Bone Marrow-Derived Cell Accumulation in the Brain in a Murine Model of Alzheimer's Disease

by

Christine Barr

B.Sc. (Kinesiology), Simon Fraser University, 2010

THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the

Department of Biomedical Physiology and Kinesiology

Faculty of Science

©Christine Barr2013

SIMON FRASER UNIVERSITY

Summer2013

All rights reserved.
However, in accordance with the *Copyright Act of Canada*, this work may be reproduced, without authorization, under the conditions for "Fair Dealing." Therefore, limited reproduction of this work for the purposes of private study, research, criticism, review and news reporting is likely to be in accordance with the law, particularly if cited appropriately.

Approval

Name: **Christine Barr**

Degree: **Master of Science**

Title of Thesis: ***Bone Marrow-Derived Cell Accumulation in the Brain in a Murine Model of Alzheimer's Disease***

Examining Committee:

Chair: Dr. Parveen Bawa

Dr. Charles Krieger
Associate Professor
Senior Supervisor

Dr. Jonathan Choy
AssistantProfessor
Supervisor

Dr. Michael Silverman
Associate Professor, BISC
Simon Fraser University
External Examiner

Date Defended/Approved: August 9, 2013

Partial Copyright Licence

The author, whose copyright is declared on the title page of this work, has granted to Simon Fraser University the right to lend this thesis, project or extended essay to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users.

The author has further granted permission to Simon Fraser University to keep or make a digital copy for use in its circulating collection (currently available to the public at the "Institutional Repository" link of the SFU Library website (www.lib.sfu.ca) at http://summit/sfu.ca and, without changing the content, to translate the thesis/project or extended essays, if technically possible, to any medium or format for the purpose of preservation of the digital work.

The author has further agreed that permission for multiple copying of this work for scholarly purposes may be granted by either the author or the Dean of Graduate Studies.

It is understood that copying or publication of this work for financial gain shall not be allowed without the author's written permission.

Permission for public performance, or limited permission for private scholarly use, of any multimedia materials forming part of this work, may have been granted by the author. This information may be found on the separately catalogued multimedia material and in the signed Partial Copyright Licence.

While licensing SFU to permit the above uses, the author retains copyright in the thesis, project or extended essays, including the right to change the work for subsequent purposes, including editing and publishing the work in whole or in part, and licensing other parties, as the author may desire.

The original Partial Copyright Licence attesting to these terms, and signed by this author, may be found in the original bound copy of this work, retained in the Simon Fraser University Archive.

Simon Fraser University Library
Burnaby, British Columbia, Canada

revised Fall 2011

Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

a. human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

b. advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

c. as a co-investigator, collaborator or research assistant in a research project approved in advance,

or

d. as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Simon Fraser University Library
Burnaby, British Columbia, Canada

update Spring 2010

Abstract

One difficulty in treating some neurological disorders is that many pharmaceuticals cannot cross the blood brain barrier to reach affected areas. Human and animal studies have shown that bone marrow transplantation can result in the engraftment of donor-derived cells in the central nervous system (CNS) under certain conditions. Understanding these conditions will allow us to optimize recruitment of bone marrow-derived cells (BMDCs) to the CNS and, in the future, use these cells as vehicles for gene delivery. Using a triple transgenic mouse model of Alzheimer's Disease (AD), I studied accumulation of amyloid-β, a pathological characteristic of AD, and association of BMDCs with amyloid-β. There were difficulties in maintaining chimerism after bone marrow transplantation in these mice. Reconstitution was achieved by depleting natural killer cell activity in the host, suggesting that hybrid resistance may be present.

Keywords: Alzheimer's Disease; bone marrow transplant; triple transgenic mouse; hybrid resistance; amyloid-β

Acknowledgements

I would like to thank my supervisor, Dr. Charles Krieger for all of the guidance throughout this entire process. I would also like to thank Dr. Jonathan Choy for the advice along the way and my external examiner, Dr. Michael Silverman for taking the time to read and critically review my thesis.

Thanks to my lab mates for the support, friendship and sharing of their knowledge with me, Coral Lewis, John Manning, Sapana Thakore and Kyle Peake.

Thanks to the staff of the Animal Care Facility for showing me the ropes and making sure my mice were always well taken care of.

And finally, thank you to my family for their loving support and encouragement.

