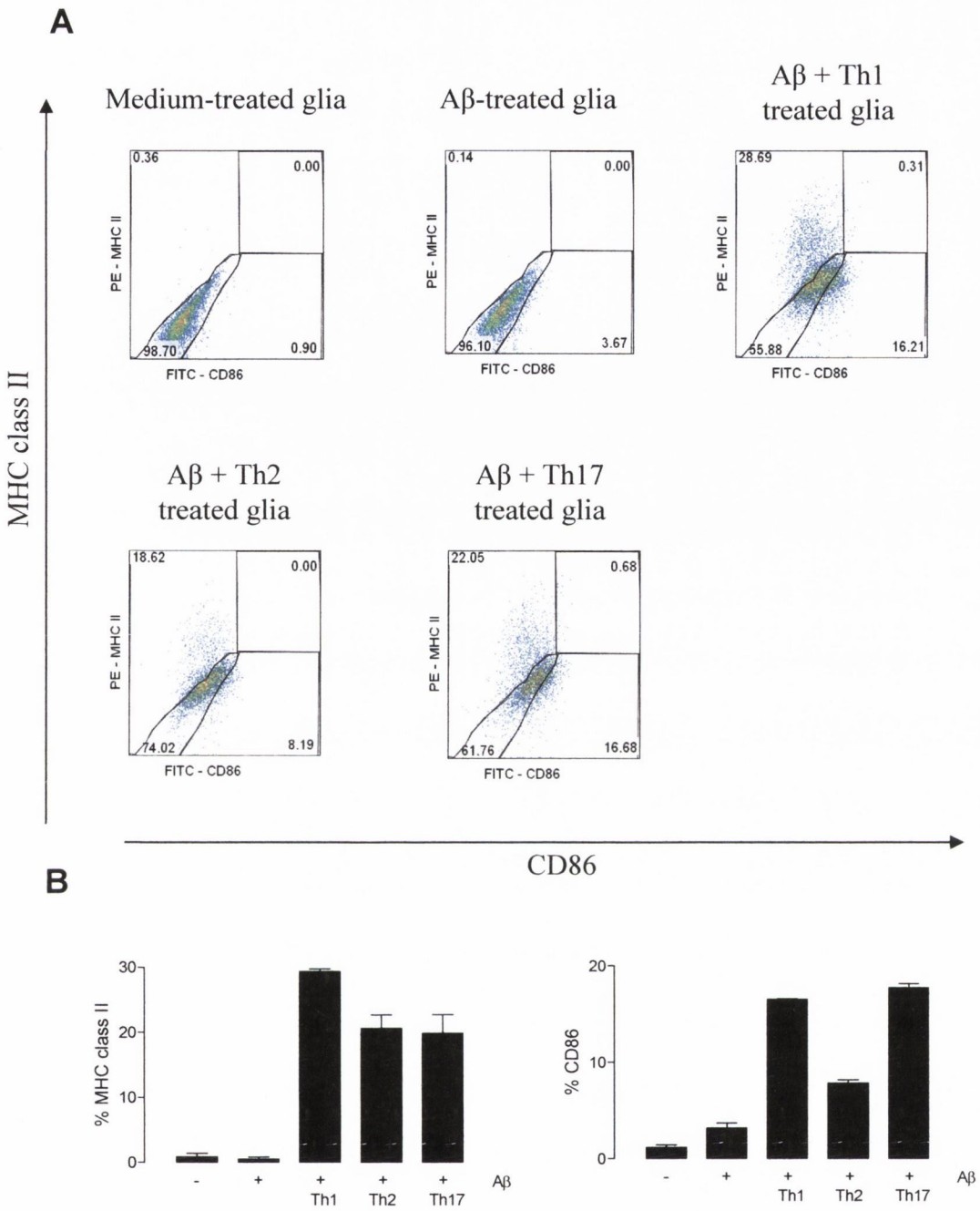


Figure 4.11. Co-culture of glia and Aβ-specific Th17 cells results in significant increase in MHC class II and CD86 expression on microglia.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.9. Mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1, Th2, or Th17 cells for 24 h. (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells expressing MHC class II or CD86 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b (n=2).

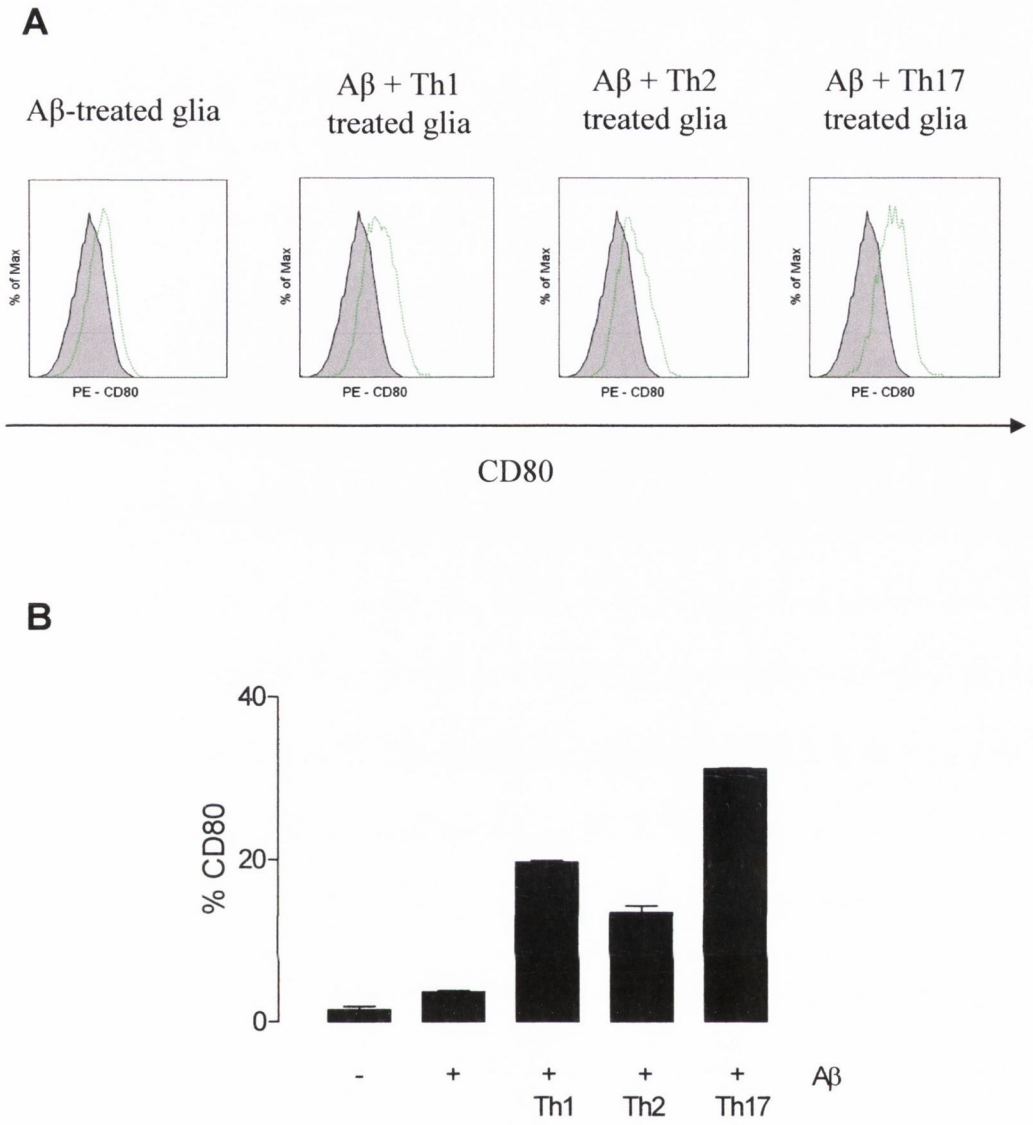


Figure 4.12. Aβ-specific Th17 cells enhance CD80 expression on Aβ-stimulated microglia.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.9. Mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1, Th2, or Th17 cells for 24 h. (A) Representative histograms are shown from each treatment group (grey histogram = untreated glia, green histogram = treated glia). (B) $CD11b^+$ cells expressing CD80 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b (n=2).

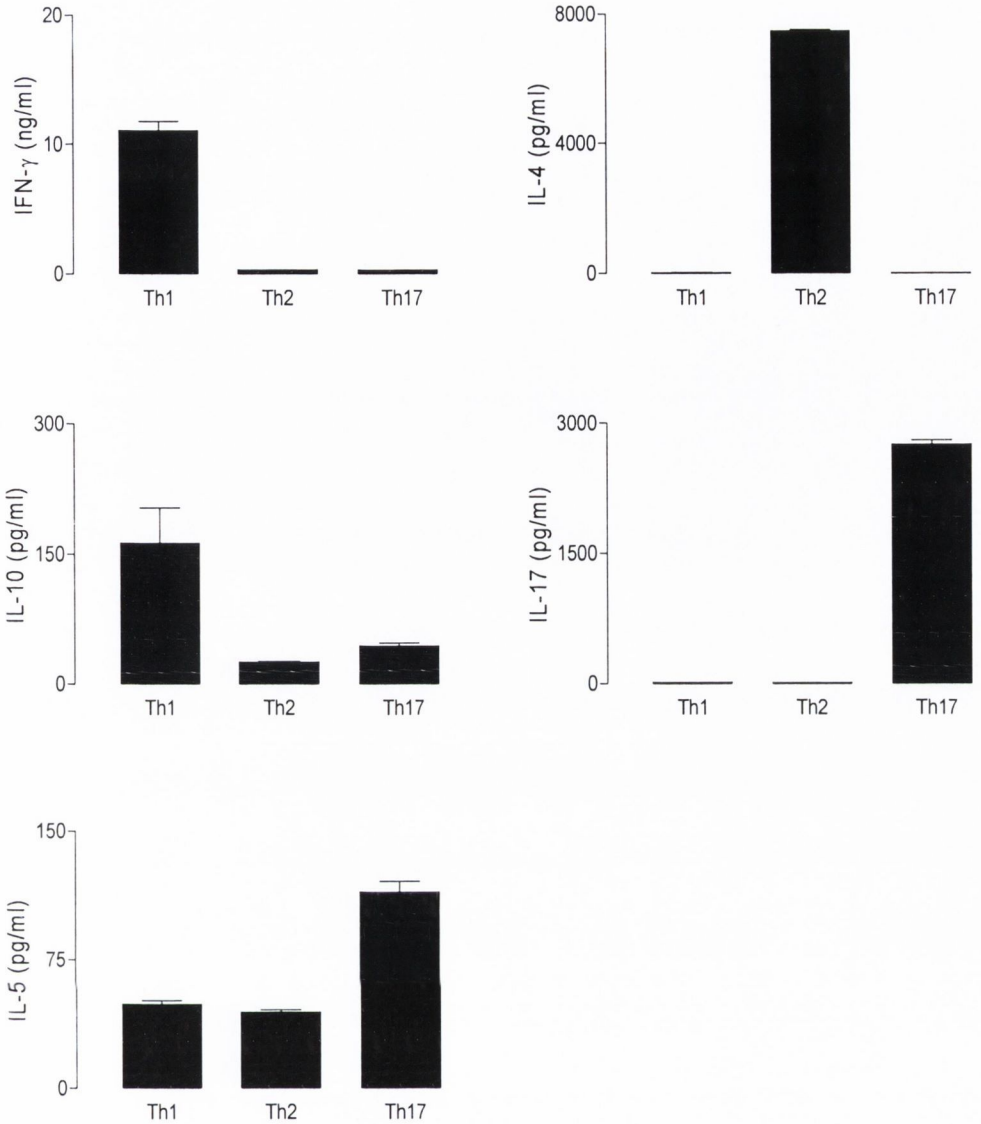


Figure 4.13. Cytokine profile of Th1, Th2, and Th17 polarised T cell lines for use in determining the effect of Th2 cells on Th1 and Th17 induced glial activation.

C57BL/6 mice were injected subcutaneously in the footpad with Aβ (75 μg/mouse) and CpG (25 μg/mouse). After 21 days, mice were injected again with Aβ and CpG. Seven days later, mice were sacrificed, popliteal lymph nodes were harvested, and the cells were stimulated with Aβ (25 μg/ml) under Th1 polarising conditions with IL-12 (10 ng/ml), under Th2 polarising conditions with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml), or under Th17 polarising conditions with IL-1β (10 ng/ml), IL-23 (10 ng/ml) and anti-IFN-γ (5 μg/ml). On day 4 of culture the supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=8).

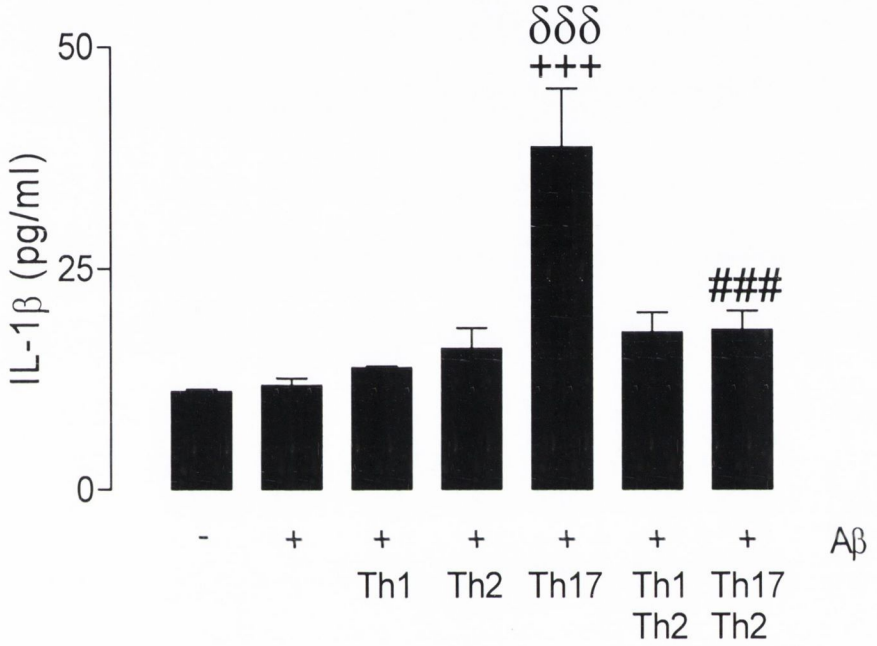


Figure 4.14. Aβ-specific Th2 cells attenuate IL-1β production induced by co-culture of glia and Aβ-specific Th17 cells.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.13. Mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1 or Th17 cells, in the presence or absence of Th2 cells. After 24 h, IL-1β production was assessed by ELISA. $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia; ; $^{\delta\delta\delta}$ $p<0.001$ versus Th1-treated mixed glia $^{\#\#\#}$ $p<0.001$ versus Th17-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=6).

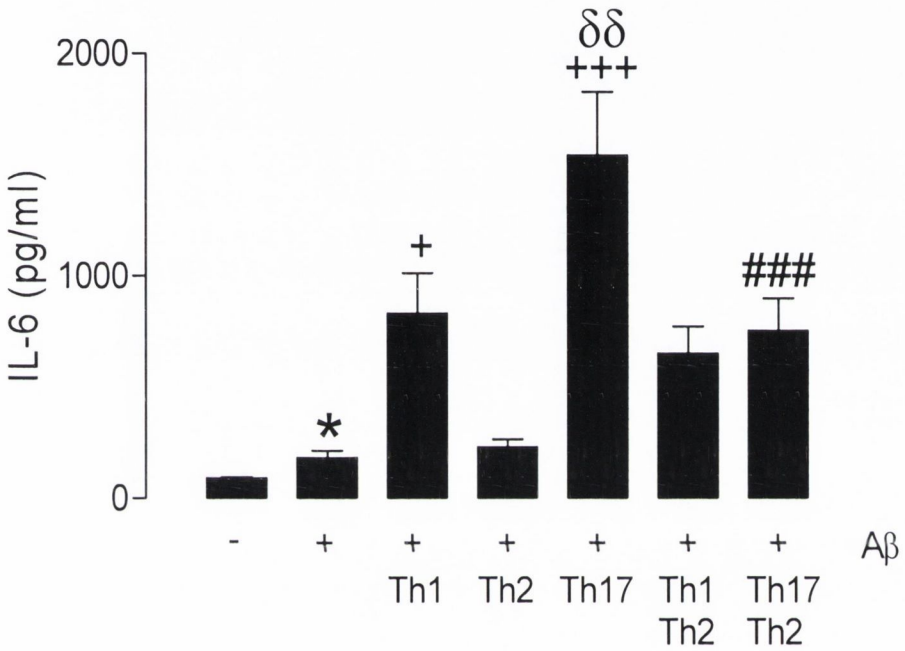


Figure 4.15. Aβ-specific Th2 cells attenuate IL-6 production induced by co-culture of glia and Aβ-specific Th17 cells.

C57BL/6 mice were immunised and T cells polarised as described in Figure 4.13. Mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml), or Aβ with polarised Th1 or Th17 cells, in the presence or absence of Th2 cells. After 24 h, IL-6 production was assessed by ELISA. * $p<0.05$ versus medium-treated mixed glia; $^{+}$ $p<0.05$, $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia; $^{\delta\delta}$ $p<0.01$ versus Th1-treated mixed glia; $^{\#\#\#}$ $p<0.001$ versus Th17-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=6).

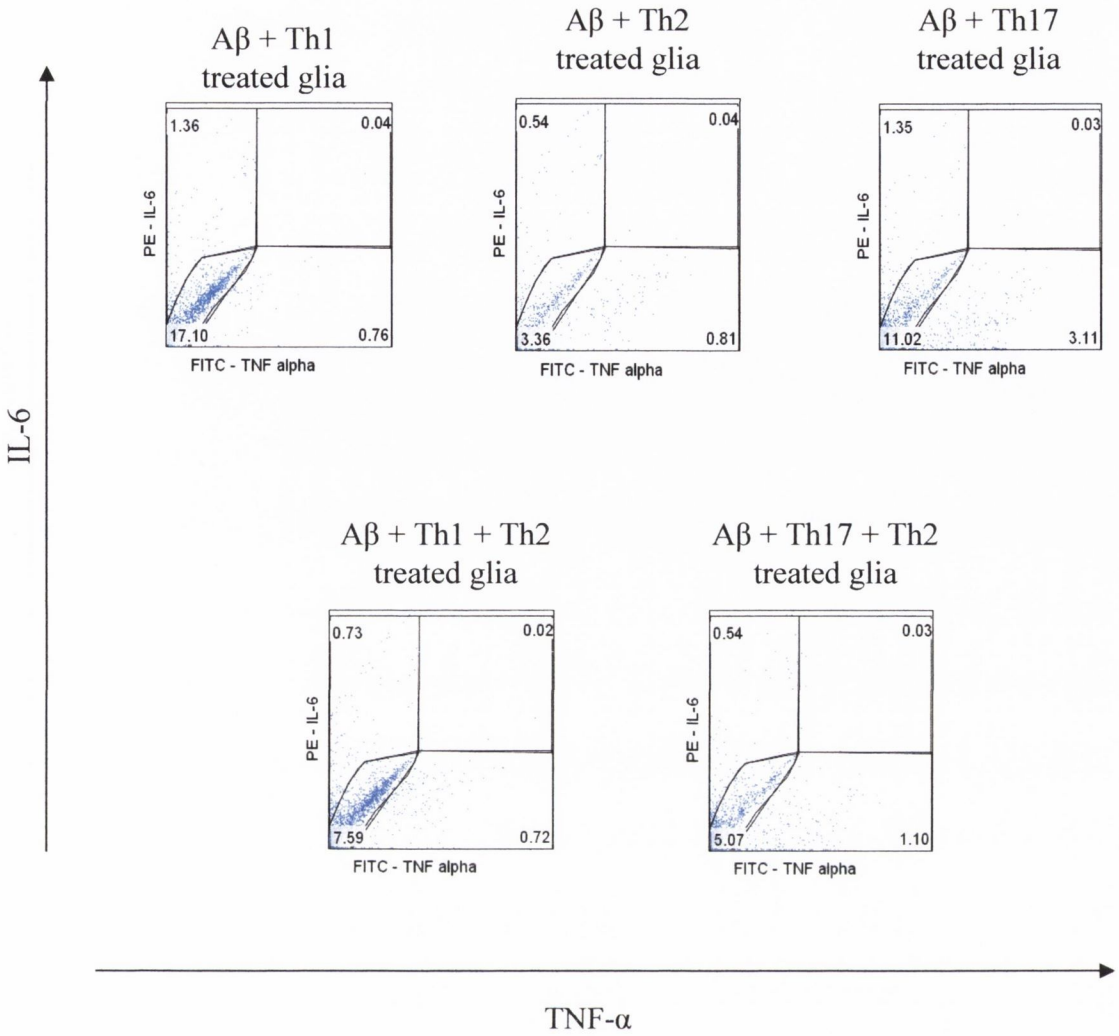
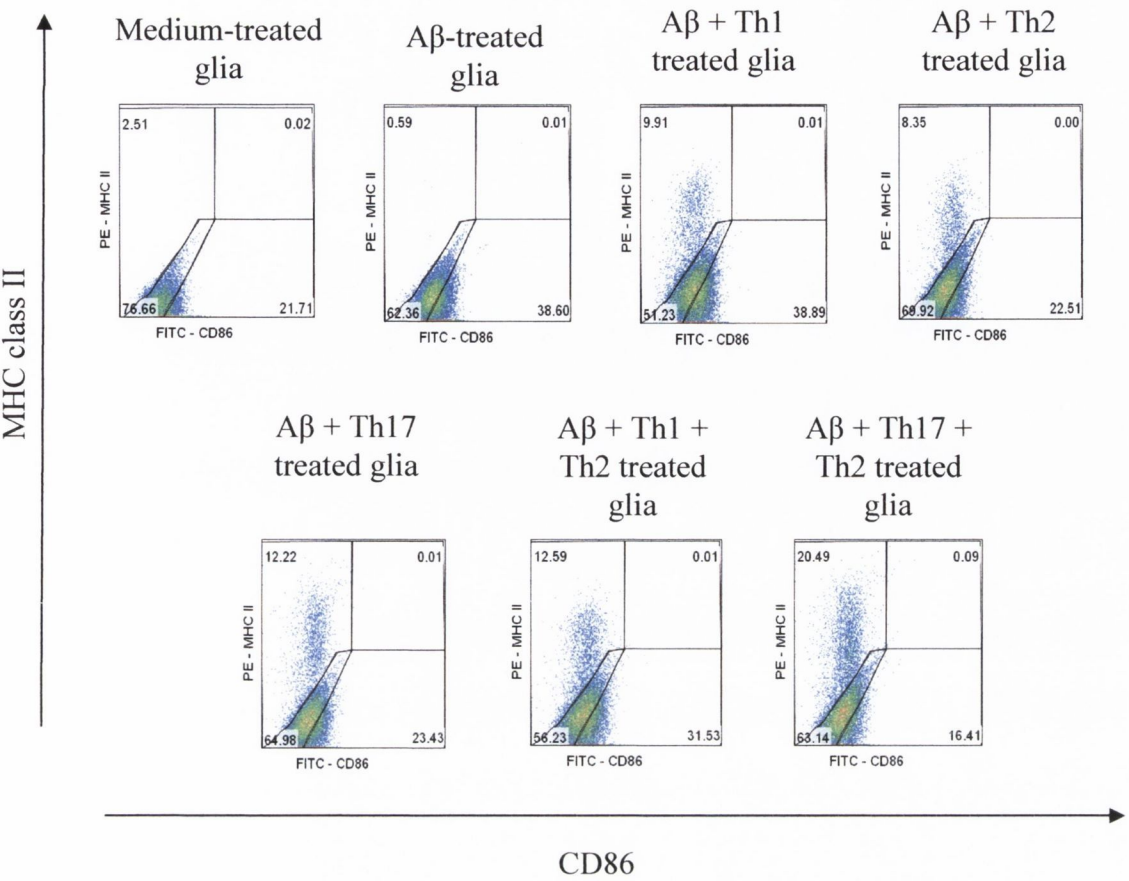


Figure 4.16. Aβ-specific T cells produce low amounts of IL-6 and TNF-α.

Aβ-specific Th1, Th2, and Th17 cells were co-cultured with mixed glial cells at a ratio of 0.5:1 for 24 h. Th1 and Th17 cells were co-cultured with mixed glial cells in the presence or absence of Th2 cells. After 8 h brefeldin A (5 μg/ml) was added to block cytokine secretion. After a further 12 h, the percentage of $CD4^+$ T cells expressing IL-6 and TNF-α was determined by flow cytometry.

A



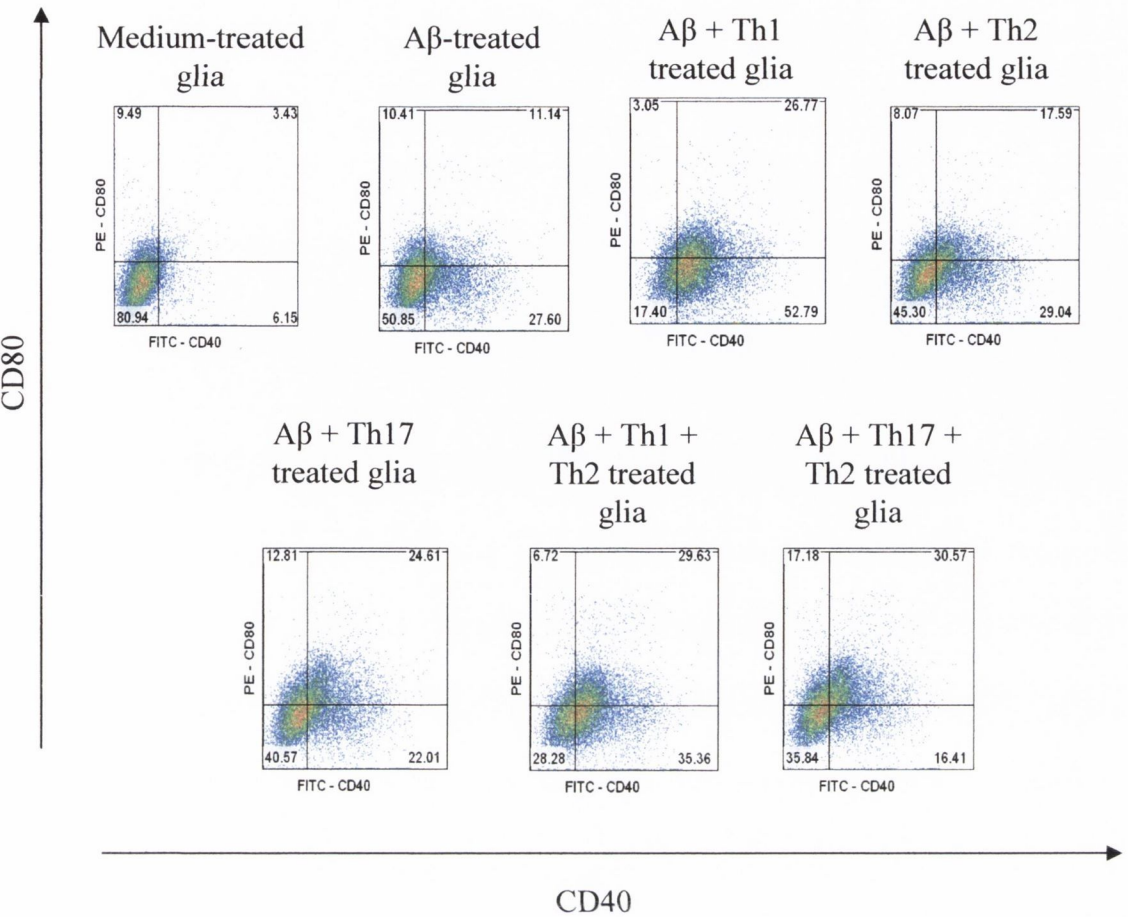
B

	Medium	Aβ	Aβ + Th1	Aβ + Th2	Aβ + Th17	Aβ + Th1 + Th2	Aβ + Th17 + Th2
MHC II	2.53	0.60	9.92	8.35	12.23	12.60	20.58
CD86	21.73	28.50	38.90	22.51	23.44	31.54	16.50

Figure 4.17. Aβ-specific Th2 cells attenuate Th1 and Th17 cell-induced upregulation of CD86 on microglia, while enhancing Th1 and Th17 cell-induced MHC class II expression.