Table of Contents

List of Tables

List of Figures

List of Acronyms

3x Tg	triple transgenic AD mouse
Aβ	amyloid beta
AD	Alzheimer's Disease
AICD	amyloid intracellular domain
APP	amyloid precursor protein
ASGM-1	asialo ganglio-N-tetraosylceramide
BACE	beta-site APP cleaving enzyme
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BM	bone marrow
BMDC	bone marrow derived cell
BU	busulfan
CCR2	chemokine receptor 2
CNS	central nervous system
COX-2	cycolooxygenase-2
CSF	cerebrospinal fluid
CY	cyclophosphamide
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cell
EGFP	enhance green fluorescence protein
fAD	familial alzheimer's disease
GDNF	glial derived neurotrophic factor
GFP	green fluorescence protein
GSK-3β	glycogen synthase kinase-3 beta
HSC	hematopoietic stem cell
ICAM-1	intercellular adhesion molecule 1
IDE	insulin-degrading enzyme
IFN-γ	interferon gamma
IL	interkeukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer-cell immunoglobulin-like receptor

KLR	killer-cell lectin-like receptor
LDL	low density lipoprotein
LPS	lipopolysaccharide
LRP-1	low density lipoprotein receptor related protein 1
LTP	long term potentiation
M-CSF	macrophage colony-stimulating factor
NFT	neurofibrillary tangle
NGF	nerve growth factor
NK cell	natural killer cell
NMDAR	N-methyl-D-aspartate receptor
NSAID	non-steroidal anti-inflammatory drug
NO	nitric oxide
PFA	paraformaldehyde
PS1	presenilin-1
RAGE	receptor of advanced glycation end products
ROS	reactive oxygen species
TBI	total body irradiation
TGF-β	transforming growth factor beta
TNFα	tumor necrosis factor alpha
XLP	X-linked lymphoproliferative disease

Introduction

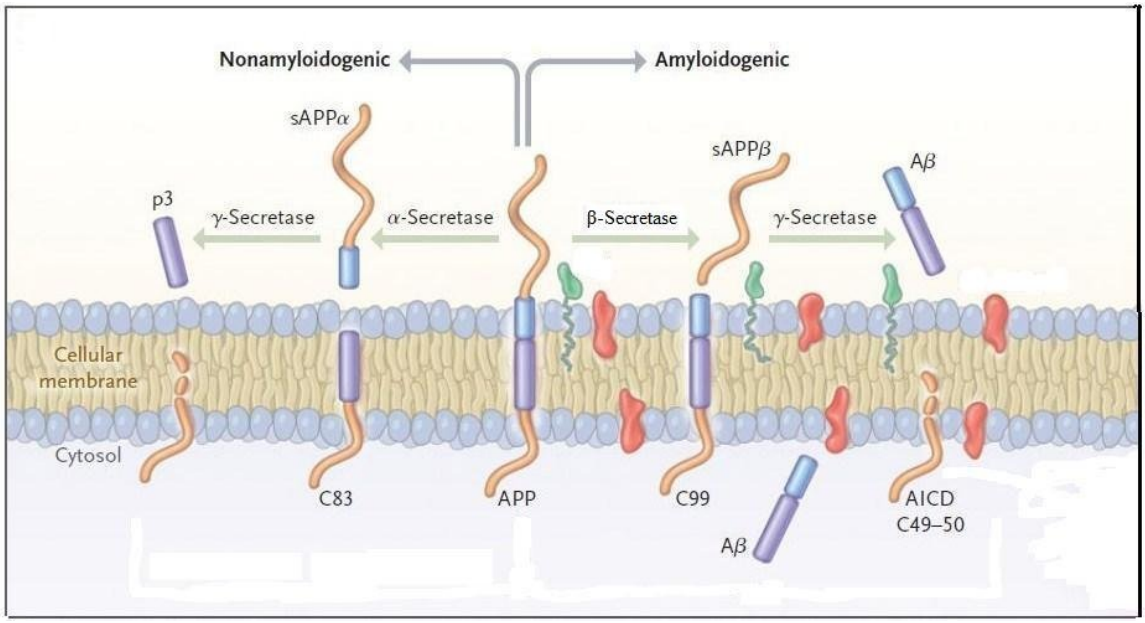
Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease affecting memory and cognitive functions, where diagnosis is confirmed post mortem by the presence of two histological findings. One is the accumulation of senile plaques of amyloid-beta (Aβ), a protein generated through the processing of amyloid precursor protein (APP), and the other consistent pathological findings are neurofibrillary tangles (NFT) composed of intracellular hyperphosphorylated tau, a protein which normally associates with tubulin, and is involved in axonal transport(Rodriguez-Martin et. al. 2013). AD is the leading cause of dementia in older adults, accounting for about 60% of patients with dementia. Other causes for dementia such as fronto-temporal dementia, multi-infarct dementia, or Huntington's disease can be distinguished from AD neuropathologically and usually clinically (Ihl et. al. 2011). In AD, there is a gradual onset of memory impairment and cognitive dysfunction, and AD is generally fatal within 3 to 9 years after initial diagnosis. The leading risk factor associated with AD is age, with incidence doubling for every 5 years past age 65 (Querfurth et. al. 2010). There are rare cases of early-onset autosomal dominant familial AD (fAD), accounting for less than 1% of total AD incidence. The majority of cases of AD are late onset and not heritable through autosomal dominant transmission, though there are certain alleles which increase susceptibility to AD (Querfurth et. al. 2010).