Aβ-specific Th1, Th2, and Th17 cells were co-cultured with mixed glial cells at a ratio of 0.5:1 for 24 h. Th1 and Th17 cells were co-cultured with mixed glial cells in the presence or absence of Th2 cells. After 24 h, expression of MHC class II and CD86 on $CD11b^+$ cells was assessed by flow cytometry. (A) Dotplots of MHC class II vs CD86 expression are shown from each treatment group. (B) A summary of this flow cytometry data is presented in table form.

A



B

	Medium	Aβ	Aβ + Th1	Aβ + Th2	Aβ + Th17	Aβ + Th1 + Th2	Aβ + Th17 + Th2
CD80	12.92	21.55	29.82	25.66	37.42	36.35	47.75
CD40	9.58	38.74	79.56	46.63	46.62	64.99	46.98

Figure 4.18. Aβ-specific Th17 cells enhance Aβ-induced CD80 expression on microglia which is augmented by Th2 cells, whereas Th1 cells enhance Aβ-induced CD40 on microglia which is suppressed by Th2 cells.

Aβ-specific Th1, Th2, and Th17 cells were co-cultured with mixed glial cells at a ratio of 0.5:1 for 24 h. Th1 and Th17 cells were co-cultured with mixed glial cells in the presence or absence of Th2 cells. After 24 h, expression of CD80 and CD40 on $CD11b^+$ cells was assessed by flow cytometry. (A) Dotplots of CD80 vs CD40 expression are shown from each treatment group. (B) A summary of this flow cytometry data is presented in table form.

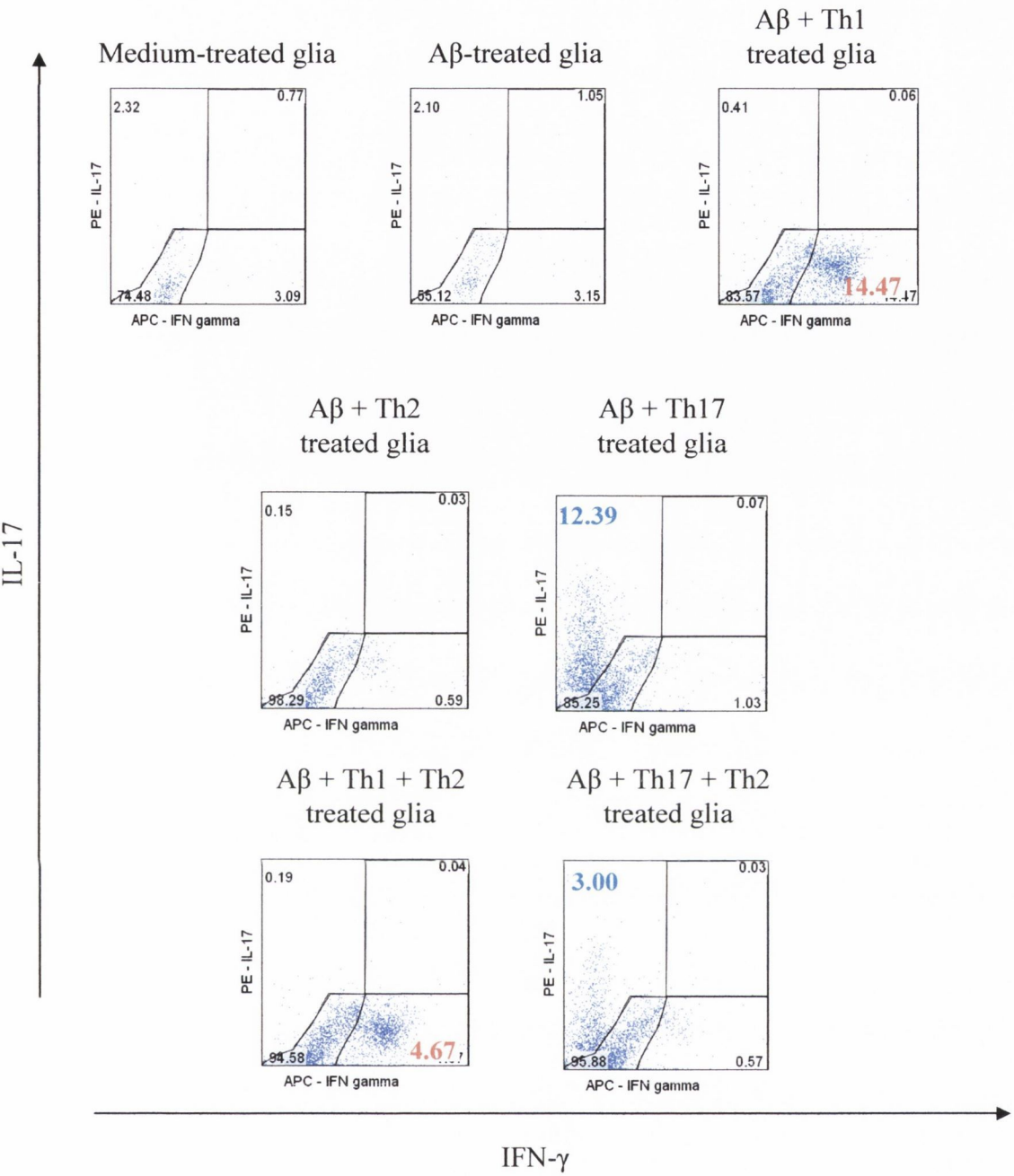


Figure 4.19. Glia act as an APC for Aβ-specific Th1 and Th17 cells, promoting IFN-γ and IL-17 production, which is inhibited by Th2 cells.

Aβ-specific Th1, Th2, and Th17 cells were co-cultured with mixed glial cells at a ratio of 0.5:1 for 24 h. Th1 and Th17 cells were co-cultured with mixed glial cells in the presence or absence of Th2 cells. After 8 h brefeldin A (5 μg/ml) was added to block cytokine secretion. After a further 12 h, the percentage of $CD4^+$ T cells expressing IFN-γ and IL-17 was determined by flow cytometry. Dotplots of IFN-γ vs IL-17 expression are shown from each treatment group.

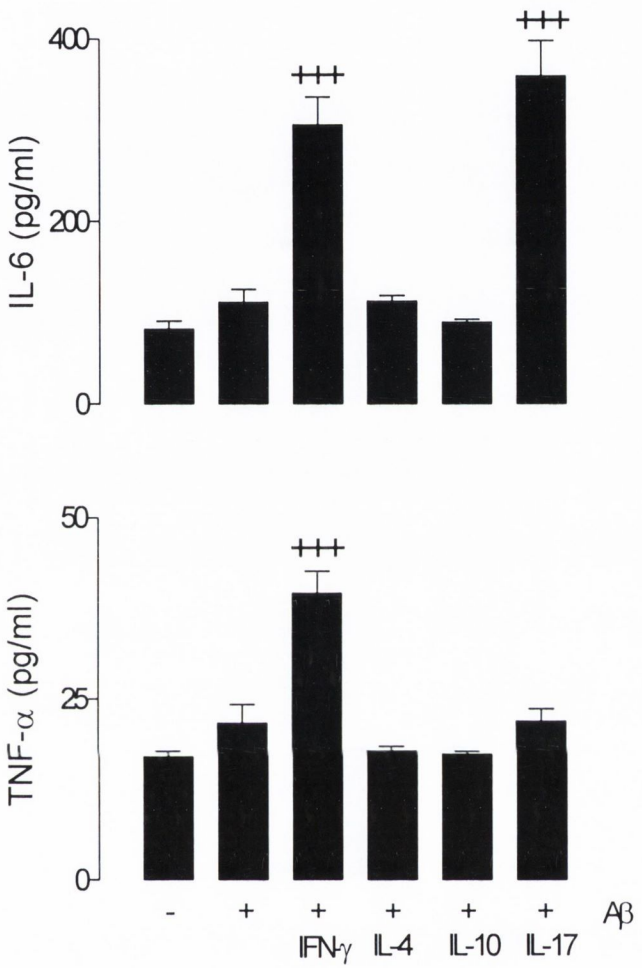


Figure 4.20. IFN-γ and IL-17 enhance Aβ-induced proinflammatory cytokine production from mixed glia.

Mixed glia from 0-1 day old C57BL/6 mice were treated with Aβ (40 μg/ml) in the presence or absence of IFN-γ (20 ng/ml), IL-4 (100 ng/ml), IL-10 (100 ng/ml), or IL-17 (20 ng/ml). After 24 h of culture, cytokine concentrations of IL-6 and TNF-α in supernatants were measured by ELISA. $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=5).

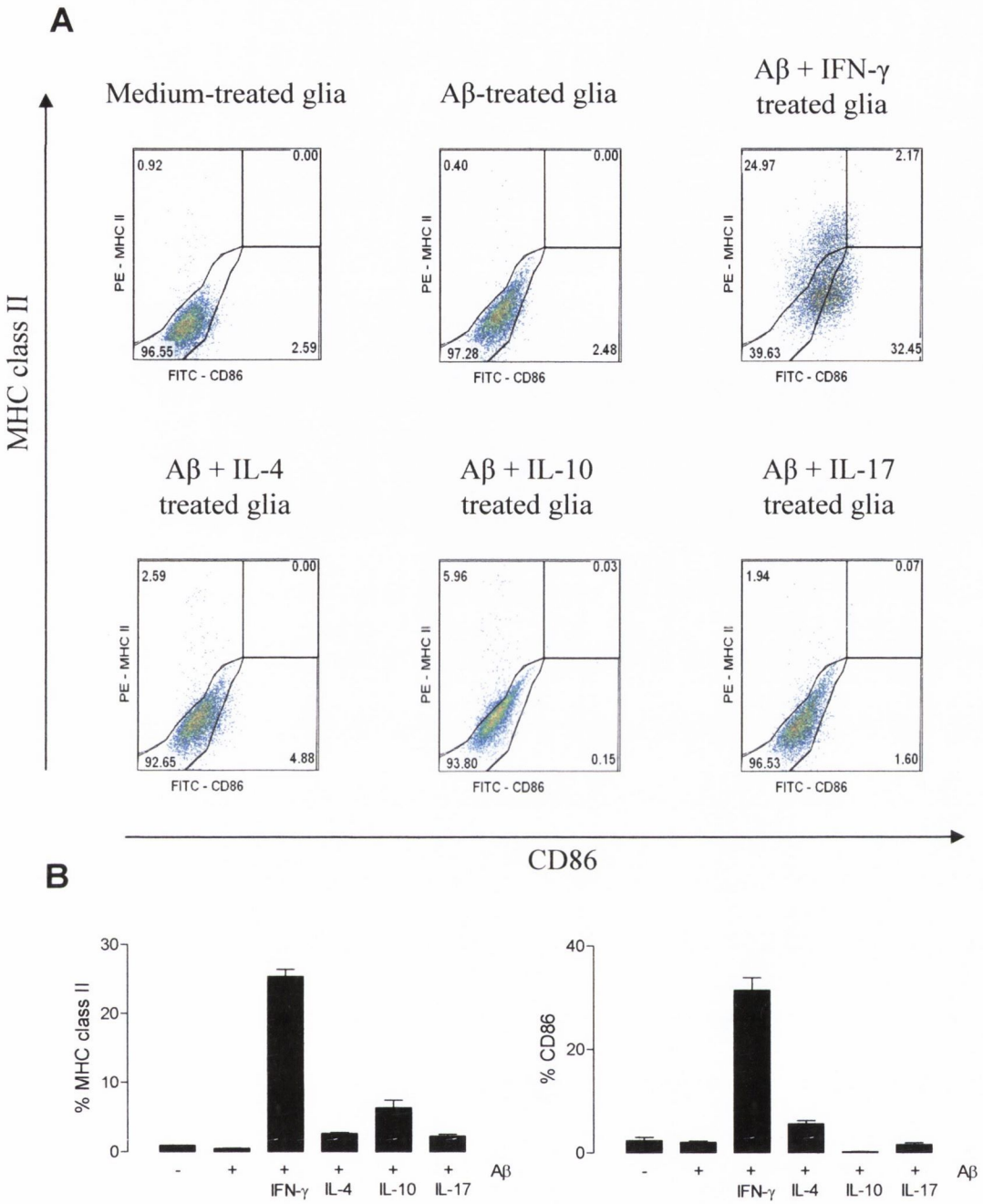


Figure 4.21. IFN-γ increases expression of MHC class II and CD86 on microglia.

Mixed glia from 0-1 day old C57BL/6 mice were treated for 24 h with Aβ (40 μg/ml) in the presence or absence of IFN-γ (20 ng/ml), IL-4 (100 ng/ml), IL-10 (100 ng/ml), or IL-17 (20 ng/ml). (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells expressing MHC class II or CD86 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b (n=2).

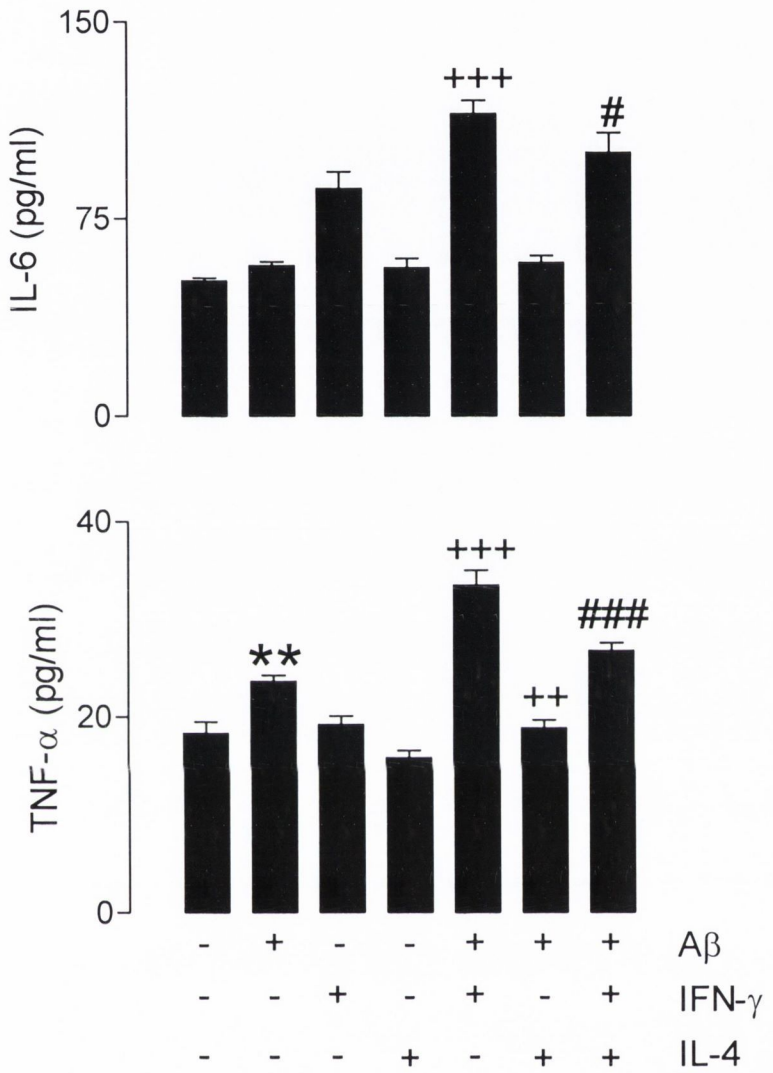


Figure 4.22. IL-4 attenuates Aβ + IFN-γ-induced production of IL-6 and TNF-α from mixed glia.

Mixed glia from 0-1 day old C57BL/6 mice were treated with Aβ (40 μg/ml), IFN-γ (10 ng/ml), or IL-4 (10 ng/ml) for 24 h. Cytokine concentrations of IL-6 and TNF-α in supernatants were measured by ELISA. ** $p<0.01$ versus medium-treated mixed glia; ++ $p<0.01$, +++ $p<0.001$ versus Aβ-treated mixed glia; # $p<0.05$, ### $p<0.001$ versus Aβ + IFN-γ-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=5).

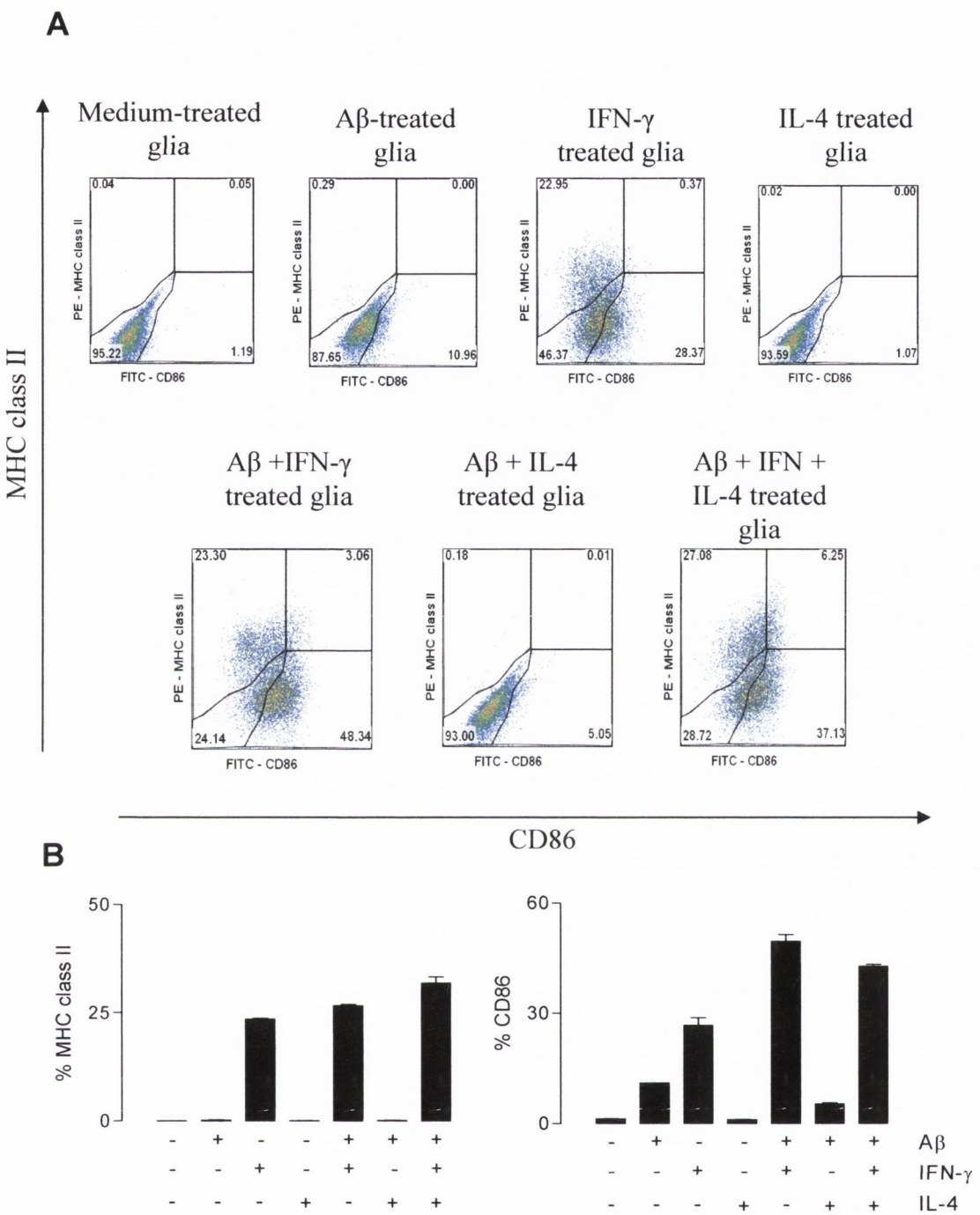


Figure 4.23. IL-4 attenuates Aβ-induced expression of CD86 on microglia.

Mixed glia from 0-1 day old C57BL/6 mice were treated for 24 h with Aβ (40 μg/ml), IFN-γ (10 ng/ml), or IL-4 (10 ng/ml). (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells expressing MHC class II or CD86 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b (n=2).

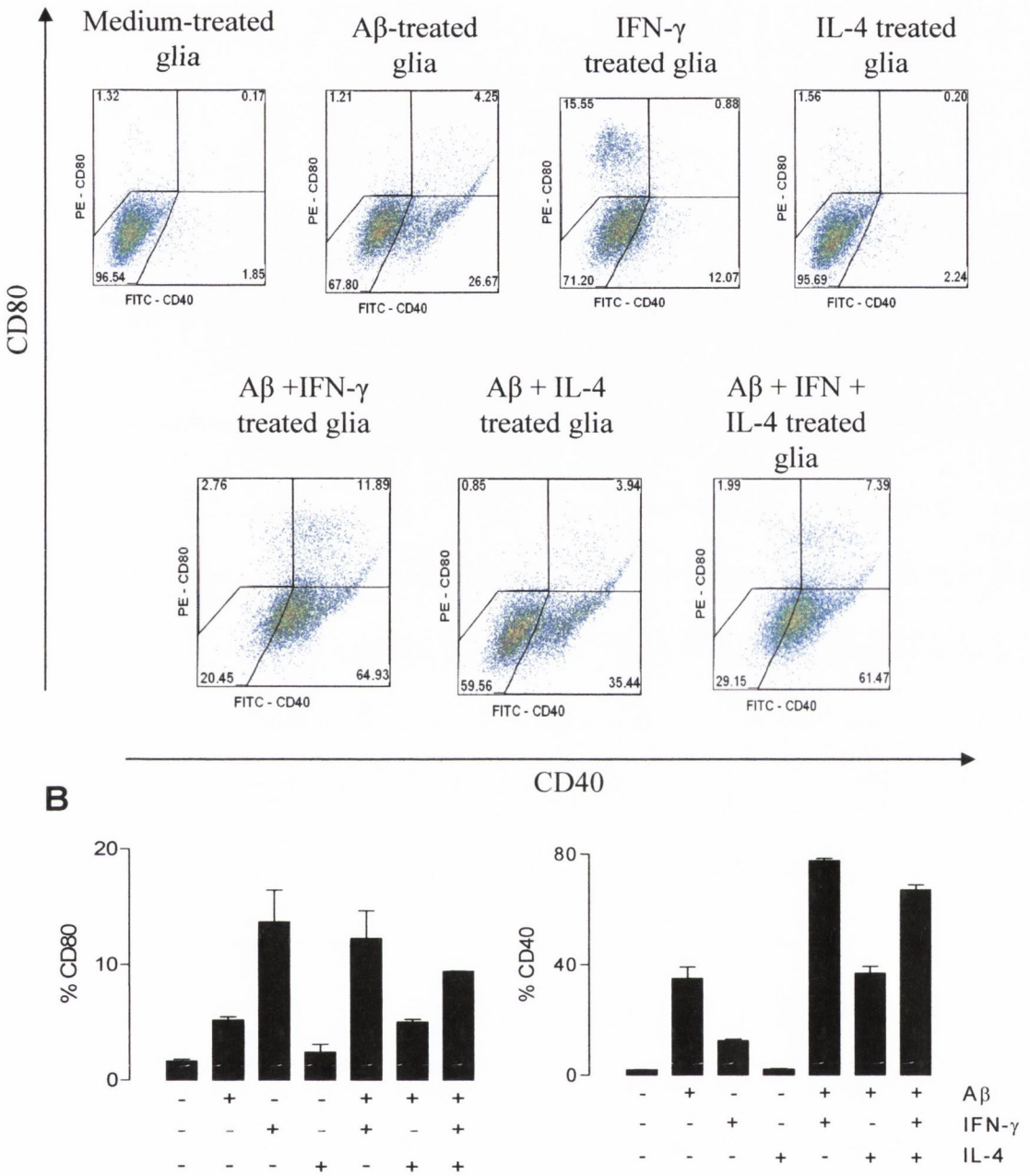


Figure 4.24. IL-4 attenuates Aβ + IFN-γ-induced expression of CD40 on microglia.

Mixed glia from 0-1 day old C57BL/6 mice were treated for 24 h with Aβ (40 μg/ml), IFN-γ (10 ng/ml), or IL-4 (10 ng/ml). (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells CD80 or CD40 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b (n=2).

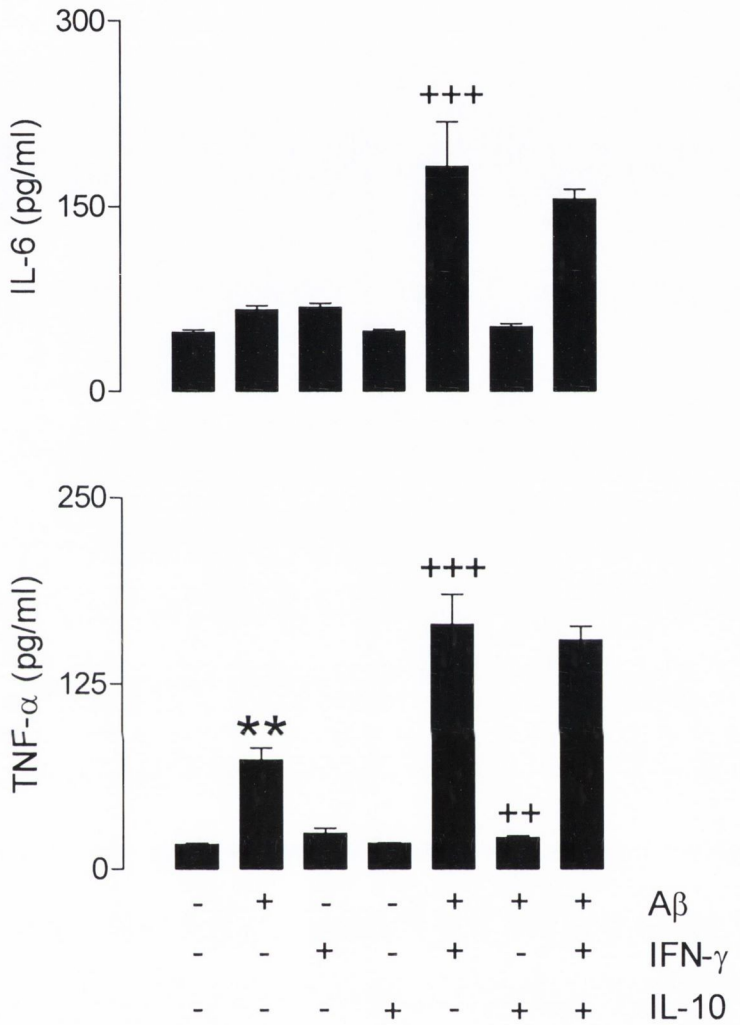


Figure 4.25. IL-10 attenuates Aβ-induced production of TNF-α from mixed glia.

Mixed glia from 0-1 day old C57BL/6 mice were treated with Aβ (40 μg/ml), IFN-γ (10 ng/ml), or IL-10 (10 ng/ml) for 24 h. Cytokine concentrations of IL-6 and TNF-α in supernatants were measured by ELISA. ** $p<0.01$ versus medium-treated mixed glia; $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$ versus Aβ-treated mixed glia (one-way ANOVA). Values are expressed as means ± SEM (n=5).