Amyloid-beta

APP is a transmembrane protein found in many tissues and is highly concentrated at neuronal synapses (Priller et. al. 2006). The normal function of APP has not been clarified; however, the proteolytic pathway leading to the formation of Aβ has been studied extensively (Fig. 1).

Figure 1. Proteolytic pathway of amyloid precursor protein



The nonamyloidogenic pathway (left side of figure) does not result in the formation of amyloid-beta (Aβ) and begins with cleavage of APP by α-secretase. The amyloidogenic pathway (right side of figure) begins with cleavage by β-secretase. Both pathways are then completed with further cleavage by γ-secretase. AICD, amyloid intracellular domain; sAPP, soluble amyloid precursor protein.Adapted from Querfurth and LaFerla 2010.

In the healthy central nervous system (CNS), APP is processed by one of two secretases, producing soluble fragments (Neugroschl and Sano 2010). The non-amyloidogenic pathway (Fig. 1, left side of panel 'nonamyloidogenic') begins with cleavage of APP by α-secretase to form soluble APPα and transmembrane C83. C83 is then further cleaved by γ-secretase giving a p3 fragment and the amyloid intracellular domain (AICD). The amyloidogenic pathway (Fig.1, right side of panel 'amyloidogenic'),

which predominates in AD, begins with β-secretase (BACE) cleavage of APP. This forms soluble APPβ and a transmembrane C99 fragment. As in the non-amyloidogenic pathway, γ-secretase then cleaves C99 into Aβ and AICD (Neugroschl and Sano 2010).

Normal neuronal activity increases the production of Aβ at synapses. This finding has been hypothesized to dampen neuron excitation, preventing hyperactivity (Kamenetz et. al. 2003). At the synapse, Aβ is degraded by neprilysin and insulin-degrading enzyme (IDE), which prevents accumulation and oligomerization (Querfurth and LaFerla 2010). Clearance of Aβ, into circulation, can also occur via receptor-mediated transport through endothelial cells expressing Aβ receptors (Deane et. al. 2004). The main receptor for Aβ removal from the CNS is the Low Density Lipoprotein (LDL) receptor related protein 1 (LRP1), while the main receptor for Aβ influx to the CNS is the receptor of advanced glycation end products (RAGE), which is expressed on microglia, neurons and endothelial cells (Chen et. al. 2007). Normally, there is a balance between Aβ influx and efflux; however, in the AD brain, there is an upregulation of RAGE and downregulation of LRP1 (Chen et. al. 2007). Soluble Aβ is easily transported or degraded; however, Aβ can begin to form oligomers if not cleared quickly. These oligomers then accumulate intracellularly as well as extracellularly, and may be necessary to seed accumulation of Aβ(Meyer-Luehmann et. al. 2006).

From C99, γ-secretase can cleave in different locations, resulting in varying lengths of Aβ peptides. Peptides range from 39 amino acids to 43 amino acids in length. Aβ42 has been shown to oligomerize more rapidly than Aβ40 (Jarrett et. al. 1993), and an increase in the ratio between Aβ42 and Aβ40, correlates with increased plaque formation in mouse models of AD (Herzig et. al. 2004). Although Aβ plaques are a characteristic of AD, the number of plaques does not correlate strongly with severity of