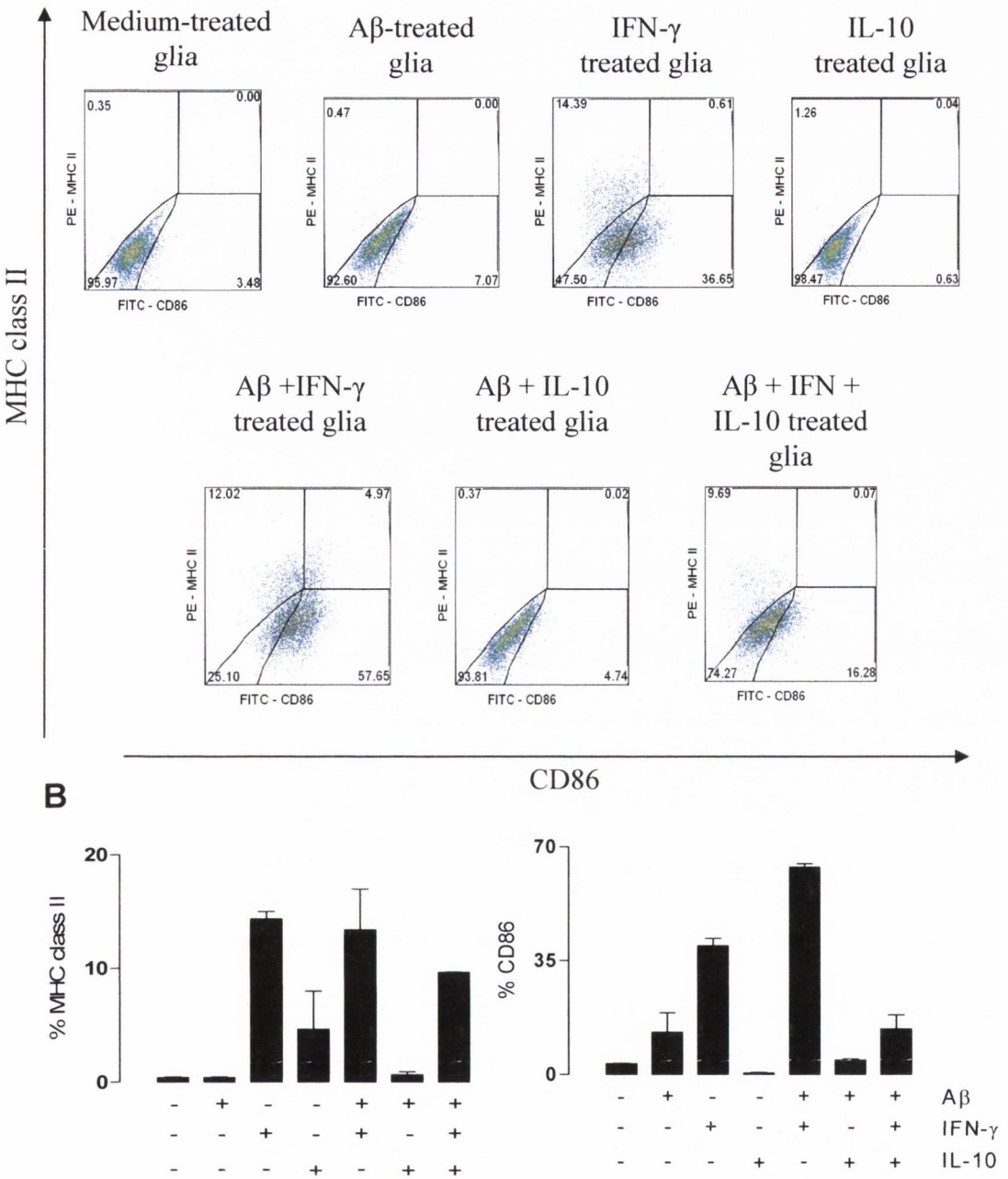


Figure 4.26. IL-10 attenuates Aβ + IFN-γ-induced CD86 expression on microglia.

Mixed glia from 0-1 day old C57BL/6 mice were treated for 24 h with Aβ (40 μg/ml), IFN-γ (10 ng/ml), or IL-10 (10 ng/ml). (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells expressing MHC class II or CD86 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b (n=2).

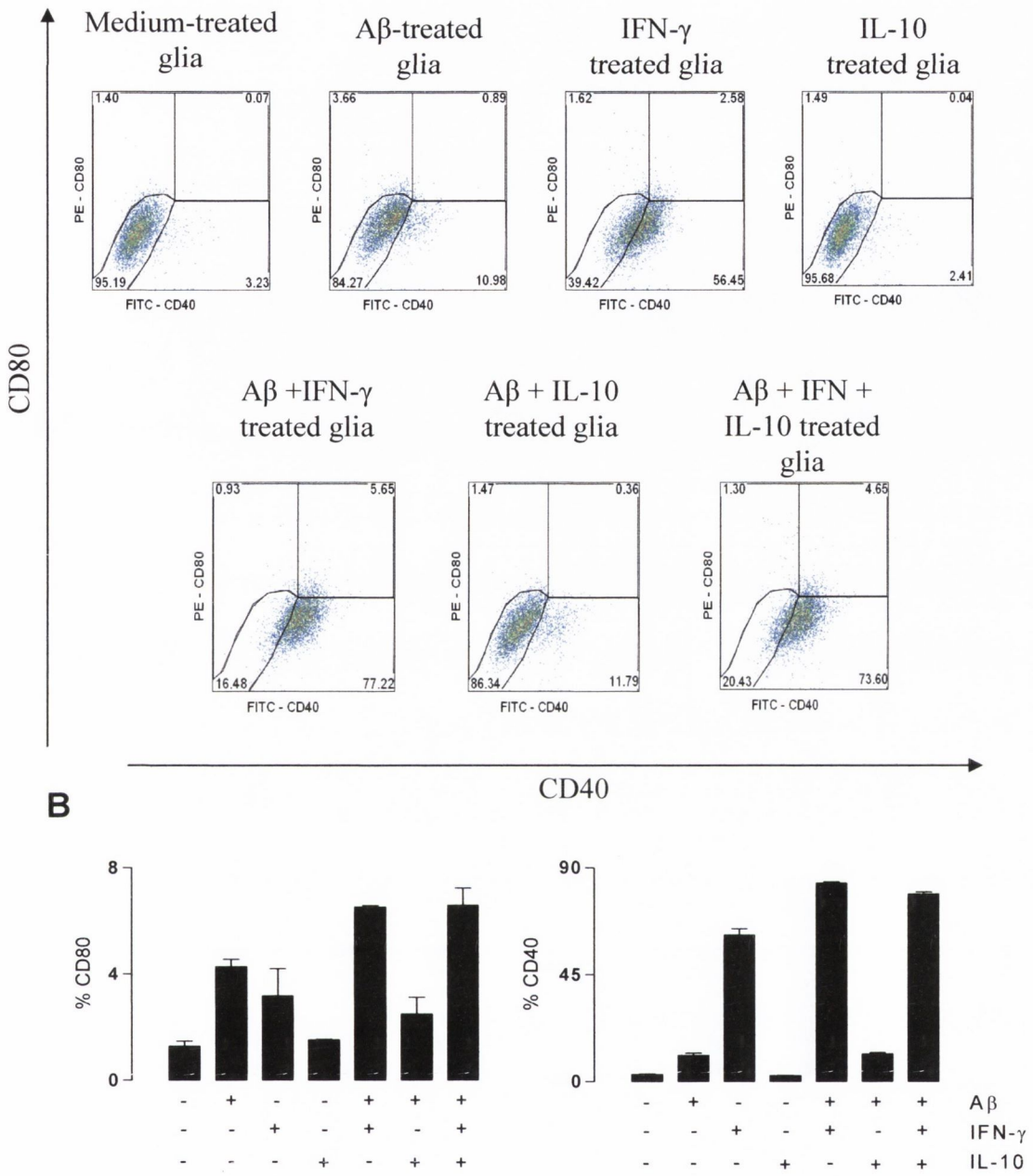


Figure 4.27. IL-10 attenuates Aβ-induced CD80 expression on microglia.

Mixed glia from 0-1 day old C57BL/6 mice were treated for 24 h with Aβ (40 μg/ml), IFN-γ (10 ng/ml), or IL-10 (10 ng/ml). (A) Representative dotplots are shown from each treatment group. (B) $CD11b^+$ cells expressing CD80 or CD40 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD11b (n=2).

Chapter 5

Aβ-specific T cells modulate memory and plaque deposition in a transgenic model of AD

5.1. Introduction

The amyloid cascade hypothesis was first proposed by Hardy and Allsop (1991), and states that an imbalance between the production and clearance of Aβ is the initiating event in the pathogenesis of AD. This imbalance leads to an increase in the level of Aβ, in particular $A\beta_{42}$, in the brain. Upon accumulation in the brain, soluble $A\beta_{42}$ is thought to undergo a conformational change to a high β-sheet content, giving it a propensity to aggregate into oligomers and fibrils in plaques. The presence of Aβ oligomers and plaques in the brain results in the activation of microglia and astrocytes, leading to inflammation, and ultimately, to neuronal death (Selkoe 2001). Following the identification of Aβ as the principal component of neuritic plaques in AD (Glenner & Wong 1984b), a number of observations have been made that lend weight to the idea that deposition of Aβ is the primary neuropathological insult in AD. There are 4 genes currently known to be involved in the development of AD: APP, PS1, PS2, and ApoE4. Mutations in APP, PS1, and PS2 have all been shown to lead to increased production of Aβ (Citron *et al.* 1997, Spasic *et al.* 2006, Selkoe 2001), while patients with 1 or 2 alleles of ApoE4 have increased Aβ deposition in the brain (Schmechel *et al.* 1993). Aggregated Aβ has been shown to be neurotoxic *in vitro*, and reversal of aggregation can prevent this toxicity (Pike *et al.* 1993, Goodman & Mattson 1994). It has been suggested that the low oligomeric forms of Aβ are the most toxic to neurons, and i.c.v. injection of conditioned medium containing Aβ oligomers inhibited LTP in rats *in vivo* (Walsh *et al.* 2002).

The advent of transgenic mouse models of AD has revolutionised our understanding of the disease process. A number of transgenic mouse models have been developed, and the majority of these are based on the overexpression of different forms of genes that are associated with the development of AD, in particular APP and PS1. Overexpression of mutant forms of APP in the brains of mice has been shown to reproduce a number of the features of AD, such as age-dependent Aβ plaque formation, microglia and astrocyte activation, and it can also induce impairments in certain aspects of cognitive function (Eriksen & Janus 2007, Morgan *et al.* 2000, Hsiao *et al.* 1996). Transgenic mice overexpressing mutant PS1 were found to have increased levels of $A\beta_{42}$ in the brain, though this did not lead to plaque formation (Duff *et al.* 1996). APP-transgenic mice are extremely useful in the study of AD, but are limited by the fact that Aβ plaques are not detectable until mice are over 1 year old (Kawarabayashi *et al.* 2001), resulting in increased mortality, and making administration of certain behavioural tests difficult. Overexpression of APP and PS1, such as in the PSAPP mouse, is associated with accelerated development of Aβ pathology, with plaques detectable as early as 3 months of age. Microgliosis and astrogliosis are evident at an early age, and this facilitates studies that would be difficult in the older, and thus more fragile single-transgenic animals. The APPswe/PS1dE9 mouse is a recently developed double-transgenic mouse co-overexpressing APP with the Swedish mutation and exon-9-deleted PS1 (Jankowsky *et al.* 2001). These mice have an increased $A\beta_{42}$:$A\beta_{40}$ ratio when compared with other transgenic mice and have robust plaque deposition by 6 months of age (Garcia-Alloza *et al.* 2006), making them an attractive model for studies on therapeutic intervention. They have also been shown to have impaired spatial and episodic-like memory (Savonenko *et al.* 2005, Jankowsky *et al.* 2005, Cao *et al.* 2007).

One therapeutic strategy that is currently the focus of much attention is Aβ immunotherapy. Active immunotherapy of AD was the first treatment approach to have a major effect on plaque burden and cognitive impairments in APP-

transgenic mice. Active immunisation with Aβ in CFA reduced plaque burden and prevented cognitive decline in transgenic mouse models of AD (Schenk *et al.* 1999, Morgan *et al.* 2000, Chen *et al.* 2007a). This immunisation protocol induced T cell and antibody responses to Aβ with no apparent ill effects. Preclinical trials in other species also showed a striking clearance of Aβ from the brain, leading to the initiation of clinical trials. Although injections of Aβ were well tolerated in the phase I trial, in the subsequent phase II trial 6% of patients developed meningoencephalitis, which is thought to be due to a Th1 cell response to Aβ (Boche & Nicoll 2008, Holmes *et al.* 2008). The results of clinical trials showed that active immunisation promoted plaque clearance from the brains of AD patients (Ferrer *et al.* 2004, Nicoll *et al.* 2003). In addition, 53% of patients developed an antibody response to Aβ, which was found to be associated with a slower rate of cognitive decline in the Zurich cohort of patients (Hock *et al.* 2003). Due to the development of meningoencephalitis, a number of studies have focused on limiting T cell responses by direct administration of Aβ-specific antibodies. However, though these antibodies have been shown to reduce plaque burden in the brains of APP-transgenic mice (Wilcock *et al.* 2003, Bard *et al.* 2000), they also result in an increased incidence of brain microhaemorrhages (Wilcock *et al.* 2004b, Racke *et al.* 2005, Pfeifer *et al.* 2002).

Although implicated in causing meningoencephalitis in AD patients, T cells may be beneficial in the clearance of Aβ from the brain. Frenkel and colleagues demonstrated that immunisation of APP-transgenic mice with glatiramer acetate, a drug used in the treatment of MS, induced clearance of Aβ from the brain, without concomitant encephalitis (Frenkel *et al.* 2005). This effect was suggested to be due to the induction of glatiramer acetate-specific T cells, which activate microglial cells, but do not cause encephalitis due to their weak cross-reactivity with CNS antigens. Further evidence that T cells may have a beneficial role in AD comes from the Arendash laboratory, who reported that i.v. injection of a mixed population of Aβ-specific T cells reversed cognitive decline in PSAPP mice (Ethell *et al.* 2006). In contrast, infusion of polarised Th1 cells

provided no cognitive benefit, suggesting that the anti-inflammatory responses induced by Th2 cells may be of more benefit in AD. Indeed, a recent paper has shown that administration of Aβ-specific Th2 cells improved performance of transgenic mice in the RAWM (Cao *et al.* 2009).

Activation of microglia has been associated with plaque clearance in transgenic mouse models of AD. In clinical trials of active immunotherapy, amyloid clearance was associated with microglia that were immunoreactive for Aβ (Wisniewski & Konietzko 2008). Furthermore, Bard and colleagues found that administration of Aβ-specific antibodies induced clearance of Aβ in APP-transgenic mice by a mechanism involving microglial phagocytosis of opsonised Aβ (Bard *et al.* 2000), though microglial-independent mechanisms of plaque removal have also been reported (Wilcock *et al.* 2003). While microglia appear to be able to phagocytose plaques, they can also become neurotoxic following exposure to Aβ. Microglia treated with Aβ have been shown to produce reactive oxygen species, nitric oxide, and proinflammatory cytokines (Akiyama *et al.* 2000, Meda *et al.* 1999, Gasic-Milenkovic *et al.* 2003). Furthermore, Giulian and colleagues reported that microglia potentiated the neurotoxic effects of Aβ *in vitro* (Giulian *et al.* 1996). These data suggest that microglia have the potential both to induce plaque clearance and mediate neurotoxicity in AD, and that the activation state of the microglial cell may be important in determining whether microglial activation has a beneficial or detrimental effect.

Recently, the role of bone-marrow-derived microglia in AD has been examined. Using bone marrow chimeras, Malm and colleagues found that macrophages infiltrated the CNS of APPswe/PS1dE9 mice and could decrease Aβ plaque burden (Malm *et al.* 2005). It has also been shown that elimination of bone-marrow-derived cells from the CNS of APP-transgenic mice resulted in an increase in size of Aβ plaques (Simard *et al.* 2006). However, recent studies have questioned the functional relevance of these models, and it has been reported that

only negligible numbers of cells from the bone marrow infiltrate the CNS under normal circumstances (Mildner *et al.* 2007).

The objectives of this chapter were to:

- Characterise the inflammatory environment present in the brains of wild-type and APPswe/PS1dE9 mice.
- Examine the effect of transfer of Aβ-specific Th1, Th2, and Th17 cells on spatial memory in a mouse model of AD.
- Determine the role of Aβ-specific T cell subsets in modulating Aβ burden in the brain and blood of APPswe/PS1dE9 mice.
- Investigate the ability of Aβ-specific T cells to induce a specific antibody response to Aβ.
- Analyse the effect of T cell transfer on the phenotype of microglia and infiltrating macrophages in the brains of APPswe/PS1dE9 mice.

5.2. Results

5.2.1. Expression of proinflammatory cytokines is increased in the hippocampus of APPswe/PS1dE9 mice

Initial evidence that microglia play a role in inflammation in the CNS of AD patients came from studies showing that IL-1β, IL-6, and TNF-α expression is upregulated in these individuals, and that it is predominantly associated with AD plaques (Griffin *et al.* 1989, Wood *et al.* 1993, Cacabelos *et al.* 1994). IL-1Ra knockout mice injected i.c.v. with Aβ show enhanced neuronal damage, implicating uncontrolled IL-1β production in the neuropathology of AD (Craft *et al.* 2005). Increased levels of TNF-α in the CSF have been shown to correlate with the subsequent development of AD (Tarkowski *et al.* 2003), while IL-1β and TNF-α polymorphisms have been linked with increased risk of developing AD (McGeer & McGeer 2001). Therefore, expression of IL-1β and TNF-α was assessed in the hippocampus of wild-type and APPswe/PS1dE9 mice. The data show that IL-1β and TNF-α mRNA expression were significantly increased in the hippocampus of APPswe/PS1dE9 mice (Fig. 5.1), indicating that there exists a more inflammatory environment in the CNS of these mice.

5.2.2. Expression of the phagocytic marker, CD68, is increased, while expression of the co-stimulatory molecule, CD40, is decreased in the hippocampus of APPswe/PS1dE9 mice

Given that proinflammatory cytokine expression was increased in the hippocampus of APPswe/PS1dE9 mice, and that microglia have been shown to produce proinflammatory cytokines in the brains of AD patients, the expression of the microglial activation markers CD68 and CD40 was assessed in the brains of wild-type and APPswe/PS1dE9 mice. Macrosialin, or CD68, is a lysosomal-

associated protein expressed by macrophages and microglia, and has been linked with the phagocytic capacity of cells (Malm *et al.* 2008, da Silva & Gordon 1999). CD40 is a co-stimulatory molecule expressed on macrophages and microglia, and is important in presentation of antigen by microglia to T cells (Aloisi *et al.* 2000b, Issazadeh 1998). Town and colleagues have proposed that microglia exist in at least 2 functionally distinct states following their activation: an antigen presenting state, and a phagocytic state (Town *et al.* 2005). To examine the effect of T cell transfer on the activation state of macrophages/microglia in the CNS of APPswe/PS1dE9 mice, CD68 and CD40 mRNA expression were assessed in the hippocampus of wild-type and APPswe/PS1dE9 mice. CD68 mRNA was significantly increased in the hippocampus of transgenic mice compared with wild-type controls, while CD40 mRNA expression was decreased in transgenic mice relative to wild-type mice (Fig. 5.2). This is consistent with an increase in microglial phagocytosis and a decrease in antigen-presentation in the CNS of APPswe/PS1dE9 mice.

5.2.3. APPswe/PS1dE9 mice are unimpaired in muscular strength and co-ordination

The APPswe/PS1dE9 mice used in this study co-overexpress a chimeric mouse/human APP695 isoform containing the K595N/M596L Swedish mutations, and a mutant human PS1 with exon 9 deleted, under the control of independent mouse prion promoters (Jankowsky *et al.* 2001, Jankowsky *et al.* 2004). This results in co-segregation of these genes. After the transgenes were introduced, these mice were maintained on a hybrid background by backcrossing to C3HeJxC57BL/6J F1 mice. Background genes that are randomly contributed by each parental strain can often interact with the mutated gene that has been inserted, which can lead to changes in behaviour and motor co-ordination not necessarily linked to the mutation (Crawley & Paylor 1997, Wolfer & Lipp 2000). Indeed, Tg2576 transgenic mice have been found to be impaired on the balance

beam and wire-hang tests (King & Arendash 2002), which may affect their performance in behavioural tests such as the Morris water maze due to poor motoric swim performance. In order to separate cognitive deficits from alterations in non-cognitive behaviours in APPswe/PS1dE9 mice, a number of sensorimotor tasks were administered to wild-type and transgenic mice 1 week prior to T cell transfer.

To assess motor co-ordination and balance, gait was analysed using the footprint test. The front and rear paws of the mouse were painted with red and black paint respectively. The mouse was immediately placed onto white paper in a narrow tunnel and allowed to run across the paper to the goal box at the opposite end. Stride length, hind-base width, and front-base width of each mouse were measured. The data show that there was no significant difference in any of these measures between groups (Fig 5.3).

Muscular strength and co-ordination were assessed using the wire-hang and inverted screen tests. In the inverted screen test, mice were placed on a wire-mesh screen, which was slowly inverted. The length of time that mice held onto the screen was recorded up to a maximum score of 60 sec. All mice were found to be able to hold onto the screen for 60 sec, and thus there were no differences between the groups (data not shown). The wire-hang test consisted of 2 one-minute trials. On the first trial, mice were suspended from a wire above a padded surface by all 4 paws. The mice were released and the length of time they held onto the wire, up to a maximum of 60 sec, was recorded. After 20 min, this process was repeated, with the mice being suspended by their 2 front paws only. The data show that there were no differences in the latency to fall between groups on either trial (Fig. 5.4). These results indicate that APPswe/PS1dE9 mice were unimpaired in muscular strength and co-ordination.

5.2.4. Characterisation of T cell lines for adoptive transfer

To determine whether Aβ-specific T cell subsets can modulate Aβ burden and spatial memory in a transgenic mouse model of AD, Aβ-specific Th1, Th2, and Th17 cells were generated for adoptive transfer to APPswe/PS1dE9 mice. Wild-type mice were injected s.c. in the footpad with Aβ and CpG and boosted 21 days later. Seven days after the boost, spleens were removed and cells were stimulated with Aβ in the presence of IL-12 for Th1 cell polarisation, dexamethasone and IL-4 to generate Th2 cells, or IL-1 and IL-23 to induce Th17 cells. After 4 days, cells were washed and injected i.v. (15×10^6 cells/mouse) into 6-7 month old APPswe/PS1dE9 mice. Approximately 15×10^4 Th1, Th2 and Th17 cells were retained, and the percentage of $CD3^+$ cells co-expressing CD4 and CD8 was measured by flow cytometry. Approximately half of the cells in each group were $CD3^+$ (Fig. 5.5A). Between 50% and 70% of $CD3^+$ cells were $CD4^+$, and approximately 25% were $CD8^+$ (Fig. 5.5B-D).

To examine the cytokine profile of Aβ-specific Th1, Th2, and Th17 cells, supernatants were removed on day 4 of culture, and IFN-γ, IL-4, IL-10, IL-17, and IL-5 production was measured by ELISA. Th1 cells produced high levels of IFN-γ, low levels of IL-5 and IL-10, and no IL-4 or IL-17 (Fig. 5.6). Th2 cells secreted high levels of IL-4, low levels of IL-5 and IL-10, and no IL-17 or IFN-γ, though the IL-4 detected could be the exogenously-added IL-4 used to generate Th2 cells. Th17 cells were found to produce high levels of IL-17, low levels of IL-5 and IL-10, and no IL-4 or IFN-γ. The results presented in chapter 4, along with work from other laboratories, have shown that Th17 cells often convert to a Th1 phenotype upon restimulation (Mathur *et al.* 2006). Furthermore, the added cytokines, especially IL-4, complicate the assessment of the T cell cytokine profile *ex vivo*. To assess the effect of antigen restimulation on the cytokine profile of Aβ-specific Th1, Th2, and Th17 cells, these cells were restimulated with Aβ and irradiated APC, in the absence of polarising cytokines, 11 days after primary antigen stimulation. After 4 days, T cell cytokine production was

determined by ELISA. Th1 cell lines produced high levels of IFN-γ, and no IL-4, IL-5, IL-10, or IL-17 (Fig. 5.7). Th2 cell lines secreted high levels of IL-4, IL-5, and IL-10, with no IL-17 or IFN-γ. Th17 cell lines produced high levels of IL-17 and IFN-γ, and no IL-4, IL-5, or IL-10. This indicated that the Th17 cell line had switched to a mixed Th1/Th17 cell line, while the Th2 cell line had the characteristic cytokine profile of Th2 cells.

To confirm these observations, T cell lines were harvested on day 4 of this second round of antigen restimulation, and stimulated with a cocktail of PMA (10 ng/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/ml) for 5 h. Th1 and Th17 cell lines were analysed for expression of IFN-γ and IL-17 on $CD4^+$ cells by flow cytometry, while Th2 cells were analysed for $CD4^+$ cell expression of IL-4 and IL-10. A large percentage of Th1 cells produced IFN-γ, while the percentage of IL-17 producing cells was very low (Fig. 5.8A). Th17 cell lines contained cells that produced IFN-γ, as well as cells that secreted IL-17 (Fig. 5.8B), while Th2 cells produced IL-4 and IL-10 in response to stimulation with PMA and ionomycin (Fig. 5.8C). These data suggest that while polarised Th1, Th2 and Th17 cells were adoptively transferred into APPswe/PS1dE9 mice, the Th17 cells may have converted to a mixed Th1/Th17 cell population *in vivo*.

5.2.5. Administration of Aβ-specific Th1 cells to APPswe/PS1dE9 mice induces impairments in spatial memory

APPswe/PS1dE9 mice have previously been shown to have deficits in spatial memory in the standard MWM task (Cao *et al.* 2007, Jankowsky *et al.* 2005), as well as in the repeated-reversal and RAWM tasks (Savonenko *et al.* 2005, Jankowsky *et al.* 2005). Recently, Ethell and colleagues reported that Aβ-specific T cells can reverse cognitive decline in an APP/PS1 mouse model of AD (Ethell *et al.* 2006). The ability of Aβ-specific Th1, Th2 and Th17 cells to alter spatial learning and memory in APPswe/PS1dE9 mice was thus examined. Three weeks

after i.v. injection of Aβ-specific T cell subsets, 7-8 month old wild-type and transgenic mice were given 1 day in the MWM to habituate to the surroundings. They were trained for 5 consecutive days with 4 one-minute trials per day administered at 5 min intervals. The pathlength and latency to find the platform over the 5 days of training were recorded. There was no difference in either latency or pathlength to find the platform between wild-type and control-treated transgenic mice (Figs. 5.9 and 5.10). APPswe/PS1dE9 mice given an injection of Aβ-specific Th1 cells had an increased pathlength to find the platform on day 5 of training (Fig. 5.9B), though their latency to find the platform was not increased (Fig. 5.10B). Administration of Aβ-specific Th2 or Th17 cells had no effect on either pathlength or latency to find the platform.

The day after the final day of training, mice were given a single 60-sec probe trial in which the platform was removed from the MWM. Animals were placed into the centre of the maze, and the percentage of time and pathlength spent by each animal in the quadrant previously containing the platform was recorded. There were no differences found between control-treated wild-type and transgenic mice in either the percentage of time or pathlength spent in the quadrant previously containing the platform (Fig. 5.11). APPswe/PS1dE9 mice that had been given an i.v. injection of Aβ-specific Th1 cells spent significantly less time and pathlength in the quadrant previously containing the platform (Fig. 5.11). Administration of Th2 or Th17 cells had no effect on either of these measures. The number of times each animal swam across the area previously containing the platform was also recorded, and it was found that there was no difference in the number of crosses over the platform between groups (Fig. 5.12). These results indicate that there is no impairment in spatial memory in 7-8 month old APPswe/PS1dE9 mice. In addition, Aβ-specific Th1 cells induced deficits in cognitive function.

5.2.6. Aβ-specific Th1 cells increase Aβ burden in the cortex of APPswe/PS1dE9 mice: assessment by Aβ-specific ELISA

APPswe/PS1dE9 mice have been shown to have early and aggressive Aβ plaque formation in comparison to other APP/PS1 transgenic mouse models, and have a substantial number of Aβ plaques in the brain by 6 months of age (Jankowsky *et al.* 2004). In the present study, Aβ-specific T cell subsets were assessed for their ability to alter Aβ levels in the cortex of transgenic mice. SDS-soluble Aβ containing monomeric and some species of oligomeric Aβ (DeMattos *et al.* 2002, Stenh *et al.* 2005), and SDS-insoluble Aβ containing mainly fibrillar Aβ were extracted from snap-frozen cortical tissue obtained from wild-type mice and APPswe/PSEN1dE9 mice treated with Aβ-specific Th1, Th2, or Th17 cells. The concentrations of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ in each of these fractions were determined by multi-spot ELISA. Comparison of Aβ concentrations in tissue from wild-type and APPswe/PS1dE9 mice showed that transgenic mice had increased levels of soluble $A\beta_{40}$ and $A\beta_{42}$, as well as insoluble $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ (Figs. 5.13 and 5.14). Transfer of Aβ-specific Th1 cells increased soluble $A\beta_{40}$ and $A\beta_{42}$ concentrations and insoluble $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ concentrations in the cortex of transgenic mice. Infusion of Aβ-specific Th17 cells reduced concentrations of soluble $A\beta_{40}$ in the cortex of transgenic mice, but had no effect on the levels of $A\beta_{38}$ or $A\beta_{42}$. Transfer of Aβ-specific Th2 cells had no effect on either soluble or insoluble levels of $A\beta_{38}$, $A\beta_{40}$, or $A\beta_{42}$ (Figs. 5.13 and 5.14). The data demonstrate that Aβ-specific Th1 cells increased Aβ load in the cortex of APPswe/PS1dE9 mice. There is also some indication that Th17 cells can reduce soluble, but not insoluble Aβ.

5.2.7. Aβ-specific Th1 cells increase plaque burden in the brains of APPswe/PS1dE9 mice: histological assessment of Aβ burden

Previous studies have shown that thioflavin S-positive Aβ plaques are markedly increased in APPswe/PS1dE9 mice at between 6 and 8 months of age (Garcia-Alloza *et al.* 2006, Savonenko *et al.* 2005). The influence of Aβ-specific Th1, Th2, and Th17 cells on plaque number in the hippocampus and in the whole brain of transgenic mice was examined using a histological approach. Saggital brain sections were prepared from wild-type and APPswe/PSEN1dE9 mice treated with Aβ-specific Th1, Th2, or Th17 cells. These sections were then stained with Congo red for detection of Aβ plaques in the brain. There were no Congo red-positive Aβ plaques detected in sections taken from wild-type mice (Fig. 5.15B). There was an increase in the number of Aβ plaques in the brains of Th1 cell-treated mice, though this did not reach statistical significance ($p=0.07$). Transgenic mice injected with Th1 cells had significantly higher numbers of Aβ plaques in the brain than mice injected with Th17 cells (Fig. 5.15A). Aβ-specific Th2 cells had no effect on plaque number in the brains of transgenic mice. T cell administration had no effect on the number of Congo red positive Aβ plaques in the hippocampus of transgenic mice (Fig. 5.16). The data suggest that Th1 cells induced increases in plaque number in regions of the brain other than the hippocampus of APPswe/PS1dE9 mice.

5.2.8. Serum Aβ levels are not significantly altered by Aβ-specific T cell transfer

To assess the ability of Aβ-specific T cells to alter Aβ levels on the blood of APPswe/PS1dE9 mice, serum taken from wild-type and transgenic mice was analysed for concentrations of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ by multi-spot ELISA. APPswe/PS1dE9 mice had circulating $A\beta_{40}$ and $A\beta_{42}$ levels of approximately 2500 pg/ml and 150 pg/ml, respectively. There was no significant difference in

$A\beta_{38}$, $A\beta_{40}$, or $A\beta_{42}$ levels in mice transferred with Aβ-specific Th1, Th2 or Th17 cells (Fig. 5.17). However, there was a trend towards increased concentrations of $A\beta_{40}$ and $A\beta_{42}$ in transgenic mice that received Th1 cells.

5.2.9. Effect of T cell transfer on Aβ-specific IgG subclasses

The ratio of IgG1 to IgG2a antibodies is an indirect measure of a Th1 or Th2 cell-type response *in vivo*. IFN-γ has been shown to promote IgG2a production and inhibit IgG1 production, while IL-4 induces production of IgG1 while inhibiting IgG2a (Coffman *et al.* 1988, Snapper & Paul 1987, Finkelman *et al.* 1990). A high production of IgG1 over IgG2a is therefore indicative of a predominantly Th2 response, while a high production of IgG2a over IgG1 suggests a prevailing Th1 response. Analysis of serum IgG concentration showed that transfer of T cells had no effect on Aβ-specific serum IgG production (Fig. 5.18). Aβ-specific IgG1 production was increased in mice injected with Th1, Th2, or Th17 cells. However, Aβ-specific IgG2a production was decreased in mice injected with Th2 cells when compared with mice injected with Th1 cells (Fig. 5.18). In addition, the ratio of IgG1:IgG2a was lower in mice injected with Th1 cells (1.4:1), than in mice infused with Th2 or Th17 cells (1.8:1 and 1.7:1 respectively; Fig. 5.18). These results show that administration of T cells leads to the development of an antibody response *in vivo*, and that transfer of Aβ-specific Th2 or Th17 cells induced a more polarised IgG1 response than transfer of Aβ-specific Th1 cells.

5.2.10. Expression of IFN-γ and IL-17 on $CD3^+$ cells in the brains of wild-type and APPswe/PS1dE9 mice

To assess whether i.v. injection of T cells into APPswe/PS1dE9 mice resulted in increased T cell activation in the brain, mononuclear cells were isolated from the brains of wild-type and transgenic mice, and the percentage of $CD3^+$ cells co-

expressing IFN-γ or IL-17 was measured by flow cytometry. The data show that there are $CD3^+$ cells in the brains of both wild-type and transgenic mice (Fig. 5.19). There was no significant difference in the percentage of cells co-expressing IFN-γ or IL-17, though there was a trend towards increased expression of IFN-γ and IL-17 on $CD3^+$ cells in the brains of transgenic mice treated with Th1 cells. The percentage of $CD3^+$ cells in the brain was not affected by treatment with Aβ-specific T cells (data not shown).

5.2.11. APPswe/PS1dE9 mice have increased infiltrating macrophages, and this is attenuated by treatment with T cells

Microglia can be distinguished from macrophages on the basis of their level of CD45 expression. Ramified microglia constitutively express low levels of CD45. Microglia respond to inflammation and injury by upregulating their expression of CD45 to an intermediate level (Sedgwick *et al.* 1991). In contrast, infiltrating macrophages express high levels of CD45 (Ford *et al.* 1995). Mononuclear cells were isolated from the brains of wild-type and transgenic mice, and the percentage of microglia ($CD11b^+CD45^{int}$), and infiltrating macrophages ($CD11b^+CD45^{hi}$) were quantified by flow cytometry. The percentage of microglia in the brain was not affected by treatment with Aβ-specific T cells (Fig. 5.20). Analysis of MHC class II and CD86 expression on microglia showed that T cell transfer did not modulate expression of these molecules (Fig. 5.21). The percentage of infiltrating macrophages in the brain was significantly increased in APPswe/PS1dE9 mice, and this was attenuated by treatment with Aβ-specific T cells (Fig. 5.22).

5.3. Discussion

This study demonstrated that at 7-8 months of age, APPswe/PS1dE9 mice had increased expression of inflammatory markers in the brain. Analysis of Aβ burden revealed that these mice had significant Aβ plaque deposition. However, APPswe/PS1dE9 mice exhibited no impairment in spatial reference memory in the MWM. Transfer of Aβ-specific Th1 cells resulted in deterioration in water maze performance, and this was associated with increased levels of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the brain. Transfer of Aβ-specific Th2 cells had no effect on cognition or Aβ burden, while Th17 cells induced a moderate decrease in soluble $A\beta_{40}$ in the brain. In addition, APPswe/PS1dE9 mice were found to have increased infiltrating macrophages in the brain relative to wild-type controls, and this was attenuated by transfer of Aβ-specific T cells.

There is considerable evidence to suggest that an increase in the level of Aβ in the brain is central to the pathogenesis of AD. In particular, studies of FAD have shown that all genes so far associated with the development of AD lead either to increased production or deposition of Aβ (Citron *et al.* 1997, Selkoe 2001). AD was one of the first neurodegenerative diseases to be associated with microglial activation, and the amyloid cascade hypothesis postulates that the accumulation of Aβ in the brains of AD patients leads to microglial activation and subsequent neuronal death (Hardy & Higgins 1992). In support of this hypothesis, Benzing and colleagues have detected IL-1β and TNF-α-immunopositive microglia at the sites of Aβ plaque deposits in APP-transgenic mice (Benzing *et al.* 1999). The importance of TNF-α signalling in the pathogenesis of AD has been demonstrated in a study by He and colleagues, who showed that deletion of the TNFR1 gene in APP-transgenic mice resulted in decreased plaque formation in the brain (He *et al.* 2007). In the present study, IL-1β and TNF-α mRNA expression were significantly increased in the hippocampus of APPswe/PS1dE9

mice, suggesting that Aβ deposition is associated with increased proinflammatory cytokine production by microglia.

Microglia are competent phagocytes and have been shown to be capable of phagocytosing fibrillar Aβ *in vitro* and *in vivo* (Paresce *et al.* 1996, deWitt *et al.* 1998b, Frautschy *et al.* 1992). However, they appear to be unable to efficiently phagocytose Aβ plaques in either the AD brain or in mouse models of AD. The results of this study show that the expression of CD68 was higher in the hippocampus of APPswe/PS1dE9 mice than in wild-type mice, suggesting increased phagocytosis. It is possible that microglia become more phagocytic in response to Aβ deposition in APPswe/PS1dE9 mice, but that Aβ is being produced at a greater rate than it is being cleared, leading to an overall increase in Aβ burden. Alternatively, it may be the case that microglia are unable to efficiently phagocytose Aβ plaques in APPswe/PS1dE9 mice, despite increased CD68 expression. Indeed, it has been reported that while microglia rapidly internalise Aβ, they degrade only a fraction of the Aβ they internalise, and thus become overwhelmed by large amounts of Aβ, such as those produced in mouse models of AD (Paresce *et al.* 1997, Chung *et al.* 1999).

Binding of CD40 to its ligand, CD154, appears to be important in the interaction of microglia and T cells, and CD40-CD154 interaction in the presence of $A\beta_{42}$ was found to enhance microglial-induced neuronal injury over that induced by Aβ alone (Tan *et al.* 1999). The interaction of CD40 and CD154 has been demonstrated to decrease phagocytosis of Aβ by microglia (Townsend *et al.* 2005). Additionally, it has been shown that APP-transgenic mice with a disrupted CD40 gene have reduced Aβ burden (Laporte *et al.* 2006). The data presented in this study show that CD40 expression is decreased in the hippocampus of APPswe/PS1dE9 mice, indicating decreased APC function.

Despite strong evidence implicating Aβ in the development of AD, linkage between Aβ levels and cognitive impairment has not been conclusively

established in humans (Braak & Braak 1997, Aizenstein *et al.* 2008). In order to determine whether Aβ production is sufficient to induce memory impairment, a number of transgenic mouse models have been developed that overexpress mutant forms of the APP and presenilin genes, leading to increased production of Aβ. The first paper linking Aβ formation to cognitive deficits in transgenic animals was published by Westerman and colleagues (2002). They reported that Tg2576 mice were unimpaired in the MWM before 6 months of age, despite the presence of soluble Aβ in the brain. After 6 months, transgenic mice did not perform as well as wild-type controls in the MWM, and this correlated with an increase in the level of insoluble Aβ in the brain. Double-transgenic PS1/APP mice were shown to have an accelerated increase in levels of insoluble Aβ, leading to early onset of memory impairment. Furthermore, Liu and colleagues have demonstrated that the level of insoluble $A\beta_{42}$ in APP/PS1 mice correlates with spatial memory (Liu *et al.* 2003), suggesting that oligomeric or fibrillar Aβ are responsible for the cognitive deficits seen in AD. The results presented here show that, despite APPswe/PS1dE9 mice exhibiting a large increase in Aβ burden in the brain parenchyma, they were not impaired in spatial or reference memory in the MWM when compared with wild-type animals. It is possible that there is a disconnection between Aβ deposition and cognitive impairment in this model, but this seems unlikely, given that other studies have shown memory deficits in APPswe/PS1dE9 mice (Cao *et al.* 2007, Savonenko *et al.* 2005).

One possible reason for the lack of memory impairment in the mice used in this study is that 7-8 month old mice were not old enough for the accumulation of sufficient Aβ to have interfered with normal neural function. This is supported by data from Savonenko and colleagues, who found that 6 month old APPswe/PS1dE9 mice were not impaired in the MWM and RAWM, whereas 18 month old mice exhibited impairments in spatial and episodic-like memory (Savonenko *et al.* 2005). In contrast, it has been reported that 8 month old APPswe/PS1dE9 mice have impaired memory in the MWM (Cao *et al.* 2007, Jankowsky *et al.* 2005). It seems likely that the lack of cognitive deficits seen in

this study is also due, at least in part, to the nature of the memory task employed. It has been suggested that the impairment in memory present in APP-transgenic mice is not just age-dependent, but also task-specific (Kobayashi & Chen 2005). Two studies of behaviour in APPswe/PS1dE9 mice reported that transgenic mice performed worse in tasks measuring episodic-like memory than in tasks such as the MWM, which measures spatial memory (Jankowsky *et al.* 2005, Savonenko *et al.* 2005). This indicates that episodic-like memory is more sensitive to the effects of Aβ than spatial memory, and therefore may be a better measure of cognitive impairment in animals of this age.

Little is known about the role of T cells in AD. While there is currently no evidence for a T cell response in the brains of AD patients, it is known that activated T cells can penetrate the BBB (Wekerle *et al.* 1987), and Trieb and colleagues have demonstrated that peripheral blood lymphocytes from healthy controls, but not patients with AD, proliferate in response to Aβ (Trieb *et al.* 1996). In addition, it has been shown that Aβ-specific antibody titers are significantly decreased in patients with AD (Brettschneider *et al.* 2005, Du *et al.* 2001). It is possible that overexpression of Aβ over many years leads to T and B cell anergy, and that the adaptive immune response could potentially be beneficial in reducing Aβ levels. In support of this theory, it was found that overexpression of APP in transgenic mice led to an impaired T cell response to Aβ (Monsonego *et al.* 2001). It was shown that immunisation of mice with Aβ in CFA resulted in clearance of Aβ deposits in the PDAPP mouse, and that this clearance was associated with antibody production (Schenk *et al.* 1999, Bard *et al.* 2000), suggesting that a T cell response was generated.

In the present study, adoptive transfer of Aβ-specific Th1 cells resulted in decreased performance in the MWM 3 weeks later. Consistent with these results, Li and colleagues have shown that pre-treatment of microglia with IFN-γ is required for microglia-mediated, Aβ-induced neurotoxicity *in vitro* (Li *et al.* 2004). Mice expressing IFN-γ in the brain under the MBP promoter had increased

microglial activation, hypomyelination, and defective cerebellar development, leading to death at 2-4 weeks of age (Corbin *et al.* 1996), suggesting that IFN-γ is destructive in the CNS. Additionally, immunisation with Aβ in CFA induced Th1 cell-mediated encephalitis in APP-transgenic mice overexpressing IFN-γ in the CNS (Monsonego *et al.* 2006), though memory performance was not analysed in these experiments. In contrast to these findings, transfer of Aβ-specific Th1-enriched cells into APP-transgenic mice had no effect on subsequent performance in the RAWM (Ethell *et al.* 2006). Furthermore, Baron and colleagues found that expression of IFN-γ under the MBP increased neurogenesis in both wild-type and APP-transgenic mice (Baron *et al.* 2008). As well as inducing cognitive impairment, treatment with Aβ-specific Th1 cells increased Aβ burden in the brains of APPswe/PS1dE9 mice. These data are consistent with a report by Yamamoto and colleagues showing that IFN-γ receptor-deficient, APP-transgenic mice have reduced plaque formation at 14 months of age (Yamamoto *et al.* 2007). A number of studies have reported that IFN-γ inhibits microglial phagocytosis of Aβ (Yamamoto *et al.* 2008, von Bernhardi *et al.* 2007, Koenigsknecht & Landreth 2005). Furthermore, IFN-γ has been shown to upregulate the expression of β-secretase, resulting in increased Aβ production by neurons and astrocytes (Cho *et al.* 2007, Yamamoto *et al.* 2007). Thus, it would appear that Aβ-specific Th1 cells, by increasing production and/or reducing clearance of Aβ, act to increase the overall Aβ burden in the brain, and this may lead to the memory impairment seen in APP-transgenic mice injected with these cells.

As Th1 cells have been implicated in the meningoencephalitis seen in clinical trials involving immunisation with Aβ (Orgogozo *et al.* 2003), it has been suggested that Th2 cells may have a beneficial effect in AD. In support of this, immunisation of APP-transgenic mice with Aβ in the Th2-inducing adjuvant alum reduced Aβ levels in the brain (Asuni *et al.* 2006). Additionally, the Th2-type cytokines, IL-4 and IL-10, have been shown to increase microglial phagocytosis of Aβ, and have also been reported to decrease Aβ-induced proinflammatory cytokine production by microglia (Szczepanik *et al.* 2001, Koenigsknecht-Talboo

& Landreth 2005, Iribarren *et al.* 2007). In the only published study on the role of Aβ-specific Th2 cells in AD, Cao and colleagues found that APP/PS1 mice transferred with Th2 cells performed significantly better in the RAWM task than untreated mice (Cao *et al.* 2009). This cognitive improvement was not associated with a reduction in Aβ levels in the brain parenchyma, but it was associated with increased levels of Aβ in the blood. The disparity between the results presented here, and the improved cognitive function reported by Cao and colleagues following administration of Aβ-specific Th2 cells, may be explained by the fact that the APPswe/PS1dE9 mice used in this study were not impaired in the MWM at the age tested (7-8 months). Cao and colleagues used older mice (13 months) for their studies. In addition, they tested these mice in the RAWM, a task which measures episodic-like memory, and which has been shown to be more sensitive in detecting early memory impairment in double-transgenic mice (Savonenko *et al.* 2005). However, in this study, transfer of Aβ-specific Th2 cells did not alter Aβ levels in the brain or blood of APPswe/PS1dE9 mice. If the amyloid cascade hypothesis is correct, then these cells are unlikely to provide any cognitive benefit, as improvements in memory in APP-transgenic mice have been shown to be linked with decreased Aβ burden (Morgan *et al.* 2000, Wilcock *et al.* 2004b).

The role of Aβ-specific Th17 cells in the pathogenesis of AD is currently unknown, though these cells have been implicated in the pathogenesis of a number of autoimmune and chronic inflammatory diseases including MS, psoriasis, and rheumatoid arthritis (Chabaud *et al.* 1999, Kryczek *et al.* 2008, Langrish *et al.* 2005). The results presented in chapter 4 showed that Th17 cells induced greater levels of IL-1β and IL-6 production in co-culture with glia than Th1 cells. Therefore, it was expected that these cells would induce a more proinflammatory environment in the brain. Surprisingly, the addition of Th17 cells had no effect on performance of APPswe/PS1dE9 mice in the MWM. Furthermore, adoptive transfer of Aβ-specific Th17 cells induced a modest reduction in soluble $A\beta_{40}$ concentration, indicating that these cells may potentially be beneficial in AD.

It is somewhat surprising that Aβ-specific Th1 cells did not induce clearance of Aβ in this study. The results presented in chapter 3 showed that immunisation with Aβ in CFA induced Aβ-specific Th1 cells, and this immunisation protocol was found by Schenk and colleagues to reduce plaque burden in PDAPP mice (Schenk *et al.* 1999). In addition, Monsonego and colleagues found that immunisation of APP-transgenic mice overexpressing IFN-γ in the CNS with Aβ in CFA led to clearance of Aβ plaques from the brain (Monsonego *et al.* 2006). It is possible that the clearance of Aβ plaques following immunisation with Aβ in CFA is due to the induction of Aβ-specific antibodies, rather than Aβ-specific T cells. The results presented in chapter 3, along with data from other laboratories, have shown that immunisation with Aβ in CFA induces Aβ-specific IgG production (Schenk *et al.* 1999). Indeed, Bard and colleagues showed that passive immunisation of PDAPP mice with an Aβ-specific antibody led to clearance of Aβ plaques from the brain, in a process involving Fc-mediated phagocytosis of opsonised Aβ by microglia (Bard *et al.* 2000). In the present study, transfer of Aβ-specific T cells did not induce a significant increase in total Aβ-specific IgG concentration, and perhaps this partially explains the lack of Aβ clearance following Aβ-specific T cell administration.

These results demonstrate that Aβ-specific Th1 cells increase soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the brain parenchyma of a transgenic mouse model of AD. They also induce an impairment in spatial memory as evidenced by performance in the MWM. The data indicate that Aβ-specific Th2 cells are not beneficial in APPswe/PS1dE9 mice, while Th17 cells appear to be of limited benefit. The data show that Aβ-specific T cell subsets have distinct roles in the pathogenesis of AD. While a protective role of Aβ-specific T cells in AD cannot be ruled out, the data presented here suggest that T cells are of limited benefit in the treatment of AD.

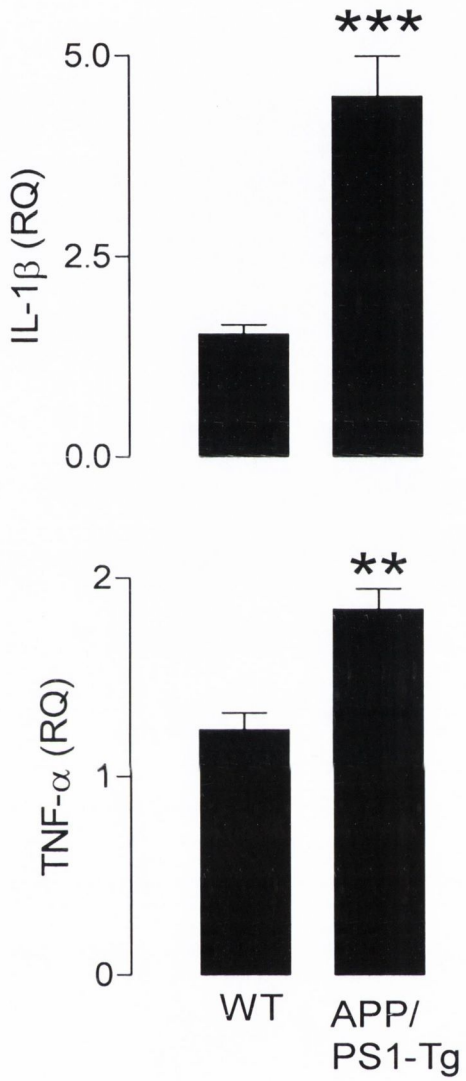


Figure 5.1. IL-1β and TNF-α mRNA expression are increased in the hippocampus of transgenic mice.

Snap-frozen hippocampal tissue from wild-type and APPswe/PS1dE9 mice was analysed for IL-1β and TNF-α mRNA expression by real-time PCR. ** $p<0.01$, *** $p<0.001$ versus tissue taken from wild-type mice (unpaired Students t-test). Values are expressed as means ± SEM (n=5-6).

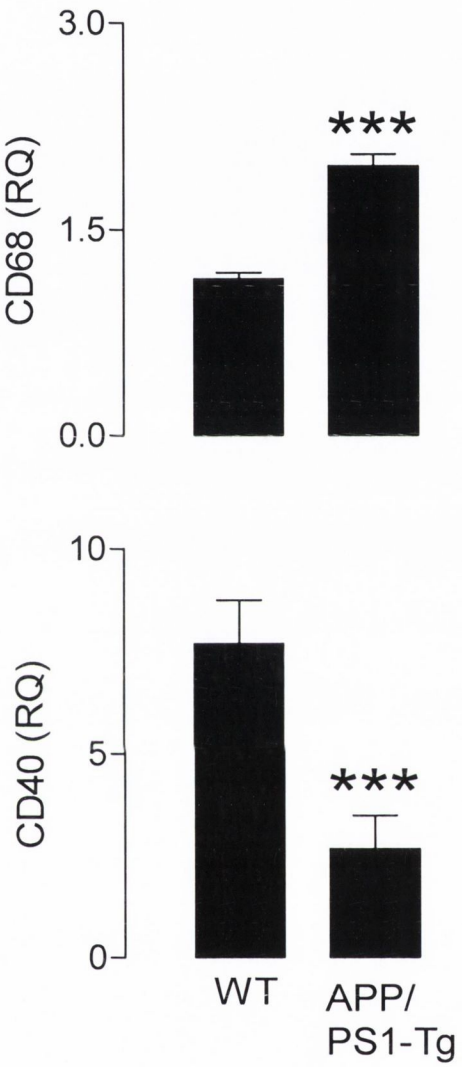


Figure 5.2. CD68 mRNA expression is increased, while CD40 mRNA expression is decreased in the hippocampus of transgenic mice.

Snap-frozen hippocampal tissue from wild-type and APPswe/PS1dE9 mice was analysed for CD68 and CD40 mRNA expression by real-time PCR. *** $p<0.001$ versus tissue taken from wild-type mice (unpaired Students t-test). Values are expressed as means ± SEM (n=5-6).

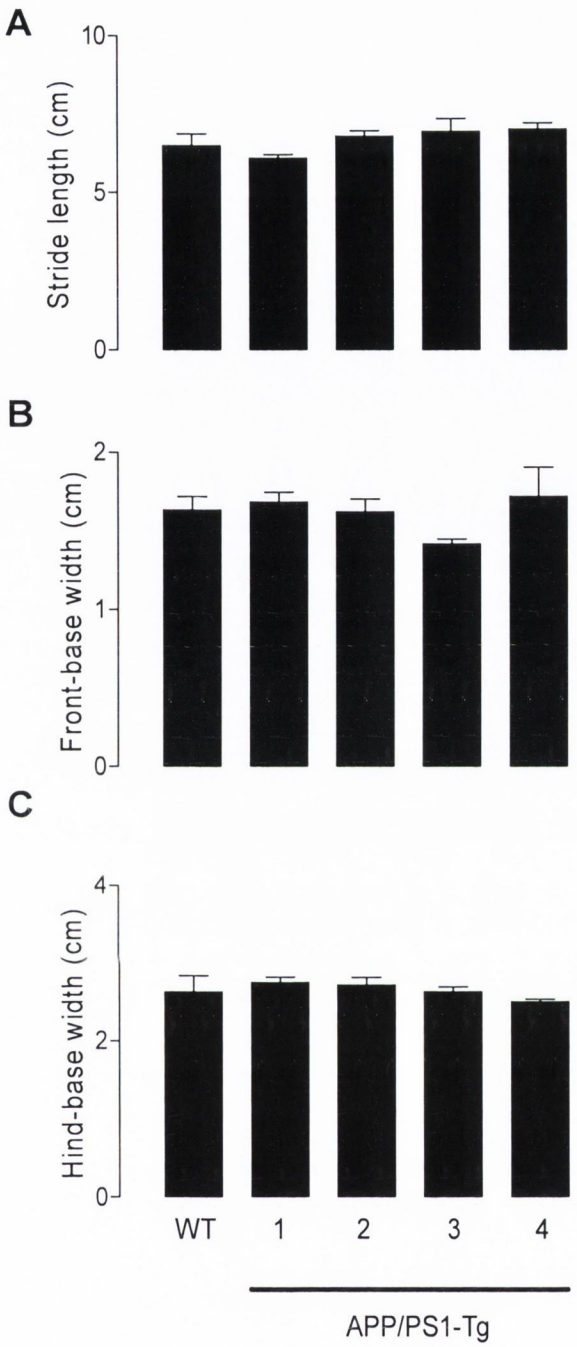


Figure 5.3. Motor co-ordination and balance are not impaired in APPswe/PS1dE9 mice.

The gait of wild-type and APPswe/PS1dE9 (APP/PS1) mice was examined 1 week prior to T cell transfer using footprint analysis. APP/PS1 mice were divided into groups 1-4, which were to be injected with vehicle, Th1, Th2, and Th17 cells respectively. Footprint analysis revealed no sign of impaired motor co-ordination in APPswe/PS1dE9 (APP/PS1) compared with wild-type mice. Stride length (A), front-base width (B), and hind-base width (C), of each mouse were assessed.