disease; instead, they seem to reach a high level early in disease, possibly before symptoms develop (Ingelsson et. al. 2004). Though the trigger for sporadic AD is unknown, one theory is referred to as 'the amyloid cascade' hypothesis (Hardy and Higgins 1992). This hypothesis was first proposed by Hardy and Higgins in 1992 and claims that as Aβ accumulates in the brain, due to decreased clearance or an increase in production, it leads to deleterious effects on the neurons and glial cells of the brain, causing neurodegeneration, vascular damage and dementia. It has been postulated that there are phenotypic changes in microglia and astrocytes (Weitz and Town, 2012). Microglia can no longer effectively clear Aβ, and are activated to secrete pro-inflammatory cytokines and neurotoxic molecules, such as reactive oxygen and nitrogen species (Chiang et. al. 2008). Additional Aβ then accumulates as it is not cleared by microglia, which leads to further activation and recruitment of microglia, and the activated microglia may have a neurotoxic effect in areas of Aβ accumulation.

Hyperphosphorylated Tau

A second histological characteristic of AD are NFTs composed of hyperphosphorylated tau. Tau is a soluble protein associated with microtubules in neurons, which maintains the stability of microtubules, and promotes vesicle transport(Querfurth and LaFerla 2010). Tau interacts with tubulin and can be phosphorylated at many different sites. Hyperphosphorylation of tau leads to an insoluble form of the protein, which lacks its normal affinity for tubulin, decreasing the stability of microtubules and the transport capabilities within neurons. Insoluble, hyperphosphorylated tau accumulates within neurons, forming the characteristic NFTs (Santacruz et. al. 2005). Mutations in the tau gene in humans are associated with frontotemporal dementia and not AD (Goedert and Jakes, 2005). Although there is a

correlation between the presence of NFTs and AD on pathological evaluation, the association is not as strong as for neuron loss and does not closely correlate with severity of the disease (Nelson et. al. 2012). There is also a higher level of hyperphosphorylated tau in the cerebrospinal fluid (CSF) of AD patients than in healthy, age-matched individuals; though, again, this does not correlate well with severity of disease (Wallin et. al. 2006).

Synaptic loss and neurodegeneration

The best correlation with clinical measures of cognitive decline in AD is synaptic loss, showing a greater proportional decrease than neuronal loss in AD (Terry et. al. 1991, Serrano-Pozo et. al. 2011). The mechanism behind synaptic loss remains to be elucidated, though there are many theories as to how neurodegeneration and synaptic loss occur in the AD brain. One such mechanism may be through the interaction between oligomeric Aβ and the *N*-methyl-*D*-aspartate receptor (NMDAR). Aβ oligomers bind to synapses and co-immunoprecipitate with the NR1 subunit of NMDAR in cultured hippocampal neurons (De Felice et. al. 2007). This interaction leads to increased production of reactive oxygen species (ROS) in the neuron, which can be blocked by the addition of either anti-Aβ oligomer antibodies or NMDAR antagonists. The production of ROS is mediated by an increased influx of Ca^{2+} via the NMDAR Ca^{2+} channel. Ca^{2+} chelation also blocks the increased production of ROS caused by Aβ oligomers (De Felice et. al. 2007). ROS are implicated in normal age-dependent decline in long term potentiation (LTP; Serrano and Klann, 2004); thus, higher levels of ROS production may play a role in memory impairment in AD.

A second proposed mechanism for neuronal dysfunction and neurodegeneration in AD is through impaired fast axonal transport caused by Aβ oligomers (Decker et. al.

2010). As measured by live imaging of RFP-tagged brain derived neurotrophic factor (BDNF) or YFP-tagged mitochondria, Aβ oligomers decreased axonal transport in cultured hippocampal neurons. Transport was restored with NMDAR antagonists or glycogen synthase kinase-3β (GSK-3β) inhibitors. The results suggest that Aβ oligomers act on NMDARs where the effect of Aβ binding may be mediated in part by GSK-3β to impair axonal transport. There were no observed changes in neuron morphology or in microtubule integrity, as measured by the ratio of soluble tubulin to polymerized tubulin. Impaired axonal transport in neurons may then decrease LTP and eventually lead to synaptic loss and neurodegeneration (Decker et. al. 2010).