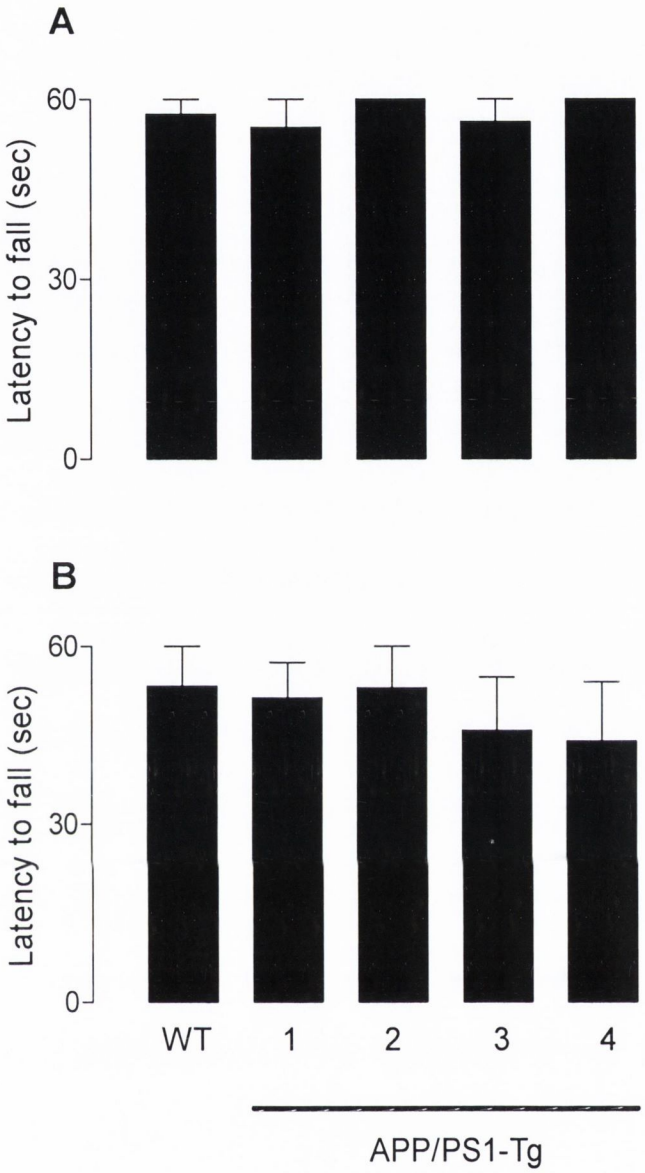


Figure 5.4. Front- and hind-paw strength are not impaired in APPswe/PS1dE9 mice.

Front- and hind-paw strength of APPswe/PS1dE9 (APP/PS1) transgenic mice and wild-type controls were assessed 1 week prior to T cell transfer using the wire-hang test. APP/PS1 mice were divided into groups 1-4, which were to be injected with vehicle, Th1, Th2, and Th17 cells respectively. The test consisted of 2 one-minute trials with an inter-trial interval of 20min. On the first trial, the mice were suspended from the wire by all 4 paws (A), and on the second trial they were suspended by their 2 front paws only (B). The time that the mice held onto the bar (latency) was recorded. Mice that crossed the wire to the poles at either end were given a maximum latency of 60 sec.

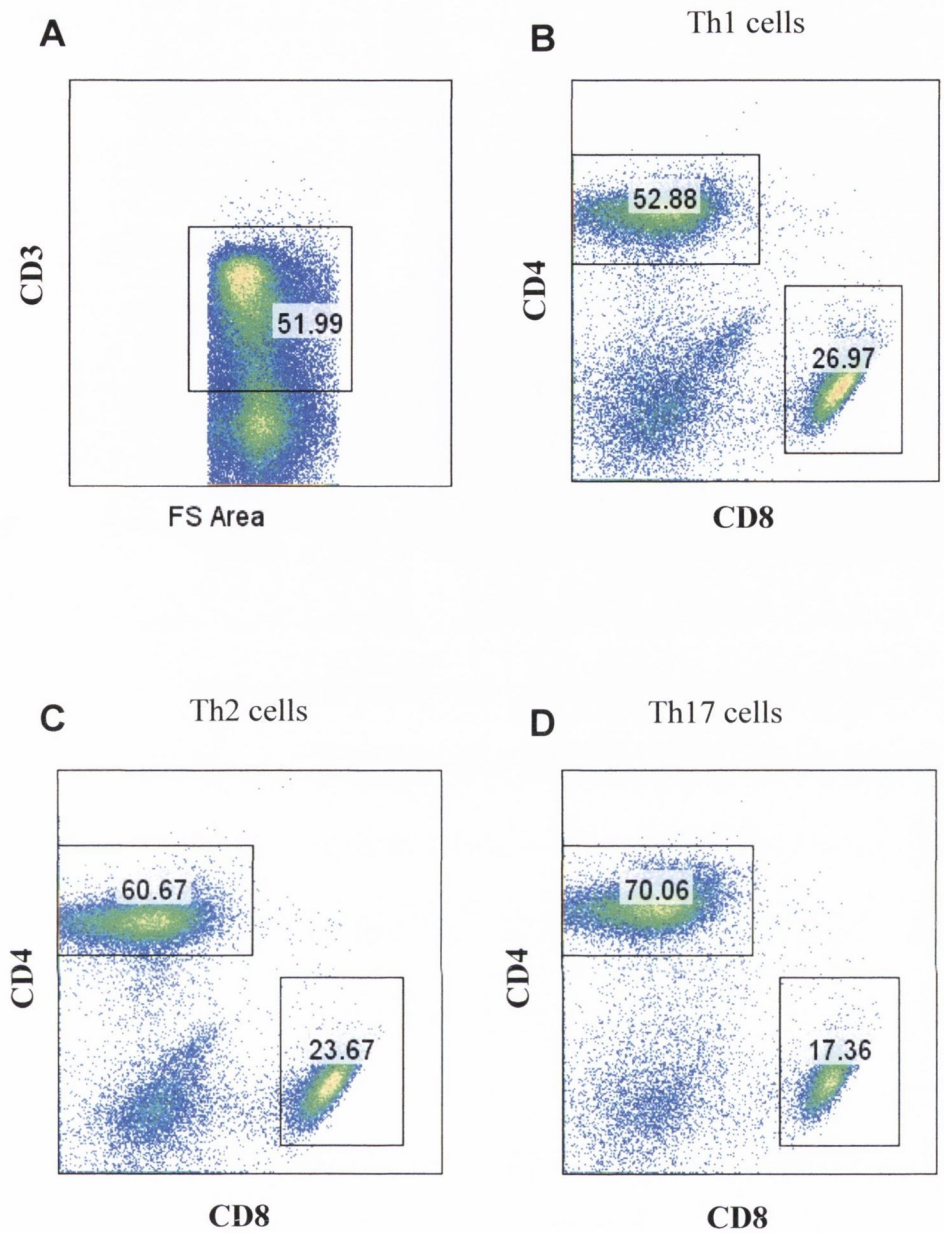


Figure 5.5. Phenotype of T cells injected into transgenic mice.

Wild-type mice were injected subcutaneously in the footpad with Aβ (75 μg/mouse) and CpG (25 μg/mouse). After 21 days, mice were injected again with Aβ and CpG. Seven days later, mice were sacrificed, popliteal lymph nodes were harvested, and the cells were stimulated with Aβ (25 μg/ml) under Th1 polarising conditions with IL-12 (10 ng/ml), under Th2 polarising conditions with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml), or under Th17 polarising conditions with IL-1β (10 ng/ml), IL-23 (10 ng/ml) and anti-IFN-γ (5 μg/ml). After 4 days, the cells were harvested and CD3, CD4, and CD8 expression was analysed by flow cytometry. (A) Percentage of $CD3^+$ cells in cultures of T cell lines. Percentage of $CD4^+$ and $CD8^+$ T cells (gated on CD3) in cultures of Th1 cell lines (B), Th2 cell lines (C), and Th17 cell lines (D). Numbers represent the percentage of cells in each gate.

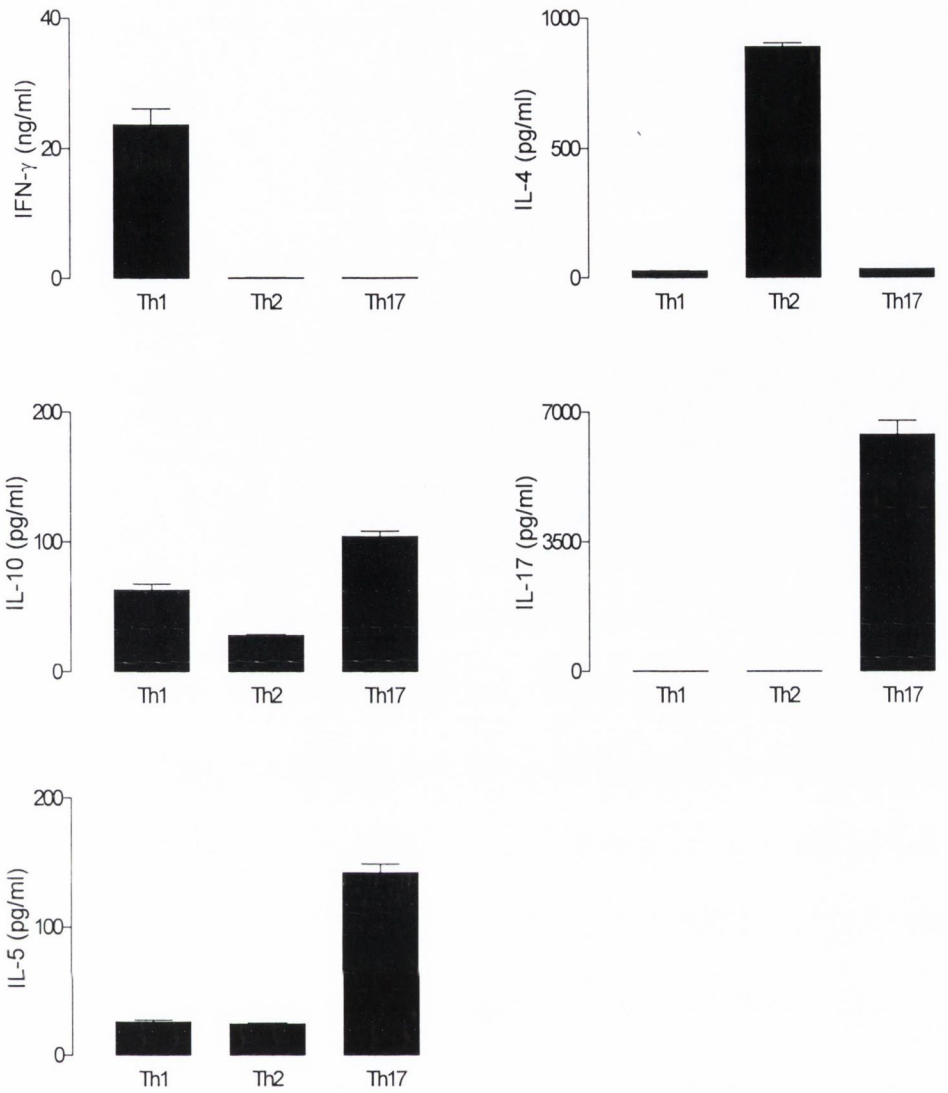


Figure 5.6. Cytokine profile of Th1, Th2, and Th17 polarised T cell lines for adoptive transfer to APPswe/PS1dE9 mice.

Wild-type mice were immunised and T cells polarised as described in Figure 5.5. On day 4 of culture the supernatants were removed for analysis of IFN-γ, IL-4, IL-10, and IL-17 concentration by ELISA. Values are expressed as means ± SEM (n=4).

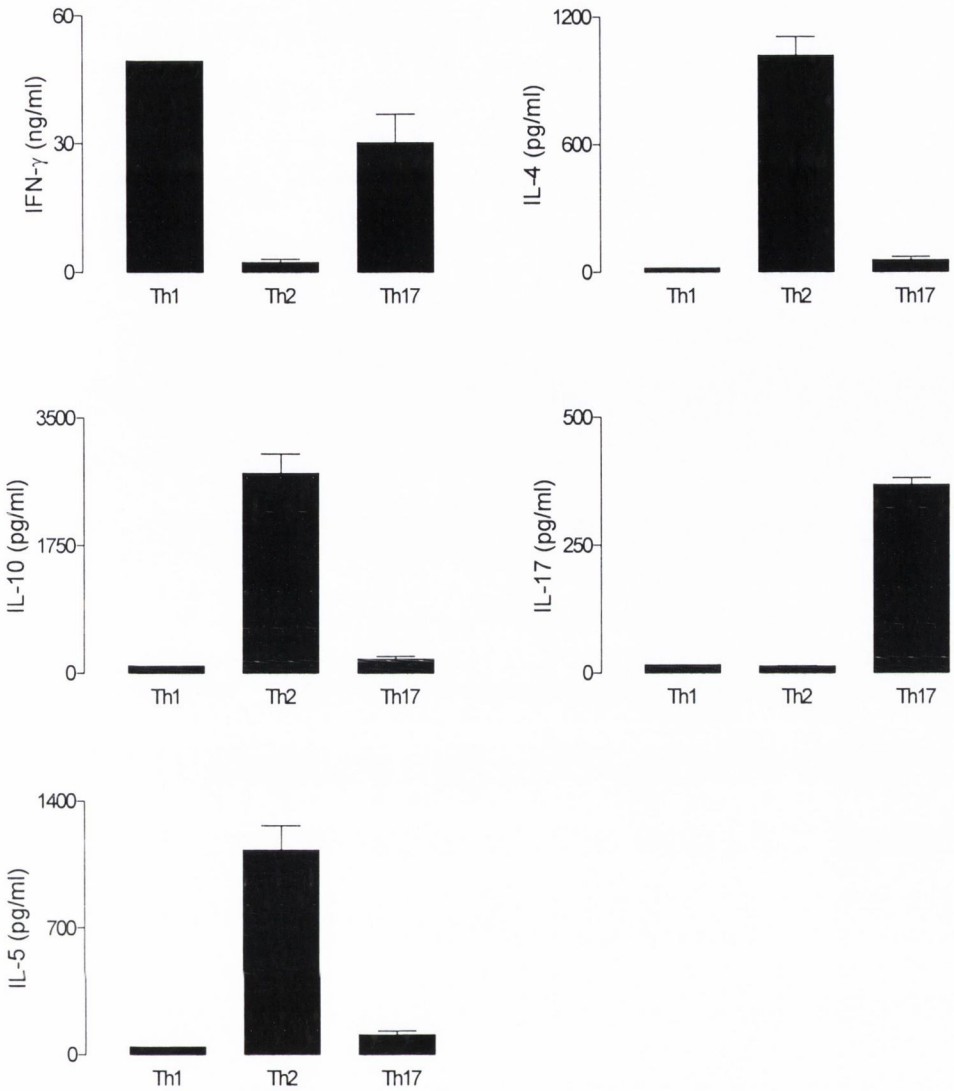


Figure 5.7. Cytokine profile of Th1, Th2, and Th17 polarised T cell lines 4 days after second round of antigen stimulation.

Wild-type mice were immunised and T cells polarised as described in Figure 5.5. On day 4 of culture, when T cells were injected i.v. into APPswe/PS1dE9 mice, 6 wells of Th1, Th2, and Th17 cell lines were retained in culture. Th1 and Th2, but not Th17 cell lines were stimulated with IL-2 (5 ng/ml) on day 4 of culture. IL-4 was added to Th2 polarised cells only. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. On day 4 of this second round of stimulation, T cell supernatants were removed for analysis of concentrations of IFN-γ, IL-4, IL-5, IL-10, and IL-17 by ELISA. Values are expressed as means ± SEM (n=3).

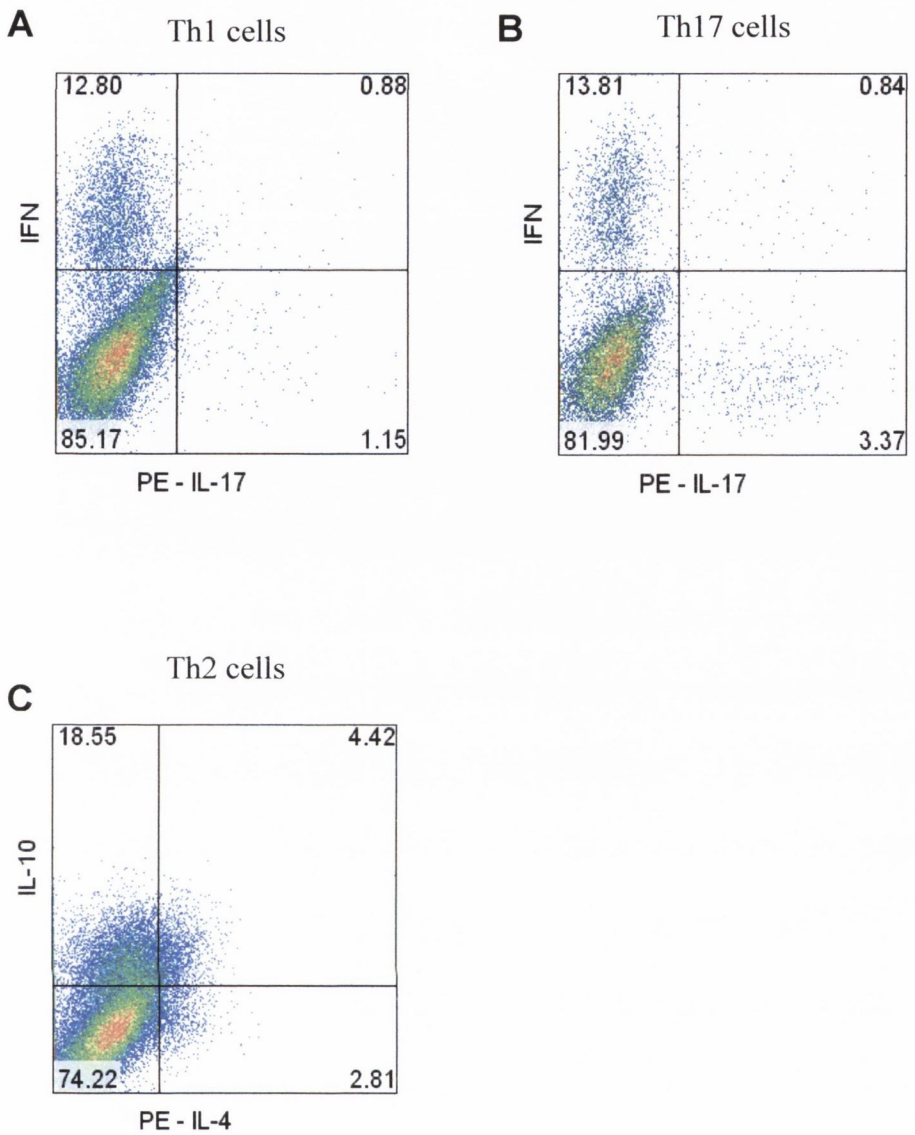


Figure 5.8. Intracellular cytokine staining of Aβ-specific T cell lines after the second round of antigen stimulation.

Wild-type mice were immunised and T cells polarised as described in Figure 5.5. On day 4 of culture, Th1 and Th2, but not Th17 cell lines were stimulated with IL-2 (5 ng/ml). IL-4 was added to Th2 polarised cells only. After a further 7 days the T cells were restimulated with Aβ and irradiated APCs without polarising cytokines. On day 4 of this second round of stimulation, T cell lines were stimulated with a cocktail of PMA (10 ng/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/ml) for 5 h. Th1 (A) and Th17 (B) T cell lines were analysed for expression of IFN-γ and IL-17 on $CD4^+$ cells by flow cytometry. Th2 cell lines (C) were analysed for expression of IL-10 and IL-4 by flow cytometry.

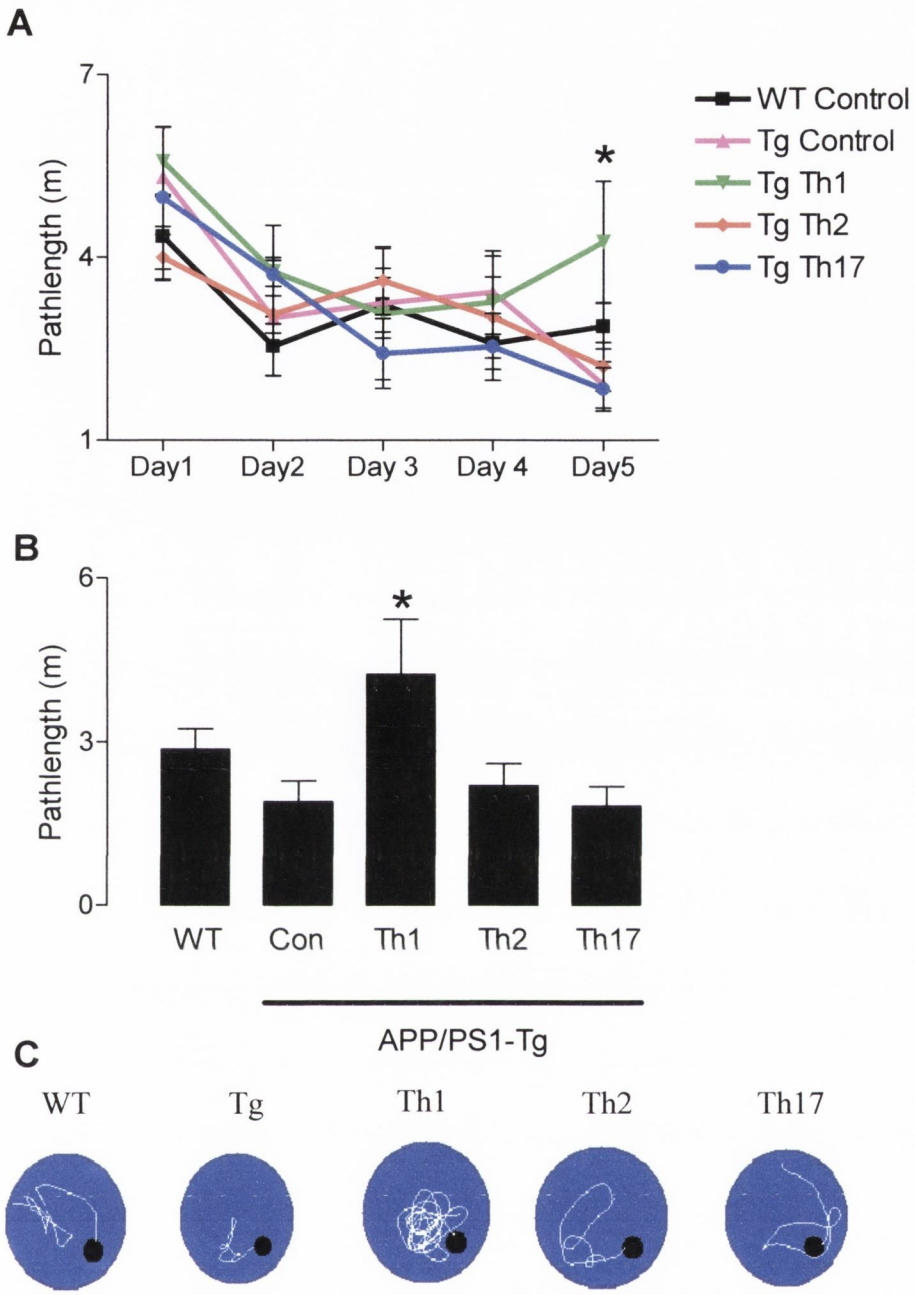


Figure 5.9. Transfer of Aβ-specific Th1 cells to APPswe/PS1dE9 mice increases the pathlength to the platform on day 5 of training in the Morris water maze.

APPswe/PS1dE9 mice were tested for spatial memory in the Morris water maze 2 weeks after administration of Aβ-specific T cells. Following 1 day of habituation in the water maze, mice were trained for 5 consecutive days to find the hidden platform. (A) Pathlength (in metres) to find the platform for all groups over 5 days of training. (B) Pathlength to find the platform on day 5 of training. (C) Representative traces of path taken by mice in each treatment group on day 5. * $p<0.05$ versus control-treated transgenic mice (one-way ANOVA). Values are expressed as means ± SEM (n=5-6).

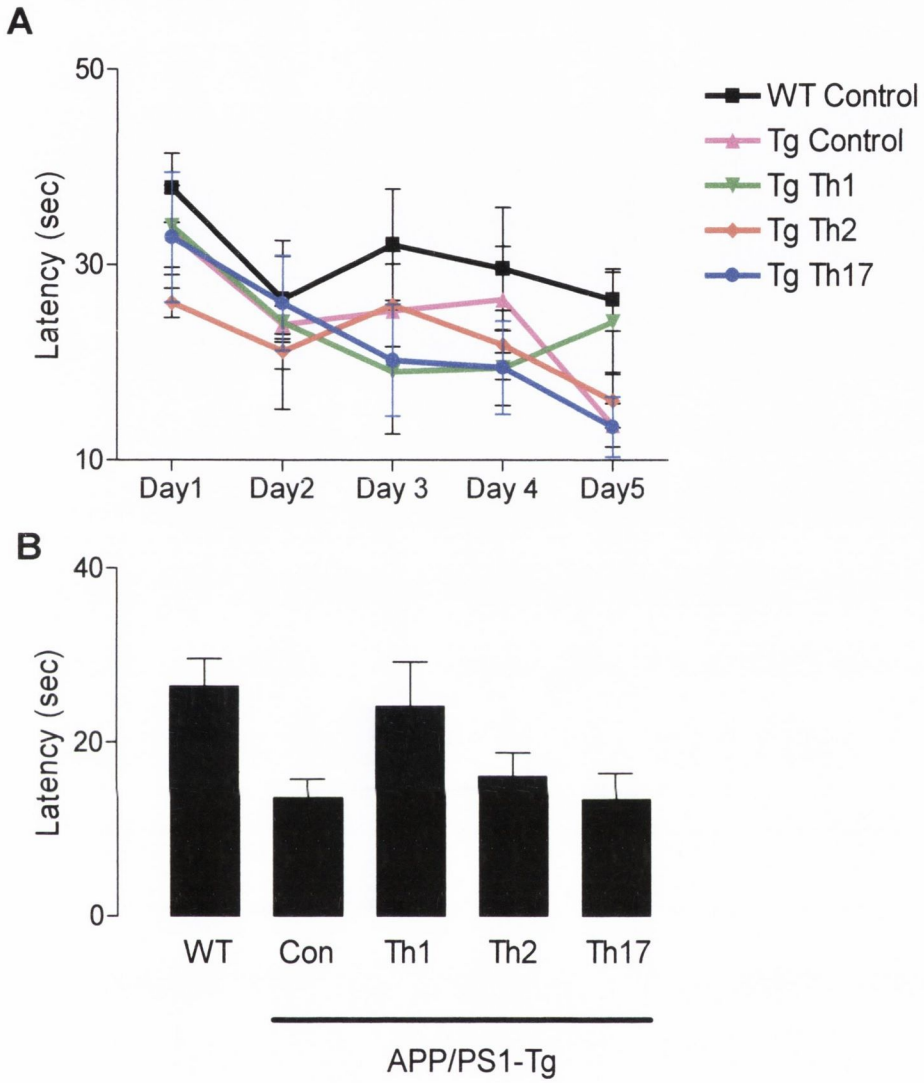


Figure 5.10. Aβ-specific T cell transfer has no effect on latency to platform in APPswe/PS1dE9 mice.

APPswe/PS1dE9 mice were tested for spatial memory in the Morris water maze two weeks after administration of Aβ-specific T cells. Following 1 day of habituation in the water maze, mice were trained for 5 consecutive days to find the hidden platform. (A) Latency (in seconds) to find the platform for all groups over 5 days of training. (B) Latency to find the platform on day 5 of training. Values are expressed as means ± SEM (n=5-6).

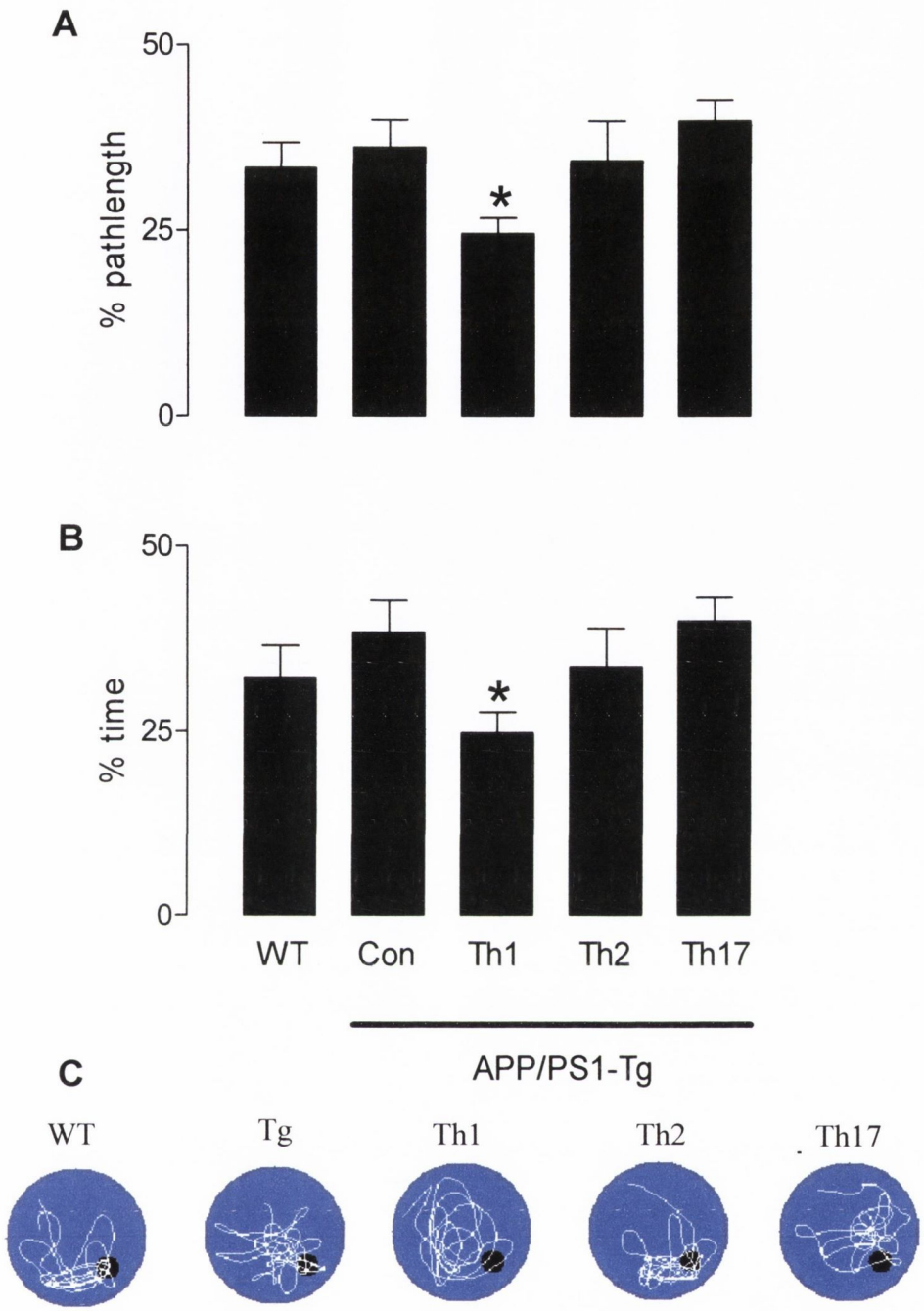


Figure 5.11. Transfer of Aβ-specific Th1 cells to APPswe/PS1dE9 mice results in impaired performance in the probe trial of the Morris water maze.

APPswe/PS1dE9 mice were tested for spatial memory in the Morris water maze two weeks after administration of Aβ-specific T cells. The day after the final day of training, the platform was removed and mice were given a single 60-sec probe trial. The percentage of time (A) and pathlength (B) each animal spent swimming in the quadrant previously containing the platform was measured. (C) Representative traces of path taken by mice in each treatment group on day 5. * $p<0.05$ vs control-treated transgenic mice (one-way ANOVA). Values are expressed as means ± SEM (n=5-6).

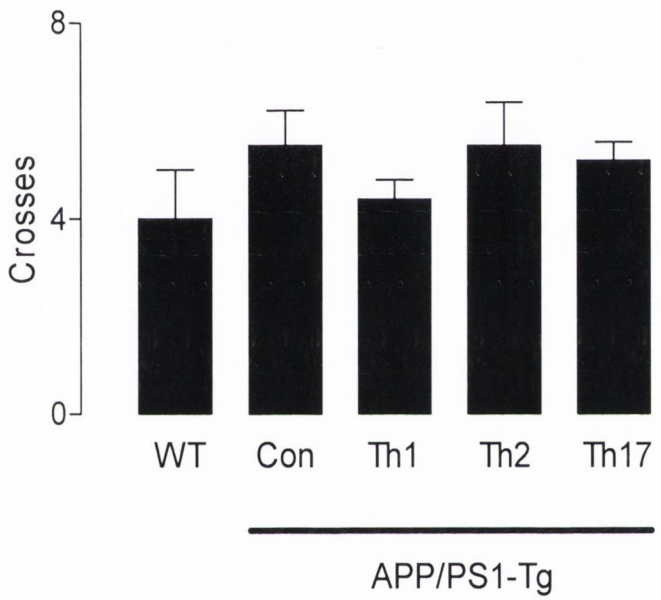


Figure 5.12. Number of crosses over platform is unchanged in treated mice.

APPswe/PS1dE9 mice were tested for spatial memory in the Morris water maze two weeks after administration of Aβ-specific T cells. The day after the final day of training, the platform was removed and mice were given a single 60-sec probe trial. The number of times each animal swam across the area previously containing the platform was measured. Values are expressed as means ± SEM (n=5-6).

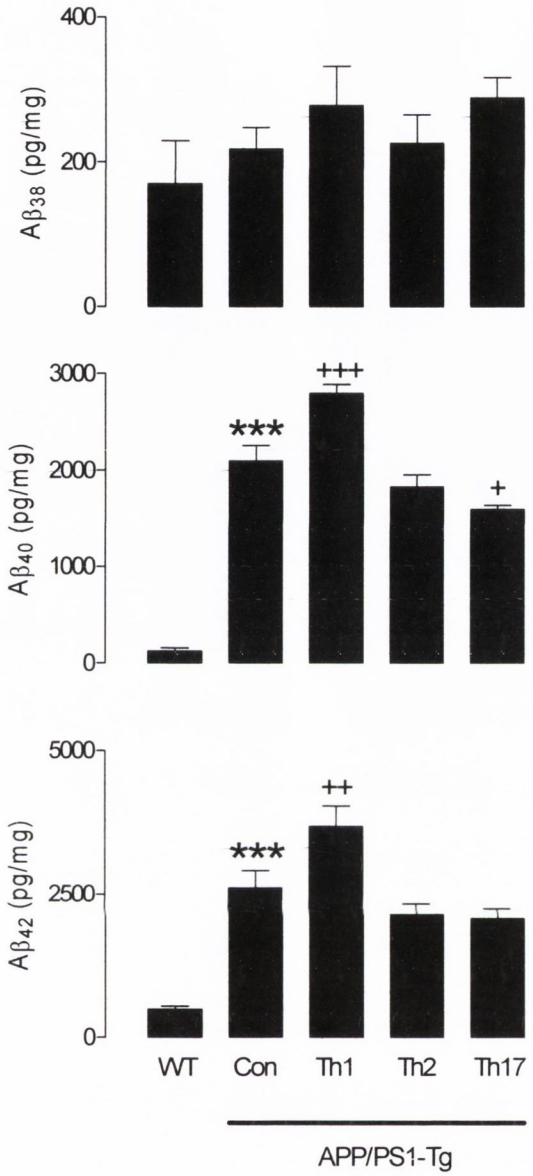


Figure 5.13. Aβ-specific Th1 cells increase soluble $A\beta_{40}$ and $A\beta_{42}$ concentration, whereas Aβ-specific Th17 cells decrease soluble $A\beta_{40}$ concentration in the cortex of APPswe/PS1dE9 mice.

Soluble $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ concentrations in the cortex of wild-type and APPswe/PS1dE9 mice were determined by multi-spot ELISA. *** $p<0.001$ versus wild-type mice; $^{+}$ $p<0.05$, $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$ versus control-treated transgenic mice (one-way ANOVA). Values are expressed as means ± SEM (n=5-6).

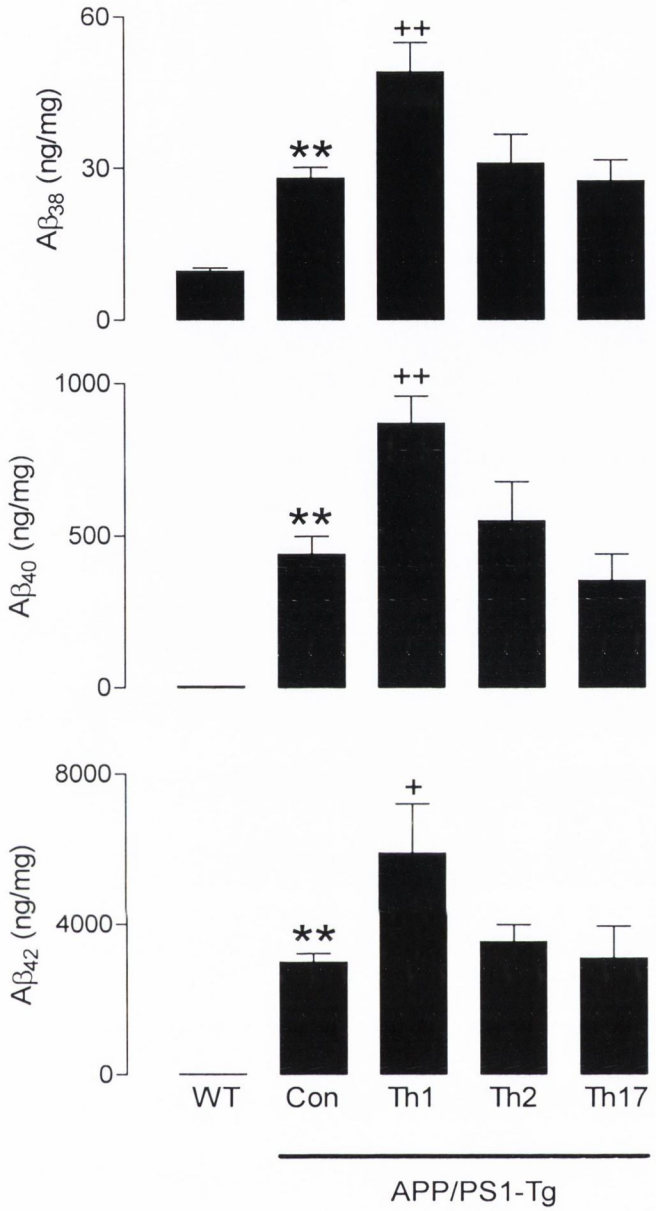


Figure 5.14. Aβ-specific Th1 cells increase insoluble $A\beta_{40}$ and $A\beta_{42}$ concentration in the cortex of APPswe/PS1dE9 mice.

Insoluble $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ concentrations in the cortex of wild-type and APPswe/PS1dE9 mice were determined by multi-spot ELISA. ** $p<0.01$ versus wild-type mice; $^{+}$ $p<0.05$, $^{++}$ $p<0.01$ versus control-treated transgenic mice (one-way ANOVA). Values are expressed as means ± SEM (n=5-6).

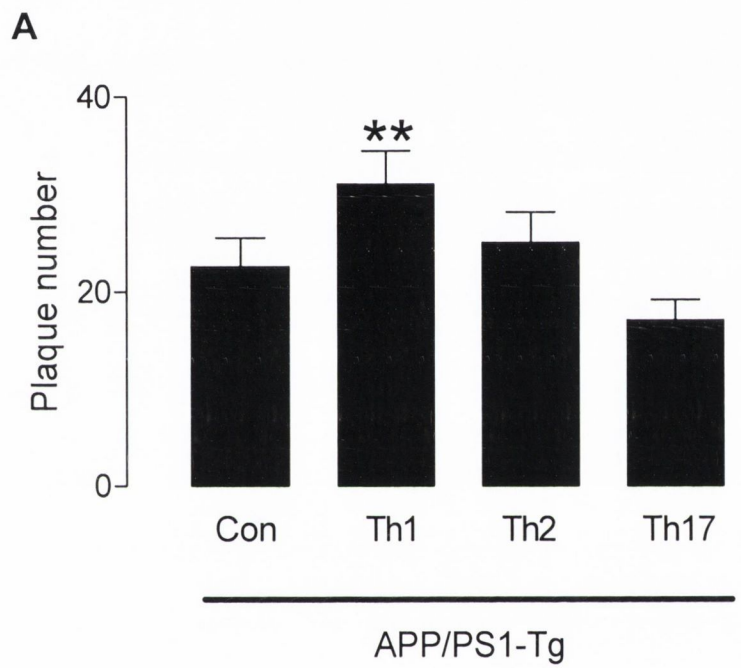
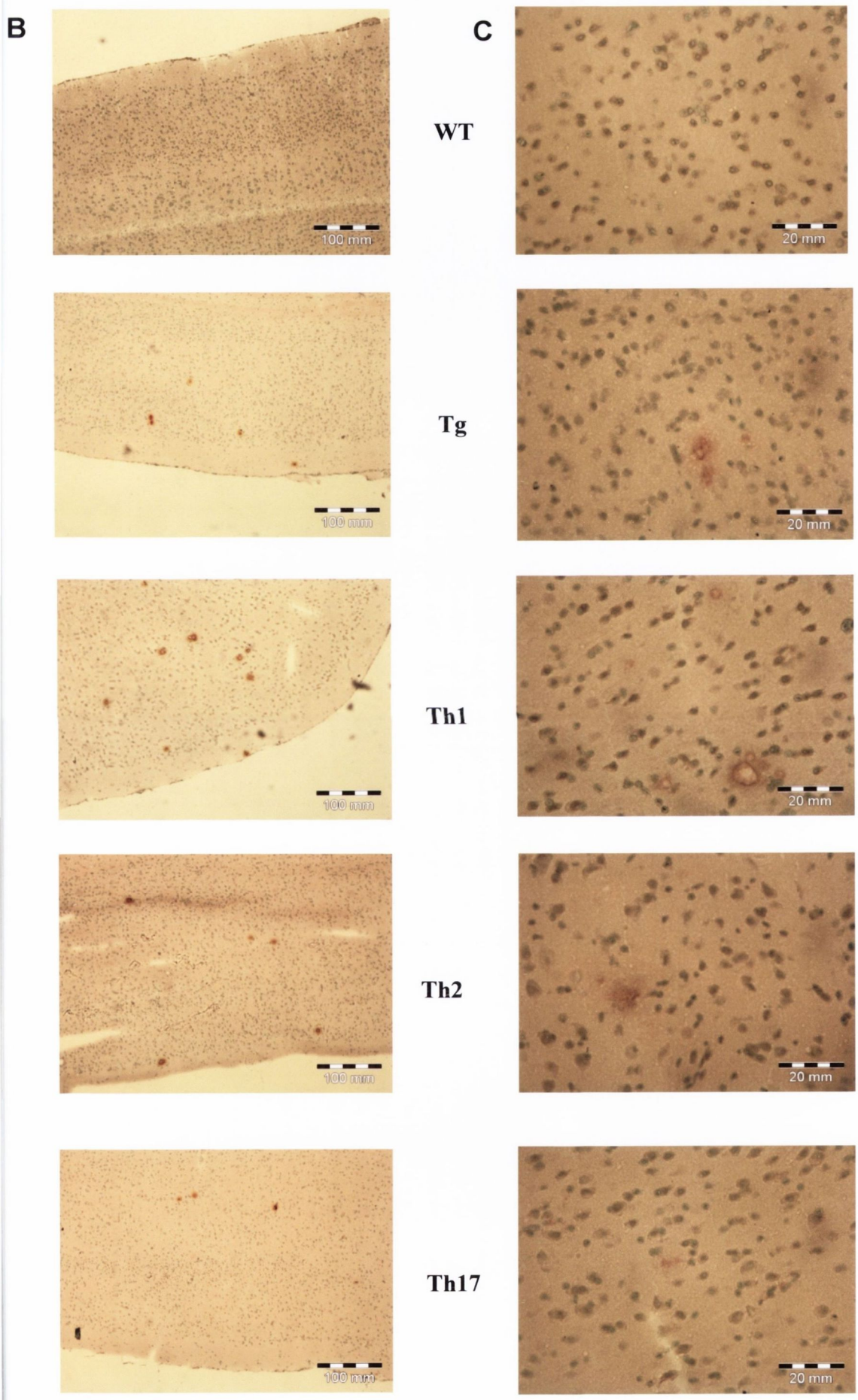


Figure 5.15. Aβ-specific Th1 cells increase the number of Congo red-positive Aβ plaques in the brains of transgenic mice.

Cryostat sections prepared from APPswe/PS1dE9 mice treated with Aβ-specific T cells were stained with Congo red for detection of Aβ plaques in the brain. (A) Average counts of plaque number in the entire section across groups. (B and C) Representative images of Congo red stained sections from each group; (B) 4x magnification, (C) 20x magnification. ** $p<0.01$ versus Th17-treated transgenic mice (one-way ANOVA). Values are expressed as means ± SEM (n=5-6).


B
C
WT
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Th17
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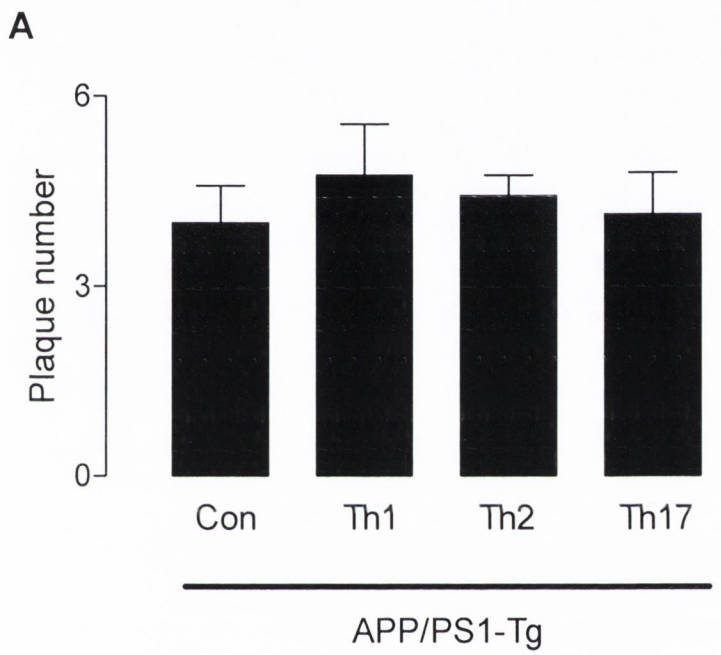
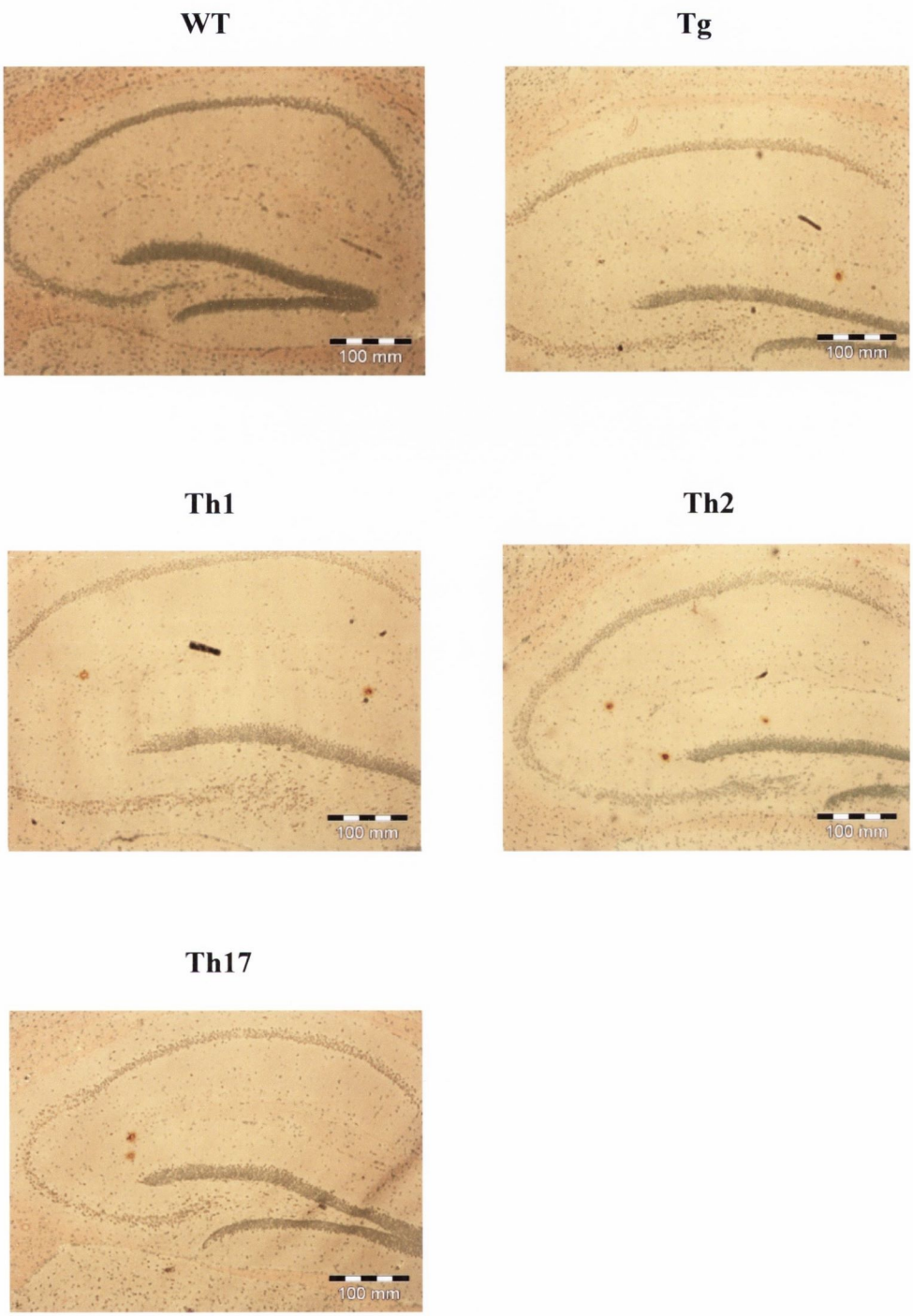


Figure 5.16. T cell transfer has no effect on Congo red-positive Aβ plaques in the hippocampus of transgenic mice.

Cryostat sections prepared from APPswe/PS1dE9 mice treated with Aβ-specific T cells were stained with Congo red for detection of Aβ plaques in the hippocampus. (A) Average counts of plaque number in the hippocampus across groups. (B) Representative images of Congo red stained hippocampal sections from each group. (4x magnification) Values are expressed as means ± SEM (n=5-6).

B



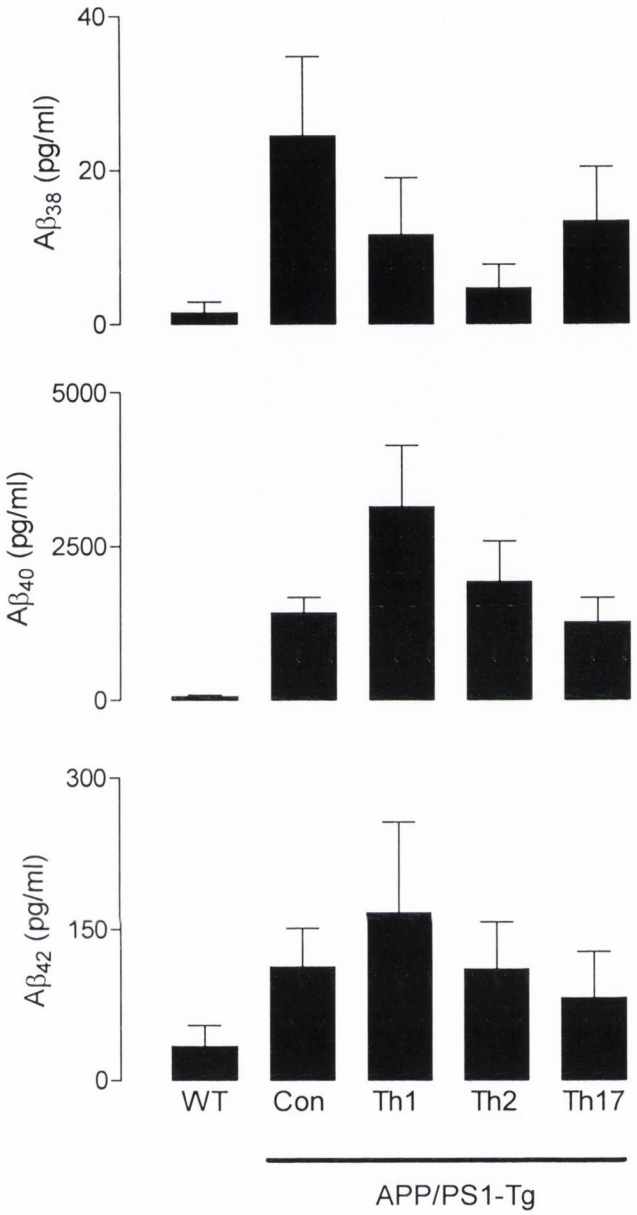


Figure 5.17. Concentration of Aβ in blood of mice treated with Th1, Th2, or Th17 cells.

Serum taken from wild-type and APPswe/PS1dE9 mice was analysed for concentrations of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ by multi-spot ELISA. Values are expressed as means ± SEM (n=5-6).

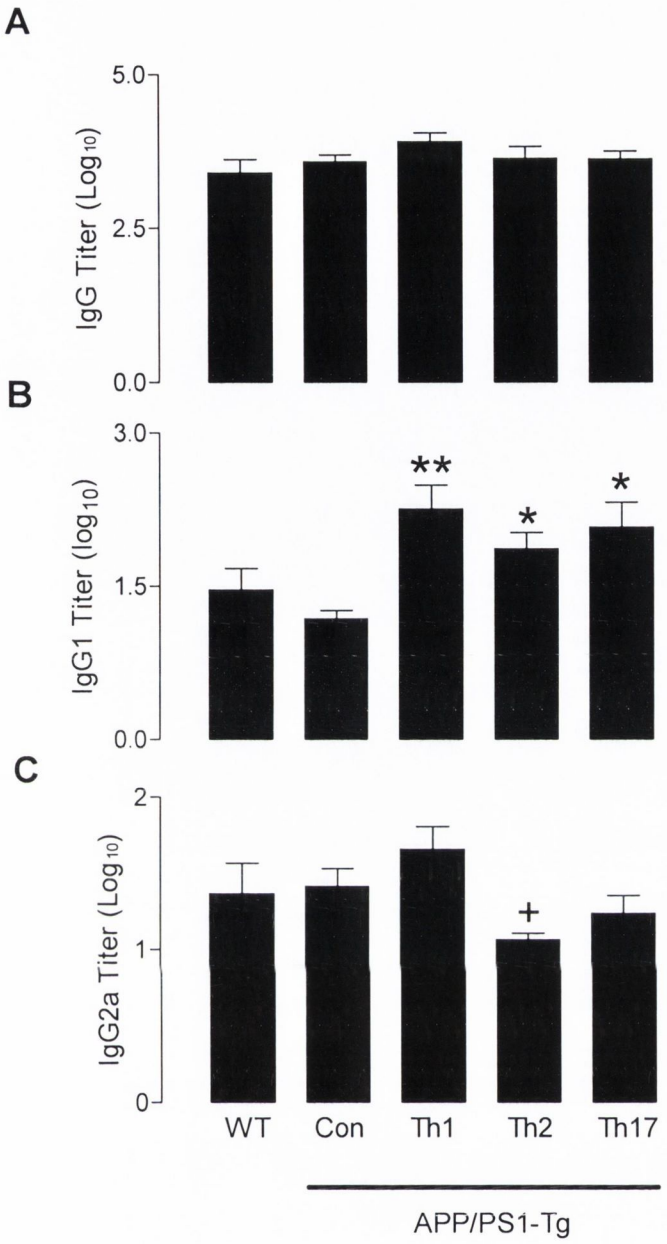


Figure 5.18. Treatment with Aβ-specific T cells enhances Aβ-specific IgG1 production.