Inflammation in AD

The role that inflammation plays in AD is far from understood. It has been hypothesized both that inflammation is a driving force in the pathology of AD and that inflammatory responses in the brain are neuroprotective. There is little doubt that neuroinflammation exists in AD, as shown by increased expression of inflammatory molecules, such as interleukin (IL)-1α, IL-1β, cyclooxygenase-2 (COX-2) and NF-κB, in the brain of AD patients compared to age-matched controls as well as increased microglia and astrocyte activation at autopsy (Akiyama et. al. 2000). However, it is unclear whether this inflammation is a protective response to increased levels of Aβ deposition in the brain, or if it is causative in the progression of the disease. Epidemiological studies support a detrimental role for inflammation. Long-term non-steroidal anti-inflammatory drug (NSAID) users, such as those with arthritis, show a lower incidence of AD than the general population (Wyss-Coray 2006). Use of NSAIDs in animal models of AD have been somewhat successful, reducing Aβ plaque numbers and microglial activation in many cases (Lim et. al. 2000). However, clinical trials using

NSAIDs in humans have not been so successful, showing little effect on disease progression when treatment begins after onset of symptoms (Hoozemans andO'Banion, 2005).

Mouse models of AD also show characteristics of neuroinflammation, and so have been used to manipulate components of inflammation in order to study effects of increasing or decreasing inflammation on the progression of AD. IL-12 and IL-23 are inflammatory cytokines and share a common subunit, p40. In a study by vom Berg *et. al.*(2012), p40 knockout mice, which do not express either IL-12 or IL-23, had decreased Aβ load, decreased astrocyte activation and a decreased number of microglia (vom Berg et. al. 2012). The same effect was seen when using p40 neutralizing antibodies, though to a lesser extent. These mice also showed improved cognitive functions compared to age-matched AD mice. Humans with AD have increased levels of p40 in CSF and binding of IL-12 to its receptor on astrocytes has been shown to decrease the astrocytes ability to degrade Aβ*in vitro*, so this pathway may present a potential target for therapeutics (vom Berg et. al. 2012).

Chronic exposure of AD mice to lipopolysaccharide (LPS), which elicits a strong immune response, especially of microglia, increases numbers of Aβ plaques; however, acute exposure can actually decrease Aβ load (Morgan et. al. 2005). This seemingly contradictory evidence can be explained if it is a dysfunctional immune response that is responsible for driving disease pathology. With chronic exposure to a stimulus, either LPS or Aβ, microglial responses may no longer target only pathological substances, but can begin to damage normal tissue as well (Wyss-Coray 2006). The fact that acutely activated microglia can improve disease supports a beneficial role of the immune response in AD. Further evidence comes from *op/op* mice, which have about two thirds

the number of microglia of wild-type mice because of a lack of macrophage colony-stimulating factor (M-CSF). These mice spontaneously develop Aβ plaques in the brain, without any AD related mutations, which will be discussed, again supporting the role of normal microglia in Aβ clearance and a beneficial effect of the immune system in AD (Kaku et. al. 2003).

Mouse Models of AD

Many mouse models have been developed to study AD. These models were developed following the identification of genetic mutations linked to rare, fAD cases. One such mutation is in the gene encoding APP, which is converted to Aβ through two sequential cleavages. Mutations that increase expression of APP in the CNS are responsible for fAD cases, such as in the duplication of the APP locus on chromosome 21, detected in 5 families (Rovelet-Lecrux et. al. 2006). Missense mutations in the APP gene can lead to over-expression of the APP protein resulting in overproduction and accumulation of Aβ and the formation of senile plaques. One such mutation is the Swedish double mutation (KM670/671NL) of APP found in members of a Swedish family with a familial form of AD and which results in a 6 to 8 fold increase in Aβ production (Citron et. al. 1992). Many other variations of mutant APP have been identified in familial AD cases.

A second genetic link to fAD is through a mutation in presenilin-1 (PS1), a component of γ-secretase, which is involved in the processing of APP to Aβ. Mutations in PS1 can result in the preferential cleavage of Aβ monomers which are more prone to accumulating, such as Aβ42 (Kumar-Singh et. al. 2006). Mutations in PS1 alone, or those found together with APP mutation in double transgenic mice both result in AD-like accumulation of Aβ plaques, with the double mutant mice developing symptoms more

rapidly than mice with either the APP mutation or PS1 mutation alone (Radde et. al. 2006).