APPswe/PS1dE9 mice were treated with Aβ-specific Th1, Th2, and Th17 cells. Mice were sacrificed 4 weeks after T cell transfer and serum was taken and analysed for Aβ-specific serum antibody total IgG (A), IgG1 (B), and IgG2a (C). * $p<0.05$, ** $p<0.01$ versus control-treated transgenic mice; $^{+}$ $p<0.05$ versus Th1-treated transgenic mice (one-way ANOVA). Values are expressed as means ± SEM (n=5-6).

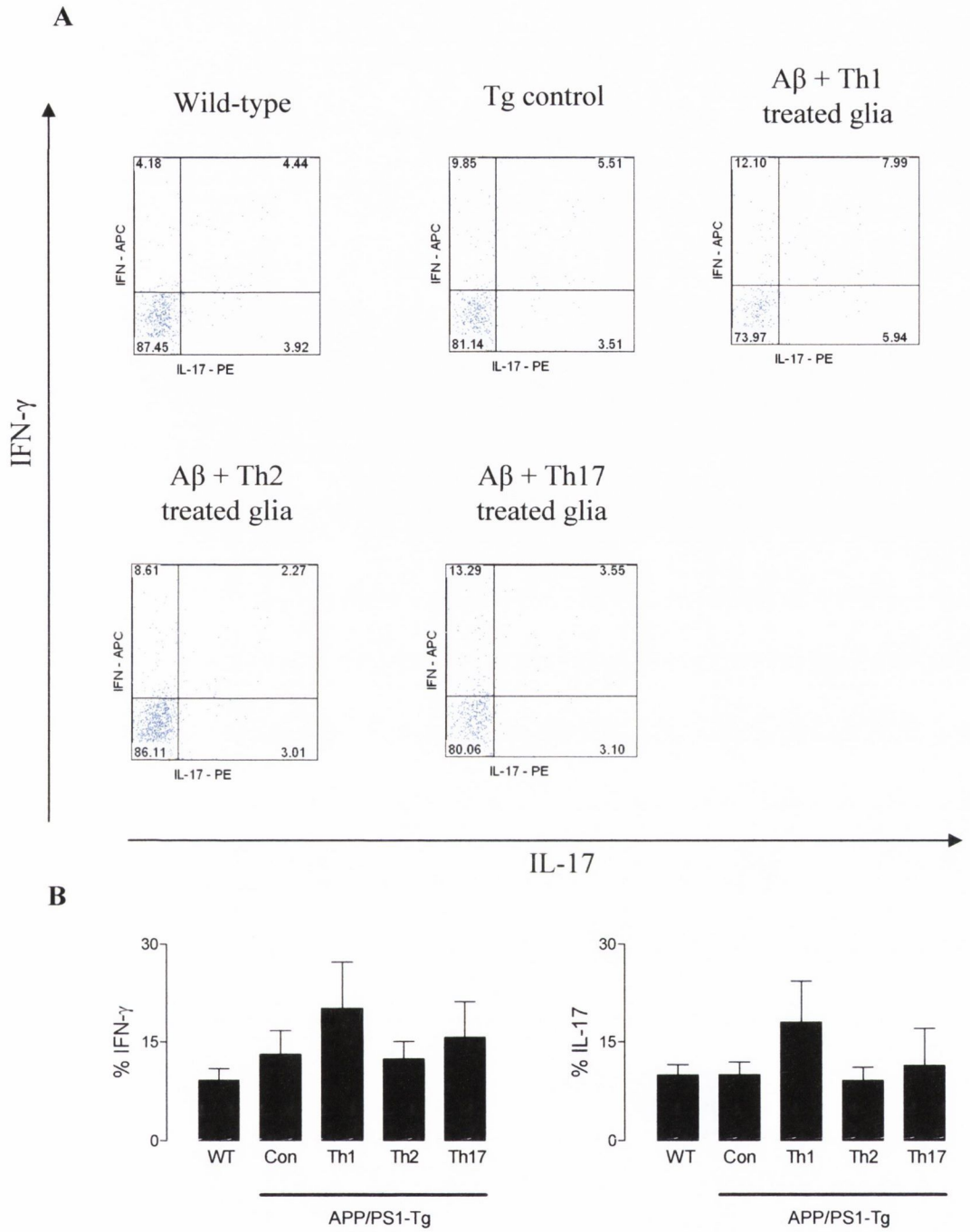


Figure 5.19. Expression of IFN-γ and IL-17 on $CD3^+$ cells in the brains of wild-type and APPswe/PS1dE9 mice.

Following treatment of APPswe/PS1dE9 mice with Aβ-specific T cells, mice were perfused, hemisected brains from 2 mice were pooled, and mononuclear cells were isolated from the CNS using Percoll gradients. (A) Representative dotplots are shown from each treatment group. (B) $CD3^+$ cells expressing IFN-γ or IL-17 were quantified by FACS and expressed as a percentage of the total population of cells expressing CD3 (n=3).

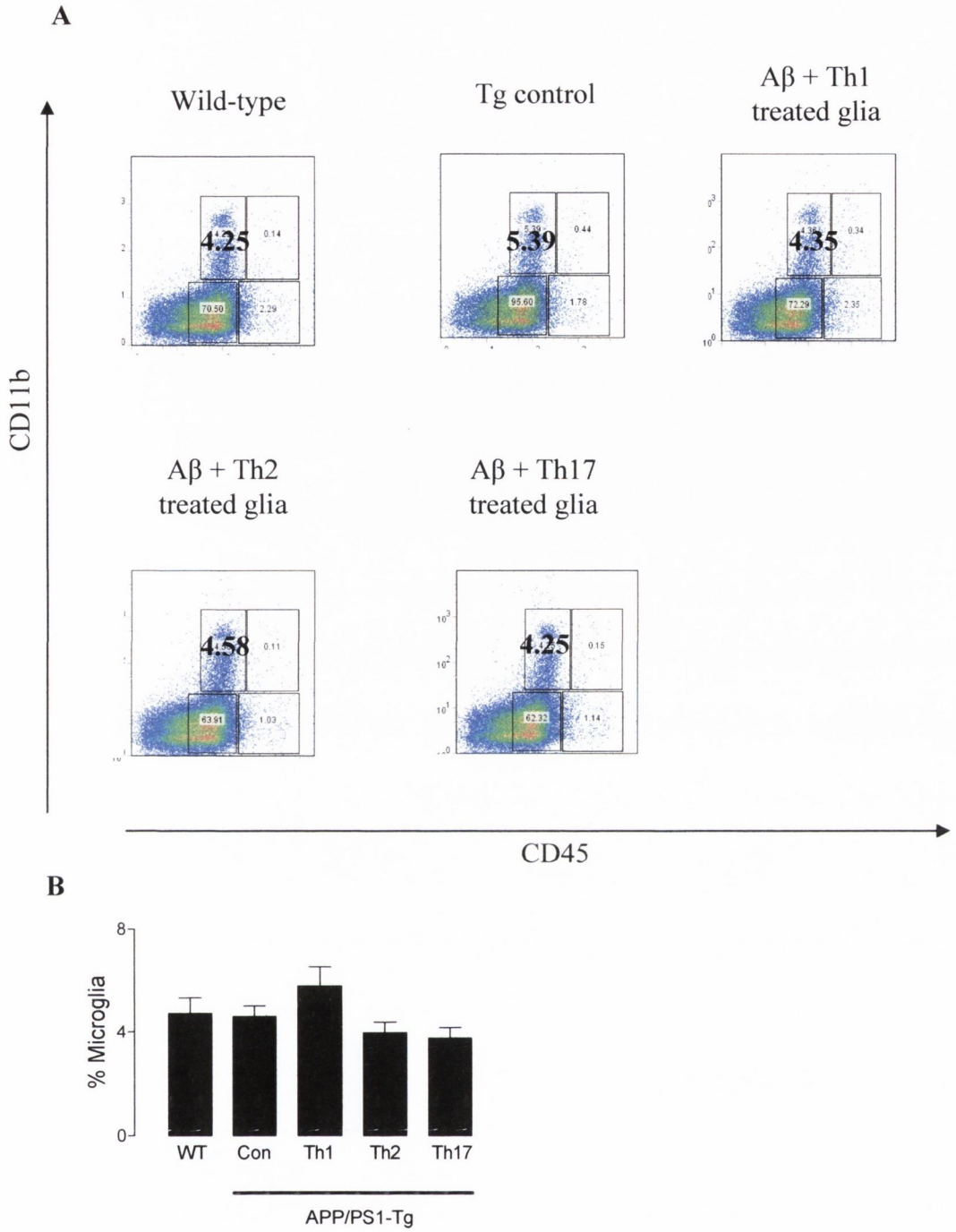


Figure 5.20. The percentage of microglia in the brain is unaffected by treatment with Aβ-specific T cells.

Following treatment of APPswe/PS1dE9 mice with Aβ-specific T cells, mice were perfused, hemisected brains from 2 mice were pooled, and mononuclear cells were isolated from the CNS using Percoll gradients. (A) Representative dotplots are shown from each treatment group. (B) $CD11b^{+}$ $CD45^{inter}$ cells were quantified by FACS and expressed as a percentage of the total population of cells in the forward scatter/side scatter gate (n=3).

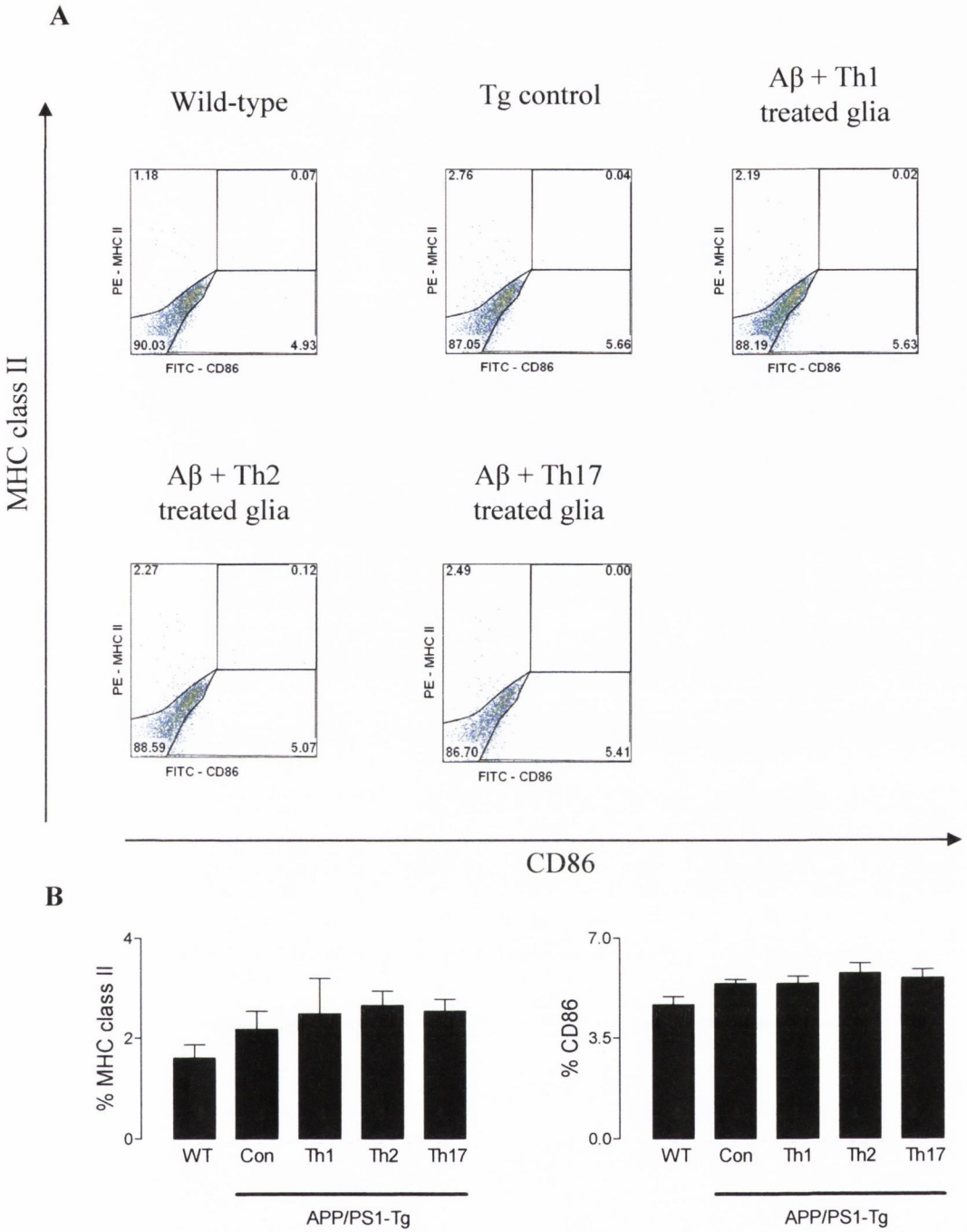


Figure 5.21. Microglial expression of MHC class II and CD86 is unaffected by treatment with Aβ-specific T cells.

Following treatment of APPswe/PS1dE9 mice with Aβ-specific T cells, mice were perfused, hemisected brains from 2 mice were pooled, and mononuclear cells were isolated from the CNS using Percoll gradients. (A) Representative dotplots are shown from each treatment group (B) $CD11b^{+}$ $CD45^{inter}$ cells expressing MHC class II or CD86 were quantified by FACS and expressed as a percentage of the total population of $CD11b^{+}$ $CD45^{inter}$ cells (n=3).

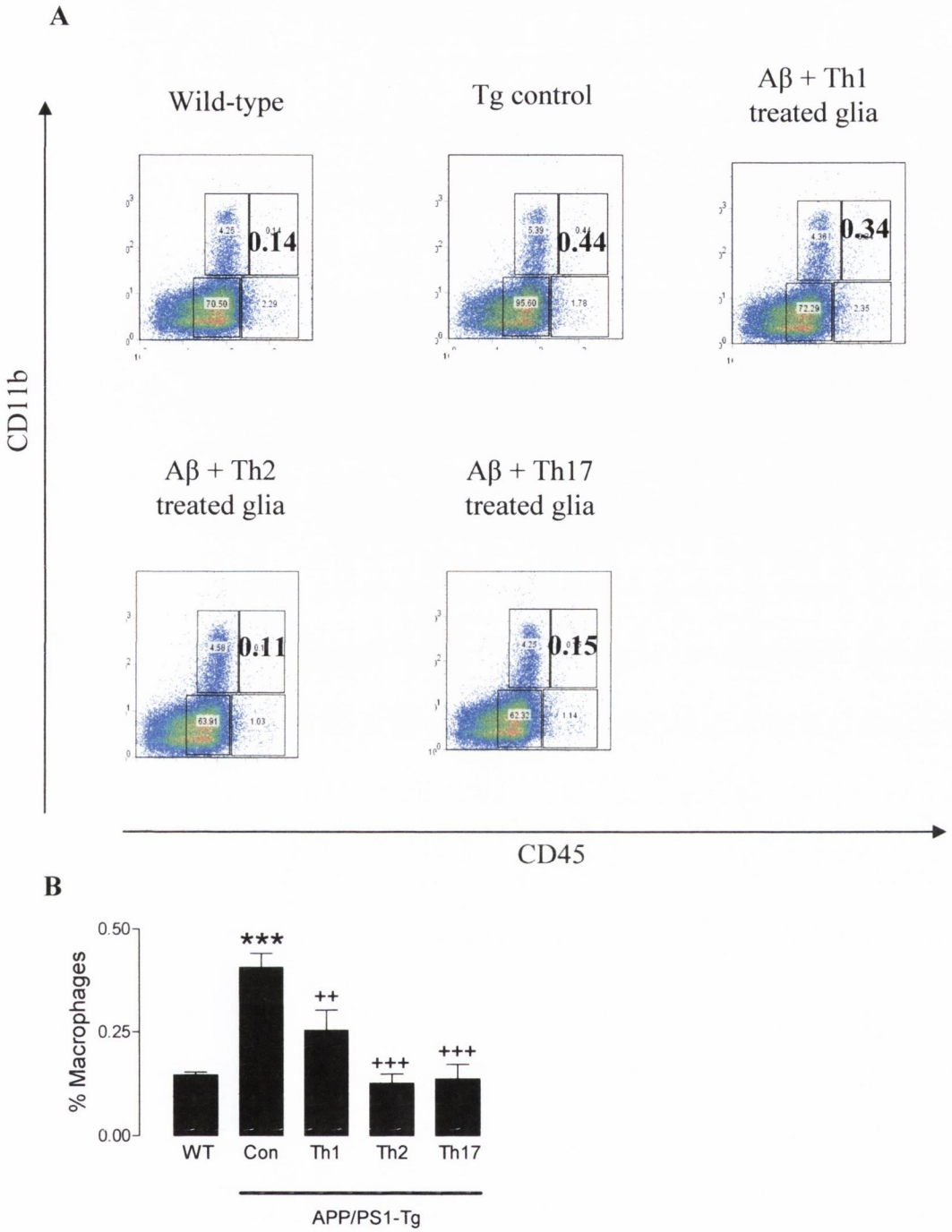


Figure 5.22. Treatment with Aβ-specific T cells attenuates the increase in infiltrating macrophages seen in APPswe/PS1dE9 mice.

Following treatment of APPswe/PS1dE9 mice with Aβ-specific T cells, mice were perfused, hemisected brains from 2 mice were pooled, and mononuclear cells were isolated from the CNS using Percoll gradients. (A) Representative dotplots are shown from each treatment group. (B) $CD11b^{+}$ $CD45^{hi}$ cells were quantified by FACS and expressed as a percentage of the total population of cells in the forward scatter/side scatter gate. *** $p<0.001$ versus cells isolated from wild-type mice; $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$ versus cells from control-treated transgenic mice. Values are expressed as means ± SEM (n=3).

Chapter 6

Hypothesis

Little is currently known about the role of T cells in AD. Though there is currently no evidence of a T cell response in the brains of AD patients, it has been shown that activated T cells of any specificity can penetrate the BBB (Wekerle *et al.* 1987), raising the possibility that they may be involved in CNS diseases, such as AD. Indeed, a number of studies have shown differences between healthy controls and AD patients in their ability to generate immune responses against Aβ (Du *et al.* 2001, Loewenbrueck *et al.* 2008, Trieb *et al.* 1996).

Chronic inflammatory activation of microglia is thought to be detrimental in AD. In support of this, microglial expression of proinflammatory cytokines has been shown to be increased in the CNS of AD patients, particularly in the areas of Aβ plaque deposition (Griffin *et al.* 1995, Lue *et al.* 2001a). In addition, treatment of cultured microglia with Aβ induces production of IL-1β, IL-6, and TNF-α (Meda *et al.* 1995, Gasic-Milenkovic *et al.* 2003). Previous work has shown that co-culture of microglia and Th1 cells induced microglial expression of MHC class II and co-stimulatory molecules, while also inducing IFN-γ and IL-2 production by the T cells (Aloisi *et al.* 1998). This thesis set out to test the hypothesis that distinct T cell subsets differentially modulate Aβ-induced microglial activation, and that this would result in alterations in Aβ plaque burden and cognitive function in a transgenic mouse model of AD. Specifically, it was postulated that administration of Aβ-specific Th1 and Th17 cells would enhance proinflammatory cytokine production by microglia, thus exacerbating symptoms of AD, while administration of Aβ-specific Th2 cells would result in decreased plaque pathology and improved cognitive function.

Th1 cells are generally considered to be proinflammatory, and their prototypic cytokine, IFN-γ, is a potent inducer of proinflammatory cytokine secretion by microglial cells, especially when in the presence of Aβ (Meda *et al.* 1995, Goodwin *et al.* 1995). The role of Th17 cells in AD has not yet been examined. However, Th17 cells are associated with the pathogenesis of a number of inflammatory diseases, such as MS and rheumatoid arthritis (Chabaud *et al.* 1999, Langrish *et al.* 2005). It was thus hypothesised that these cells would increase proinflammatory cytokine production in the brain, leading to increased neuronal death. In contrast to Th1 and Th17 cells, Th2 cells are generally considered to be anti-inflammatory. The Th2-type cytokine, IL-4, is down-regulated in the cerebral cortex of APP-transgenic mice (Abbas *et al.* 2002), and IL-4 and IL-10 have previously been shown to attenuate production of proinflammatory cytokines by microglia in response to LPS and Aβ (Chao *et al.* 1993, Sawada *et al.* 1999, Szczepanik *et al.* 2001).

The data presented here show that microglia act as an effective APC for Aβ-specific Th1 and Th17 cells, inducing production of IFN-γ and IL-17 by Th1 and Th17 cells respectively. In addition, co-culture of Aβ-specific Th1 or Th17 cells with glia resulted in increased production of IL-1β, IL-6, and TNF-α, and also enhanced expression of MHC class II, CD40, CD80, and CD86 on microglia. This indicates that the presence of these T cell subsets in the CNS is likely to induce a proinflammatory environment in the brain. Transfer of Aβ-specific Th1 cells to APPswe/PS1dE9 transgenic mice increased Aβ burden and led to impairments in cognitive function, suggesting that an inflammatory environment in the CNS exacerbates the progression of AD. However, despite their ability to enhance Aβ-induced proinflammatory cytokine production from microglia, Aβ-specific Th17 cells did not increase levels of Aβ in the brains of APP-transgenic mice, nor did they induce impairments in spatial memory. The reason for this disparity between the effects of Th1 and Th17 cells on Aβ levels is unknown, though it may be related to the fact that IFN-γ has been shown to upregulate β-secretase expression, leading to increased production of Aβ (Cho *et al.* 2007).

In contrast to the effect of Th1 and Th17 cells on Aβ-induced microglial activation, Th2 cells induced significantly less proinflammatory cytokine production and co-stimulatory molecule expression when incubated with glia. Indeed, Aβ-specific Th2 cells were found to attenuate production of IL-1β and IL-6 induced by co-culture of glia and Th17 cells. IL-4 and IL-10 were found to attenuate Aβ and IFN-γ-induced increases in glial proinflammatory cytokine production and co-stimulatory molecule expression on microglia. Others have shown that IL-4 and IL-10 induce microglial phagocytosis of Aβ (Koenigsknecht-Talboo & Landreth 2005, Michelucci *et al.* 2009). It was thus hypothesised that Aβ-specific Th2 cells would be capable of down-regulating proinflammatory cytokine production, and increasing phagocytosis by microglia *in vivo*, leading to decreased plaque levels and improvements in memory in APP-transgenic mice. The present set of studies have shown that Aβ-specific Th2 cells had no effect on either cognitive function or Aβ levels in APPswe/PS1dE9 mice. This indicates that Aβ-specific Th2 cells do not stimulate microglial phagocytosis of Aβ *in vivo*.

The results presented here show that Aβ-specific Th1 cells increase Aβ burden and thus induce impairments in cognitive function in APP-transgenic mice. As IFN-γ is implicated in mediating the effects of Th1 cells, future research will examine whether blocking IFN-γ by administration of a neutralising antibody can inhibit the effects of Aβ-specific Th1 cells on memory and Aβ burden in APPswe/PS1dE9 mice. It will also be highly important to study the effect of Aβ-specific T cell subsets on microglial phagocytosis of Aβ, and to examine the relationship between proinflammatory cytokine production and phagocytosis by microglia.

Chapter 7

General discussion

7.1. General Discussion

Alzheimer's disease is the most common neurodegenerative disease, currently affecting between 25 and 34 million individuals worldwide (Williams 2009). AD is clinically characterised by slow but progressive impairments in memory and other cognitive functions, with diagnosis relying primarily on excluding other dementias (McKhann *et al.* 1984). The most obvious risk factor for AD is aging: approximately 1 percent of individuals aged 65 to 69 years have AD, but this increases to between 40 and 50 percent in persons over 95 years of age (Hy & Keller 2000). Apart from aging, other risk factors associated with disease include reduced mental and physical activity late in life, low educational and occupational attainment, coronary heart disease, smoking, and obesity (Blennow *et al.* 2006). There is no cure for AD, and current treatments are largely symptomatic. These include acetylcholinesterase inhibitors, which keep levels of the neurotransmitter acetylcholine high in the brain, and memantine, an NMDA antagonist, which blocks excessive NMDA receptor activity, thus preventing excitotoxic cell death (Chen & Lipton 1997).

The dominant theory for the development of AD is the amyloid cascade hypothesis (Hardy & Allsop 1991, Hardy & Higgins 1992). According to this hypothesis, an increase in Aβ in the brain is the initiating event in AD, and triggers subsequent tau tangle formation, microglial activation, and eventually neuronal loss, resulting in cognitive decline. Support for the amyloid cascade hypothesis comes from studies of pathology, genetics, and biochemistry of AD.

All 4 genes known to be involved in the development of AD either increase the production (APP, PS1, PS2) or deposition (ApoE4) of Aβ (Citron *et al.* 1997, Selkoe 2001, Schmechel *et al.* 1993). Patients with Down's syndrome, who possess a third copy of the APP gene, develop Aβ plaques and neuropathology at a much earlier age than AD patients (Lemere *et al.* 1996). Furthermore, fibrillar Aβ has been shown to be neurotoxic both *in vitro* and *in vivo*, and reversal of aggregation can prevent this toxicity (Pike *et al.* 1993, Urbanc *et al.* 2002).

As Aβ is postulated to be the primary neuropathological insult in AD, the majority of current therapies have focused either on inhibiting production or aggregation of Aβ, or increasing clearance of Aβ from the brain. One of the most promising therapeutic strategies being explored for the treatment of AD is Aβ immunotherapy. Immunotherapy of AD incorporates an array of strategies designed to modulate the immune system to induce clearance of Aβ. The first paper reporting the use of immunotherapy in AD was by Schenk and colleagues, who showed that active immunisation of APP-transgenic mice with Aβ in CFA prevented Aβ deposition in young mice, and induced clearance of Aβ in older animals (Schenk *et al.* 1999). This immunisation protocol was subsequently demonstrated to prevent cognitive impairment in the PDAPP mouse model of AD (Morgan *et al.* 2000, Janus *et al.* 2000). Peripheral injections of Aβ-specific antibodies were found to have similar effect on memory and Aβ burden, and it has been suggested that clearance of Aβ in active immunisation studies was primarily due to the induction of a humoral response (Bard *et al.* 2000, Wisniewski & Konietzko 2008). The finding that active immunisation with Aβ induced a marked reduction in Aβ load in preclinical trials on multiple species led to the initiation of clinical trials of an Aβ vaccine. Phase I clinical trial showed that multiple injections of the Aβ synthetic peptide, AN-1792 in the adjuvant QS-21, were well tolerated, and over half of the patients studied developed antibody responses to Aβ (Bayer *et al.* 2005). However, in Phase II clinical trials, 6 percent of patients given the vaccine developed meningoencephalitis, which is thought to be

mediated by Th1 cells specific for Aβ (Mathews & Nixon 2003, Boche & Nicoll 2008, Monsonego *et al.* 2006).