A triple transgenic model was created by Oddo *et. al.* (2003), which contains human transgenes with mutations in APP, PS1 and tau (3x Tg mice). This is the first model to develop both characteristics of AD, Aβ plaques and hyperphosphorylated tau NFTs. These mice were developed by introducing transgenes of APP^{swe} and tau^{P301L} into embryos of PS1 mutant knock in mice ($PS1^{M146VKI}$) on a hybrid 129/C57BL6 background. These mice were then bred until they were homozygous for all three transgenes. Because offspring with genes cointegrated at the same site were used, the genes are unlikely to independently assort, meaning all offspring express all three transgenes, and genotyping of offspring is not necessary. These mice overexpress APP and tau by 6 to 8 fold and show an increase in the ratio of Aβ42 to Aβ40. Extracellular Aβ has been reported to begin accumulating at around 6 months of age, and to be readily evident by 12 months. NFTs are seen in the hippocampus and cortex beginning around 12 months (Oddo et. al. 2003). Overall, these mice appear normal with transgenes not affecting fertility or weight and no significant decrease in lifespan, with mice surviving beyond two years. These mice are homozygous for all 3 transgenes, so there is complete penetrance of disease phenotype with the same characteristics in both male and female mice, unlike in some other models of AD where differences between genders can be observed.

Microglia

Origin

Microglia are widely believed to be of myelomonocytic origin, as evidenced by the fact that mice deficient in PU.1, a transcription factor essential for the development of myeloid and lymphoid cells, do not develop monocytes, tissue macrophages or parenchymal microglia, though other glial cells and neuron numbers are not affected (Beers et. al. 2006). Furthermore, $CD45^{+}$ (hematopoietic marker) $CD11b^{+}$ $CX3CR1^{+}$ (microglia/macrophage markers) cells have been observed in the developing brain starting at E9.5 in $Cx3cr1^{GFP/+}$ knock in mice, indicating that myeloid progenitors populate the CNS early in embryonic development (Ginhoux et. al. 2010). These early yolk sac cells have been characterized as $F4/80^{bright}$ $CD45^{+}$ $CD11b^{low}$ cells, and were shown to develop independently of the hematopoietic stem cell (HSC) transcription factor Myb (Schulz et. al. 2012). Using conditional deletion of Myb in adult mice through the Cre-LoxP system, Schulz et. al. were able to ablate monocytes and granulocytes and replace them with donor cells. Microglia, Langerhans cells and Kupffer cells were not replaced by cells of donor origin, as identified by CD45.2. However, 10% of macrophages in the pancreas and spleen and more in the kidneys and lungs were replaced by cells of donor origin.

Function

Microglia are the resident immune cells of the CNS, surveying the healthy CNS with motile processes. In response to damage, microglia have a role in synapse removal, and possibly synaptogenesis, depending on the signals that are present, showing a specific, graded activation in response to different stimuli (Graeber 2010).

Microglia can be cytotoxic in response to pathogens in the CNS, by releasing pro-inflammatory cytokines such as interferon γ (IFN-γ) and tumor necrosis factor α (TNFα) as well as free oxygen radicals, nitric oxide (NO) and proteases, which can directly damage cells. Microglia can also phagocytose both pathogens and debris through the expression of surface receptors, such as Fc receptors and complement receptors (Kreutzberg 1996). Activated microglia are not necessarily damaging to the CNS, but can instead be neuroprotective, through the release of transforming growth factor β (TGF-β) and the growth factors nerve growth factor (NGF) and basic fibroblast growth factor (bFGF; Nakajima et. al. 1992). Activated microglia have been seen to interact with neurons, without leading to synaptic loss or neurodegeneration in *in vivo* mouse studies using transcranial, thin skull imaging and enhanced green fluorescence protein (EGFP) expressed under control of the Iba-1 promoter (Wake et. al. 2009). Non-functional microglia have also been implicated in some diseases. For example, Nasu-Hakola disease, characterized by early onset cognitive dementia, is a recessive disease caused by genetic mutations in microglial signaling proteins, leading to non-functional microglia (Thrash et. al. 2009).

Role in AD

In the case of AD, some observations have suggested that activated microglia are cytotoxic, leading to neurodegeneration; whereas other investigators have claimed that dysfunctional microglia have a decreased ability to phagocytose Aβ deposits, which, might lead to neurodegeneration and synaptic loss (Chakrabarty et. al. 2010, Weitz et. al. 2012). Cultured microglial cells stimulated with Aβ produce significant levels of TNFα, and when co-incubated with IFN-γ, produced NO_2^-. When microglia were co-cultured with neurons, and then stimulated with Aβ, significant neuronal death was seen