Though it caused meningoencephalitis in AD patients, active immunisation with Aβ induced Aβ-specific antibodies in over half the immunised patients, and the evidence indicates that immunisation resulted in clearance of Aβ plaques from the brain (Boche & Nicoll 2008, Holmes *et al.* 2008). Furthermore, analysis of a small cohort of patients from the trial found that patients with plaque-reactive Aβ-specific antibodies showed significantly slower rates of cognitive decline than participants without detectable Aβ-specific antibodies (Hock *et al.* 2003). As a result of these findings, the focus of Aβ-based immunotherapy has now shifted to passive immunotherapy to avoid potential adverse T cell responses. Passive immunotherapy reduces Aβ burden in transgenic mice (Wilcock *et al.* 2004a, Bard *et al.* 2000), but also has been reported to cause meningoencephalitis and increase the incidence of CAA (Wilcock *et al.* 2004b, Lee *et al.* 2005).

Despite the drawbacks of Aβ-based immunotherapeutic strategies, they clearly show that the immune system can generate Aβ-specific immune responses that clear Aβ plaques and improve cognitive function. While Aβ-specific Th1 cells are thought to cause meningoencephalitis, they have also been reported to have a beneficial effect in clearance of Aβ plaques, probably through their activation of microglia. Immunisation of APP-transgenic mice with GA in CFA activated microglial-induced clearance of Aβ, which was associated with an increased number of IFN-γ secreting cells in the brain, suggesting that Th1 cells can activate microglia to phagocytose Aβ (Frenkel *et al.* 2005). Other subsets of T cells may induce plaque clearance without an associated inflammatory response. Th2 cells produce the anti-inflammatory cytokines IL-4 and IL-10, and immunisation of APP-transgenic mice with Aβ in the Th2-promoting adjuvant alum induced significant reductions in Aβ plaque burden in the brain, which appeared to be associated with an increase in microglial activation (Asuni *et al.*

2006). In addition, infusion of Aβ-specific Th2 cells improved cognitive performance of PSAPP mice in the RAWM (Ethell *et al.* 2006, Cao *et al.* 2009). Different T cell subsets would be expected to have differential effects on microglial activation, and thus on clearance of Aβ. This thesis has explored the modulatory effects of Aβ-specific Th1, Th2 and Th17 cells on Aβ-induced microglial activation *in vitro* and *in vivo*. These studies also determined the role of Aβ-specific T cell subsets in altering Aβ burden and memory impairments seen in a transgenic mouse model of AD.

Previous studies have shown that Aβ induces proinflammatory cytokine production from mixed glial cultures *in vitro* (Floden & Combs 2006, Ii *et al.* 1996, Murphy *et al.* 1998). The data presented here show that treatment of glia with Aβ induced production of IL-6 and TNF-α, as well as microglial expression of CD80, CD86, and CD40. However, Aβ was unable to induce robust production of IL-1β from mixed glia. Treatment of glia with Aβ in combination with LPS induced significantly greater levels of IL-1β release than LPS or Aβ alone, supporting the hypothesis that Aβ activates the inflammasome, but is unable to induce pro-IL-1β production (Halle *et al.* 2008).

To examine the interaction between T cells and Aβ-treated glia, initial studies focused on the generation of polarised Aβ-specific T cells *in vivo*, as this protocol could potentially be used to generate Aβ-specific T cell subsets in a mouse model of AD without the need for adoptive transfer. Immunisation with Aβ and CpG followed by a boost with Aβ in IFA induced a predominantly Th1 response to Aβ, while immunisation with Aβ in CFA followed by boosting with Aβ in IFA induced a mixed Th1/Th17 response. Treatment of Aβ-stimulated glia with either of these T cell populations resulted in increased production of IL-6 and TNF-α, as well as microglial expression of CD86 and MHC class II. However, there was little difference in cytokine production, or MHC and co-stimulatory molecule expression induced by T cells from the different immunisation groups. In addition, none of the adjuvants tested were able to produce a polarised Th2

response. Previous reports have demonstrated that immunisation with Aβ in alum induced Aβ-specific antibodies (Cribbs *et al.* 2003, Asuni *et al.* 2006). However, it may be the case that Th2-promoting adjuvants are not sufficiently immunogenic to generate a significant cellular response to Aβ.

An alternative method of generating antigen-specific T cell subsets involves *ex vivo* polarisation using a combination of neutralising antibodies and polarising cytokines. Using this method, it was found that immunisation of mice with Aβ and CpG generated Aβ-specific T cells that could be polarised *in vitro* with Aβ and IL-12 to generate Th1 cells. Polarisation with dexamethasone and IL-4 generated Th2 cells, while cells polarised with IL-1 and IL-23 developed into Th17 cells.

A number of studies have shown that microglia surround Aβ plaques, both in AD patients and transgenic mouse models of AD (Itagaki *et al.* 1989, Perlmutter *et al.* 1992, Frautschy *et al.* 1998). Microglia continuously survey the brain parenchyma (Nimmerjahn *et al.* 2005, Davalos *et al.* 2005), and recent studies have reported that they are rapidly recruited to the sites of new plaque formation in APP-transgenic mice (Meyer-Luehmann *et al.* 2008, Bolmont *et al.* 2008). It has been found that microglia are capable of phagocytosing Aβ plaques *in vitro* (Paresce *et al.* 1996), and injected Aβ cores *in vivo* (Frautschy *et al.* 1992), and can secrete anti-inflammatory factors that may protect against Aβ-induced neuropathology (Butovsky *et al.* 2006). However, despite their association with Aβ plaques *in vivo*, they appear to be unable to effectively phagocytose these plaques in either AD patients or transgenic mouse models of AD. Other studies have shown that Aβ-treated microglia produce proinflammatory cytokines, nitric oxide, and reactive oxygen species (Combs *et al.* 2001, Lue *et al.* 2001b), and microglia stimulated with Aβ have been shown to produce factors that induce neuronal death (Giulian *et al.* 1996, Butovsky *et al.* 2005a). Taken together, the results from these and other studies indicate that

microglia have the potential to be beneficial or detrimental in AD, and this may depend on the activation state of the microglial cell.

Recent reports have suggested that microglia can develop variable phenotypes, and the response generated to a stimulus depends not only on the stimulus itself, but also by preceding, concomitant, and subsequent stimuli (Schwartz *et al.* 2006, Town *et al.* 2005). Town and colleagues have suggested that microglia exist in at least 2 functional states: an antigen presenting "adaptive phenotype", and an "innate phenotype" characterised by phagocytosis, and that the activation state is governed by the stimulatory environment (Town *et al.* 2005).

Co-culture of Aβ-specific Th1 cells with glia activated microglia to an "adaptive phenotype", significantly enhancing Aβ-induced production of IL-1β, IL-6, and TNF-α, as well as microglial expression of MHC class II, CD80, CD86, and CD40. Treatment of Aβ-stimulated glia with the prototypic Th1 cytokine, IFN-γ, activated microglia to a lesser extent than Th1 cells, indicating that this cytokine is only partially responsible for Th1 cell-mediated activation of microglia. Glia co-cultured with Th17 cells were also "adaptively" activated, showing increased proinflammatory cytokine production and upregulated expression of MHC class II and co-stimulatory molecules on microglia. IL-17 had little effect on Aβ-induced microglial activation, thus Th17 cells appear to induce microglial activation by factors other than this cytokine. A comparison of the of the effects of Th1 and Th17 cells on glial activation revealed that Aβ-specific Th17 cells induced greater production of IL-1β and IL-6 than Aβ-specific Th1 cells when cultured with glia, suggesting that Aβ-specific Th17 cells are likely to induce an highly inflammatory response if present in the CNS of patients with AD.

Consistent with the "adaptive phenotype" induced by co-culture of Aβ-specific Th1 or Th17 cells and glia, microglia acted as an effective APC for

induction of IFN-γ and IL-17 production by Th1 and Th17 cells respectively. Microglia have been reported to be capable of stimulating cytokine production by antigen-specific T cells (Aloisi *et al.* 2000c, Matsumoto *et al.* 1992, Li *et al.* 2007). However, this is the first time that microglia have been demonstrated to present antigen to Aβ-specific Th17 cells. A number of studies have reported that microglia only induce the activation of Aβ-specific Th1 or Th2 cells when they are pre-treated with IFN-γ (Monsonego *et al.* 2003a, Aloisi *et al.* 2000c, Li *et al.* 2007). Pre-treatment with IFN-γ was not required for activation of Th1 or Th17 cells in these studies, and this may be due to the fact that Aβ appears to be able to act both as antigen and co-stimulator, upregulating expression of CD40, CD80, and CD86 to provide the second signal required for T cell stimulation.

While Aβ-specific Th1 cells are thought to be the cause of the meningoencephalitis seen following vaccination of AD patients with Aβ, their role in plaque deposition/clearance is less certain. There is evidence in the literature to suggest that immunisation protocols promoting the generation of Aβ-specific Th1 cells result in clearance of Aβ plaques from the brains both of mice and humans (Schenk *et al.* 1999, Gilman *et al.* 2005). Additionally, immunisation with Aβ in adjuvant induced T cell-dependent clearance of Aβ in APP-transgenic mice expressing IFN-γ in the brain, suggesting Th1 cell-mediated clearance of Aβ (Monsonego *et al.* 2006). However, it has been demonstrated that injection of Aβ-specific Th1-enriched cells had no effect on Aβ burden in PSAPP mice (Ethell *et al.* 2006), and IFN-γ has been reported to inhibit microglial phagocytosis of Aβ (Koenigsknecht-Talboo & Landreth 2005). The results presented in this thesis have shown that transfer of Aβ-specific Th1 cells increased Aβ burden in the brains of APPswe/PS1dE9 mice. Additionally, mice receiving Th1 cells had impaired cognitive performance in the MWM test for spatial and reference memory, providing further evidence that Aβ is responsible for causing the neuronal death and behavioural impairments seen in AD. Though there is no clear T cell response in the brains of patients with AD, the fact that microglia induce

apoptosis of Th1 cells following their interaction may mean that these cells are a transient population in the brain, making them difficult to detect.

Th17 cells are associated with the pathogenesis of a number of inflammatory diseases, such as MS and rheumatoid arthritis (Langrish *et al.* 2005, Chabaud *et al.* 1999). Until now, the role of these cells in the pathogenesis of AD has not been examined. The results presented here show that, despite appearing to activate Aβ-stimulated microglia in a manner similar to Th1 cells, Aβ-specific Th17 cells did not increase levels of Aβ in the brains of APP-transgenic mice, nor did they induce memory impairments. In fact, Aβ-specific Th17 cells were found to reduce the concentration of soluble $A\beta_{40}$ in the cortex of these animals, suggesting that they may have a beneficial effect on clearance of Aβ from the brain. It is not known why Th1 and Th17 cells had such differing effects on Aβ levels *in vivo*, but it may be related to their effects on microglial phagocytosis of Aβ. IFN-γ has been shown to inhibit microglial phagocytosis of Aβ (Koenigsknecht-Talboo & Landreth 2005, von Bernhardi *et al.* 2007), indicating that Th1 cells also inhibit phagocytosis. It is possible that Th17 cells do not inhibit phagocytosis, although this seems unlikely, given that TNF-α has also been shown to inhibit microglial phagocytosis (Yamamoto *et al.* 2007). A more likely explanation may be that IFN-γ increases production of Aβ. Indeed, IFN-γ has been found to upregulate expression of β-secretase, leading to increased neuronal and astrocytic production of Aβ (Cho *et al.* 2007).

It should be noted that although $CD3^+$ T cells were detected in the brains of mice, no evidence was found of an increase in T cells expressing IFN-γ or IL-17 in the brain following adoptive transfer. This may indicate that Aβ-specific Th1 cells exert their effect in the periphery or in the cerbrovasculature. As these mice were culled 5 weeks after infusion of Aβ-specific T cells, it is also possible that Aβ-specific T cells entered the brain and interacted with microglia, but subsequently underwent apoptosis. Indeed, it has been shown that while microglia act as an effective APC for Aβ-specific Th1 cells, they subsequently initiate

apoptosis of Th1 cells by a nitric oxide-dependent mechanism (Ford *et al.* 1996, Monsonego *et al.* 2003a).

The data here show that, in contrast to the "adaptive" activation of microglia following treatment with Th1 or Th17 cells, Aβ-specific Th2 cells induced significantly less proinflammatory cytokine production and co-stimulatory molecule expression when incubated with glia. Chronic inflammatory activation of microglia is thought to be detrimental in AD, and IFN-γ and IL-12 are upregulated, and IL-4 down-regulated, in the cerebral cortex of APP-transgenic mice (Abbas *et al.* 2002). Aβ induces the production of the neurotoxic factor, nitric oxide, from microglia, and co-culture of microglia and Aβ-specific Th2 cells was found to inhibit this production of nitric oxide (Akama & Van Eldik 2000, Monsonego *et al.* 2003a). In addition, a study of PSAPP mice has shown that, as these mice age, their microglia switched from a phagocytic phenotype to one with cytotoxic properties, and this phenotype switch was correlated with significant neuronal death (Jimenez *et al.* 2008). Therefore, it was hypothesised that Aβ-specific Th2 cells may be able to down-regulate microglial inflammation. In these studies, Aβ-specific Th2 cells attenuated IL-1β and IL-6 production induced by co-culture of glia and Aβ-specific Th17 cells, however, they had no effect on proinflammatory cytokine production induced by Th1 cells. Furthermore, IL-4 and IL-10 attenuated Aβ and IFN-γ-induced proinflammatory cytokine production in mixed glial cultures, as well as inhibiting MHC class II and co-stimulatory molecule expression on microglia.

A number of current studies have suggested that Th2-type responses may be able to induce microglial-mediated clearance of Aβ (DaSilva *et al.* 2006, Asuni *et al.* 2006). Indeed, the Th2-type cytokines, IL-4 and IL-10, have been shown to induce microglial phagocytosis of Aβ (Michelucci *et al.* 2009, Koenigsknecht-Talboo & Landreth 2005). The present studies have shown that adoptive transfer of Aβ-specific Th2 cells had no effect on Aβ burden in the brains of APPswe/PS1dE9 transgenic mice. The lack of effect of Aβ-specific Th2 cells on

Aβ levels is consistent with results from a recent study showing that adoptive transfer of Aβ-specific Th2 cells had no effect on Aβ burden in the brains of PSAPP mice (Cao *et al.* 2009). In contrast to the results shown here, Cao and colleagues found that Aβ-specific Th2 cells attenuated cognitive impairments in PSAPP mice (Cao *et al.* 2009), although a beneficial effect of Aβ-specific Th2 cells on cognitive function could not be ruled out in these experiments, as the transgenic mice were not impaired in the MWM at the age tested here. The data presented here indicate that Aβ-specific Th2 cells do not stimulate microglial phagocytosis of Aβ. Therefore, it seems that clearance of Aβ plaques from the brain following Th2-based immunisation protocols is not mediated by Th2 cells, and perhaps Aβ-specific antibodies are more important in plaque clearance.

The results of these studies have provided important insights into the ability of Aβ-specific T cell subsets and their associated cytokines to differentially activate microglia. In addition, these results have provided evidence that Aβ-specific T cell subsets can modulate Aβ burden and cognitive function. These data show that Aβ-specific Th1 cells exacerbate the signs and symptoms of AD, and a major focus of future research will be to determine whether Aβ-specific Th1 cells play a role in the development of AD.

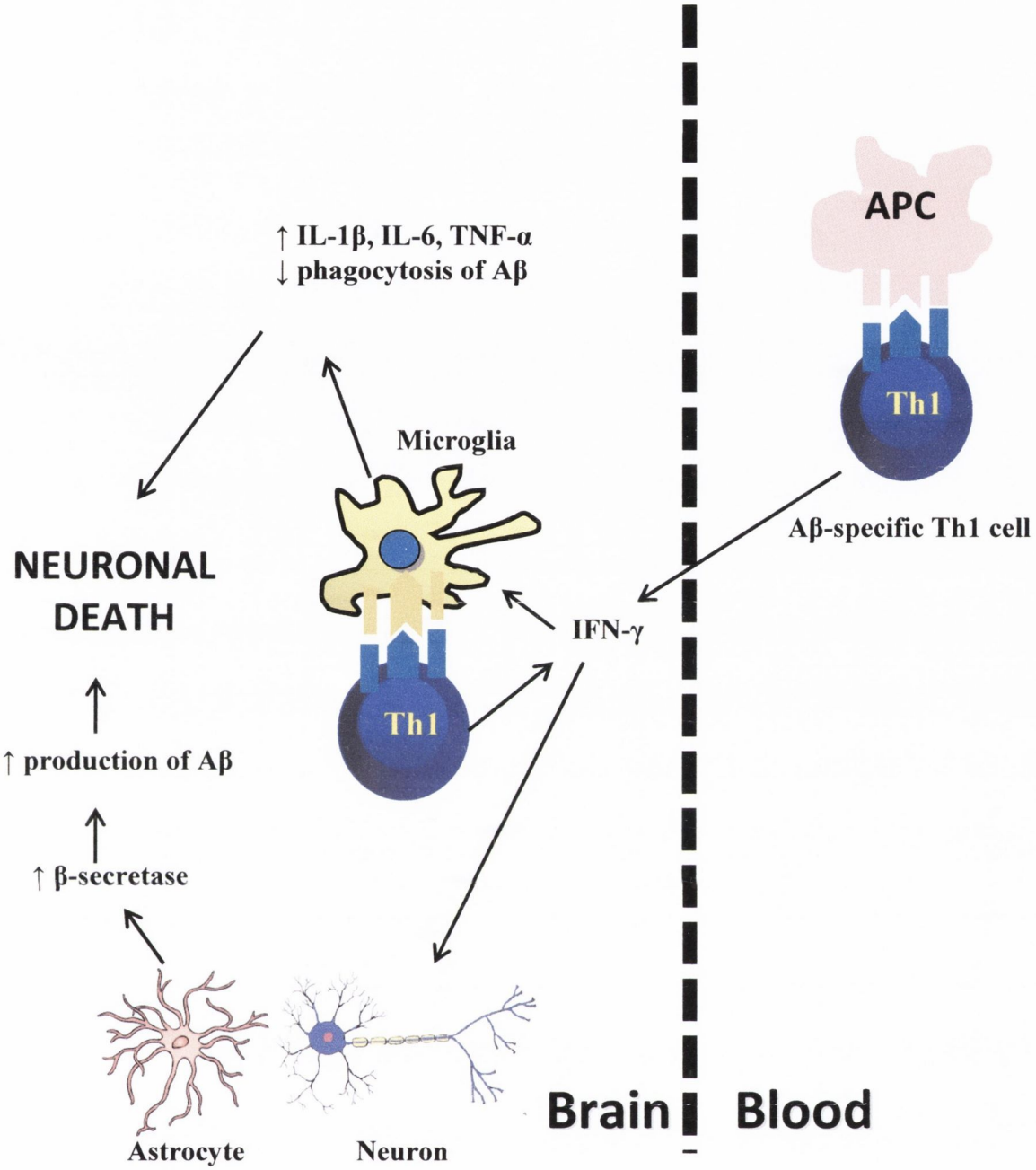


Figure 6.1. Possible role of Aβ-specific Th1 cells in the pathogenesis of AD

Aβ-specific Th1 cells are activated by dendritic cells or macrophages in the periphery, or microglia in the CNS, to produce IFN-γ. Interaction of T cells and microglia in the presence of Aβ induces secretion of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, leading to increased inflammation. Th1 cells may inhibit microglial phagocytosis of Aβ, thus allowing the peptide to accumulate. In addition, IFN-γ acts on neurons and astrocytes to increase expression of β-secretase, further increasing the production of Aβ. An increase in Aβ burden combined with increased inflammation in the CNS may lead to neuronal death, causing cognitive impairments.

Chapter 8

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Appendix I

Publications

Costelloe, C., Watson, M., Murphy, A., **McQuillan, K.**, Loscher, C., Armstrong, M. E., Garlanda, C., Mantovani, A., O'Neill, L. A., Mills, K. H. & Lynch, M. A. (2008) IL-1F5 mediates anti-inflammatory activity in the brain through induction of IL-4 following interaction with SIGIRR/TIR8. *J Neurochem,* 105**,** 1960-9.

Lyons, A., **McQuillan, K**, Deighan, B., O'Reilly, J. A., Downer, E. J., Murphy, A., Watson, M., Piazza, A., O'Connell, F., Griffin, R., Mills, K. H., Lynch, M. A. (2009) Decreased neuronal CD200 expression in IL-4-deficient mice results in increased neuroinflammation in response to lipopolysaccharide. Brain, Behaviour and Immunity. Manuscript accepted (BBI-D-09-00055).

Appendix II

Materials

Ammonium chloride	Sigma-Aldrich
Anti-mouse CD3	BD Biosciences
Aβ	Biosource
Acrodisc syringe filters	Pall Corporation
Alum	Superfos Biosector
BCA protein assay kit	Pierce
BSA	Sigma-Aldrich
Citric acid anhydrous	Sigma-Aldrich
cDNA kit	Applied Biosystems
Cell harvester	Wallac
Congo red	Sigma-Aldrich
Collagenase D	Roche
CpG	Sigma-Aldrich
Cytokines (recombinant)	R&D Systems
Dexamethasone	Sigma-Aldrich
Dimethyl sulphoxide	Sigma-Aldrich
Dulbecco's modified eagles medium	Gibco
DNA ladder	Promega
DNA loading buffer	Promega
DNase I	Sigma-Aldrich
DNase-RNase free water	Sigma-Aldrich
EDTA	Sigma-Aldrich
ELISA plates (black)	Labsystems
Ethanol	Sigma-Aldrich
Falcon tubes	Sarstedt
Foetal bovine serum	Sigma-Aldrich
Flow-check fluorospheres	Beckman Coulter

Fluorometer	Molecular Devices
Glycine	Sigma-Aldrich
Guanidine HCl	Sigma-Aldrich
Haematocytometer	Hycor Biomedical
HBSS	Gibco
Hydrochloric acid	Lennox
LPS	Alexis
L-Glutamine	Gibco
Multiskan RC ELISA plate reader	Labsystems
Magnesium chloride	Promega
MSD 96-well multi-spot 4G8 Aβ Triple ultra-sensitive assay kit	Meso Scale Discovery
Microtitre plates (96-well)	Greiner Bio-One
MJ Research Peltier Thermal Cycler-200	Biosciences
NanoDrop spectrophotometer	NanoDrop Technologies Inc
Neutralising antibodies	BD Biosciences
Nuaire Flow CO_2 incubator	Jencons
NucleoSpin® RNA extraction kit	Macherey-Nagel
Nucleotide mix	Bioline
Nylon mesh filter	BD Biosciences
OCT compound	R.A. Lamb
Penicillin/streptomycin	Gibco
Pertussis toxin	Kaketsuken
Phenyl:chloroform:isoamyl alcohol	Sigma-Aldrich
1 o-Phenyldiamine dihydrochloride tablets	Sigma-Aldrich
Phosphate buffered saline (Dulbecco's)	Sigma-Aldrich
PMA	Sigma-Aldrich
Polytron homogeniser	Kinematica
Potassium chloride	Sigma-Aldrich
Potassium dihydrogen phosphate	Lennox
Primers for genotyping	MWG Biotech

Proteinase K	Sigma-Aldrich
Pyrogene recombinant factor C endotoxin detection system	Cambrex
RPMI	Biosera
SDS	Sigma-Aldrich
Scalpel	Schwann-Mann
Scintillation fluid	Wallac
Sodium chloride	Sigma-Aldrich
Sodium azide	Sigma-Aldrich
di-sodium hydrogen orthophosphate dodecahydrate	Sigma-Aldrich
di-sodium hydrogen phosphate	Sigma-Aldrich
Sodium pentobarbital	Merial Animal Health
Reaction buffer (10X)	Promega
Stereotaxic frame	Kopf
Sulphuric acid	Lennox
Taq polymerase	Sigma-Aldrich
Taqman gene expression assays	Applied Biosystems
Tempura paint	Crafty Devils
Thioflavin T	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Trisodium citrate	Sigma-Aldrich
[^{3}H] thymidine	Amersham Biosciences
Trypsin-EDTA	Sigma-Aldrich
Tween-20	Sigma-Aldrich
UV transiluminator	Bioimaging Systems

Appendix III

Addresses

Alexis	Axxora (UK) Ltd. P.O. Box 6757 Bingham Nottingham NG13 8LS UK
Amersham Biosciences	GE Healthcare Biosciences 800 Centennial Avenue PO Box 1327 Piscataway NJ 08855 1327 USA
Applied Biosystems	Applied Biosystems Frankfurter Str. 129b 64293 Darmstadt Germany
BD Biosciences	BD Pharmingen 10975 Torreyana Road San Diego CA 92121 USA
Beckman Coulter	Beckman Coulter Inc. 4300 N Harbor Boulevard PO Box 3100 Fullerton CA 92834 USA
Bioimaging Systems	UVP 2066 W 11th Street Upland CA 91786 USA
Bioline	Bioline Ltd. 16 The Egde Business Centre

	Humber Road London NW2 6EW UK
Biosciences	Biosciences Ltd. 3 Charlemont Terrace Crofton Road Dun Laoghaire Co Dublin Ireland
Biosera	1 Acorn House The Broyle Ringmer East Sussex BN8 5NN UK
Biosource	Invitrogen 1600 Faraday Avenue Carlsbad CA 92008 USA
Caltag	Invitrogen 1600 Faraday Avenue Carlsbad CA 92008 USA
Cambrex	Cambrex 1205 11th street Charles City IA 50616
Crafty Devils	22/23 Taw Mill Business Park Howard Avenue Barnstaple Devon EX32 8QA UK
Gibco	Gibco BRL Life Technologies PO Box 9418 Gaithersburg

	MD 20898 USA
Greiner Bio-One	Greiner Bio-One 4238 Capital Drive 28110 Monroe NC USA
Hycor Biomedical	Hycor Pentlands Science Park Bush Loan Penicuik Edinburgh EH26 0PL UK
Jencons	Jencons Unit 15 The Birches Willard Way Imberhome Industrial Estate East Grinstead West Sussex RH19 1XZ UK
Kaketsuken	Kaketsuken 1-6-1 Okubo Kumamoto-shi Kumamoto 860-8568 Japan
Kinematica	Kinematica AG Luzernerstrasse 147a Littau-Lucerne 6014 Switzerland
Kopf	David Kopf Instruments 7234 Elmo Street Tujunga CA 91042 USA

Labsystems	Labsystems OY Sorvaajankatu 15 SF-FIN-00811 Helsinki Finland
Lennox	Lennox Laboratory Supplies Ltd John F. Kennedy Drive Naas Road Dublin 12 Ireland
Macherey-Nagel	Macherey-Nagel GmbH & Co. KG Postfach 10 13 52 D-52313 Düren Neumann Neander Str. 6-8 D-52355 Düren Germany
Merial Animal Health Limited	PO Box 327 Sandringham House Sandringham Avenue Harlow Business Park Harlow Essex CM19 5TG
Meso Scale Discovery	Meso Scale Discovery 9238 Gaither Road Gaithersburg Maryland 20877 USA
Molecular Devices	1311 Orleans Drive Sunnyvale CA 94089-1136 USA
MWG Biotech	MWG Biotech Anzinger Strasse 7A Ebersberg D-85560 USA

NanoDrop Technologies	NanoDrop Technologies Inc. NanoDrop products 3411 Silverside Road Bancroft Building Wilmington DE 19810 USA
Pall Corporation	Pall Corporation 2200 Northern Boulevard East Hills NY 11548 USA
Pierce	Pierce Biotechnology Inc. 3747 N Meridian Road PO Box 117 Rockford IL 61105 USA
Promega	Promega US 2800 Woods Hollow Road Madison WI 53711 USA
R & D Systems	R & D Systems 614 McKinley Place NE Minneapolis MN 55413 USA
Roche	Roche Ireland Ltd. Clarecastle Co. Clare Ireland
Sarstedt	Sarstedt Ltd. Sinnotstown Lane Drinagh Wexford Ireland

Sigma-Aldrich	Sigma-Aldrich Company Ltd. Fancy Road Poole Dorset BH12 4QH UK
Superfos Biosector	Superfos Biosector A/S 30 Frydenlundsvej DK 2950 Vedbaek Denmark
Wallac	PerkinElmer Life and Analytical Sciences Inc. 940 Winter Street Waltham Massachusetts 02451 USA