as compared to controls (Meda et. al. 1995). Microglia are seen to associate closely with Aβ plaques in AD mouse models, but the ability of endogenous microglia to effectively phagocytose Aβ has been questioned. Stalder *et. al.* (2001) examined microglia associated with Aβ plaques using electron microscopy to determine if Aβ oligomers were located within the cytoplasm or vesicles of the microglia. They found that the Aβ was extracellular, and that the microglia contained no intracellular Aβ, despite the close proximity and presence of typical phagocyte markers, such as Fc receptors, on the microglia (Stalder et. al. 2001). Microglia are able to phagocytose Aβ *in vitro* and in animal models after active or passive Aβ immunization with soluble Aβ or antibodies against Aβ, respectively (Rogers and Lue, 2001). Monocytes collected from peripheral blood of AD patients showed a decreased ability to phagocytose Aβ *in vitro* when compared to monocytes obtained in the same way from healthy individuals (Fiala et. al. 2005, Zaghi et. al. 2009).

Recently, Krabbe *et. al.* (2013) showed *in vivo* and in acute cerebral slices that microglia of AD mice are less functional than microglia from an age-matched wild type control. Using two-photon microscopy through a cranial window in a *Cx3cr1*$^{gfp/-}$ AD mouse model, microglia were seen to have impaired ability to extend processes towards an area of acute injury introduced by irradiation with a laser. The AD microglia also displayed a decreased ability to phagocytose fluorescently labeled microspheres in areas of Aβ plaque deposition in culture as well as in acute cerebral slices. When compared to age matched wild-type controls, mice with APP/PS1 mutations showed reduced phagocytosis, but only after the onset of Aβ plaque deposition, and only in areas with Aβ plaques (Krabbe et. al. 2013). This evidence suggests that microglia become dysfunctional in the presence of Aβ and can no longer effectively clear it. When

Aβ plaque load was reduced in these mice using an Aβ antibody, the microglia regained the ability to effectively phagocytose microspheres, showing that the dysfunction is temporary and function can be restored (Krabbe et. al. 2013).

BM Transplantation

Preconditioning

Bone marrow (BM) transplantation is a widely used treatment for many blood malignancies including some leukemias and lymphomas (Duran-Struuck and Dysko, 2009). It is necessary to give a preconditioning treatment to a BM transplant recipient in order to ablate existing BM and create space for donor BM to engraft. This can be done using either total body irradiation (TBI) or using chemotherapy employing a myeloablative chemotherapeutic agent such as Busulfan (BU). Both of these protocols work by destroying mitotically active cells through breakage (TBI) or cross-linking (BU) of DNA leading to apoptosis or necrosis of the cell (Duran-Struuck and Dysko, 2009, Iwamoto et. al. 2004). Irradiation is also immunosuppressive, destroying the host's immune cells, creating an environment for engraftment of allogeneic BM. However, BU is not immunosuppressive, and thus is often paired with the immunosuppressant Cyclophosphamide (CY) in allogeneic transplants to prevent graft rejection in the case of incomplete MHC matching (Galambrun et. al. 2013).

Hybrid Resistance

Hybrid resistance is a phenomenon whereby F_1 hybrid mice reject parental BM transplants. This goes against conventional thinking where MHC compatibility is co-dominantly inherited; meaning recipient F_1 hybrid mice can accept BM from either

parental strain, as neither would be recognized as foreign. Hybrid resistance was first observed in 1971 (Cudkowicz and Bennett, 1971). It was originally believed to be antibody mediated, where a radioresistant cell type bound Fc regions activating the cell to kill the antibody bound target cell. However, observations of hybrid resistance in SCID mice, which do not develop T or B cells, and thus no antibodies, demonstrated that the rejection is not antibody mediated (Murphy et. al 1987). Murphy *et. al.* (1987) determined that the resistance was instead mediated by natural killer (NK) cells.

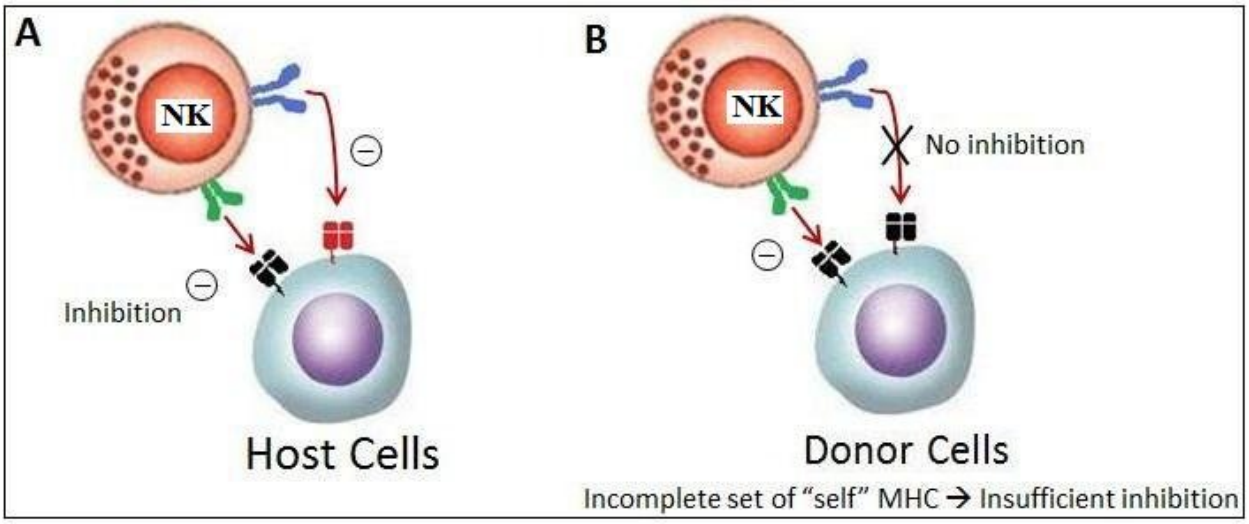
NK cells were first characterized by their ability to kill certain tumor cells and later discovered to be the same cells responsible for hybrid resistance (Herberman et. al. 1975). In both contexts, NK cell function can be explained by the "missing self" theory (Ljunggren and Karre 1985). NK cells express activating as well as inhibitory receptors on their surface. There are two families of receptors, killer cell immunoglobulin-like receptors (KIRs) and killer cell lectin-like receptors (KLRs), either of which can be activating or inhibiting depending on motifs in the intracellular region (Barao and Murphy, 2003). While humans express both KIRs and KLRs, mice express only KLRs, relying especially on the Ly49 receptors (Yokoyama et. al. 1991). Inhibitory receptors contain an immunoreceptor tyrosine-based inhibition motif (ITIM), which, when phosphorylated, blocks signaling of nearby molecules by localizing phosphatases to the cell membrane (Mason et. al. 1997). Activating receptors associate with an intracellular adaptor protein, such as DAP12, which contains an immunoreceptor tyrosine-based activation motifs (ITAM), which can turn on signaling pathways when phosphorylated (Lanier et. al. 1998).

Activation of NK cells is not as clear as inhibition; however, some ligands involved with cell activation have been identified. These include various adhesion molecules which are upregulated due to inflammation such as ICAM-1, VCAM-1 and

LFA-3 (Bakker et. al. 2000). NK cells also express Fc receptors and can be activated in an antibody-dependent cell-mediated manner (Al-Adra et. al. 2011). Another activating receptor on NK cells is 2B4 (CD244), which binds to CD48 expressed by leukocytes (Bakker et. al. 2000). The CD48/2B4 interaction has been shown to modulate HSC quiescence and, recently, $CD48^{-/-}$ mice have been seen to develop lymphoma with tumors being capable of reconstituting non-irradiated recipients (Boles et. al. 2011). The loss of CD48/2B4 signaling is associated with X-linked lymphoproliferative disease (XLP), in which patients display defective antiviral and anti-tumor responses (Boles et. al. 2011). These findings implicate NK cells in the destruction of infected or modified blood cells, via CD48/2B4 signaling, in a non-MHC restricted manner, supporting their role in hybrid resistance.

It is the balance between activation and inhibition that determines whether the NK cell will release its perforin and granzyme containing granules, leading to apoptosis of the target cell (Bakker et.al. 2000). NK inhibitory receptors bind MHC class I molecules on the surface of cells. If the MHC I is recognized as self by the NK cell receptor, the inhibitory receptor is activated and weakens signaling of activating receptors, and the cell is not killed. However, if the MHC I is altered or expressed at lower levels, there is decreased, or no inhibitory signaling. In this case, activating signals predominate and the cell will be killed. In hybrid resistance, the host NK cells will have two different sets of inhibitory receptors. The parental donor BM cells will only have one set of MHC. Only half of the inhibitory receptors of the NK cells will recognize the parental BM cells as self, so there will be a decrease in inhibitory signals, which will no longer override the activating signals on the NK cells. As a result, the donor BM cells will be killed by host NK cells leading to rejection of the BM transplant (Fig. 2).

Figure 2. Incomplete set of self MHC in hybrid resistance



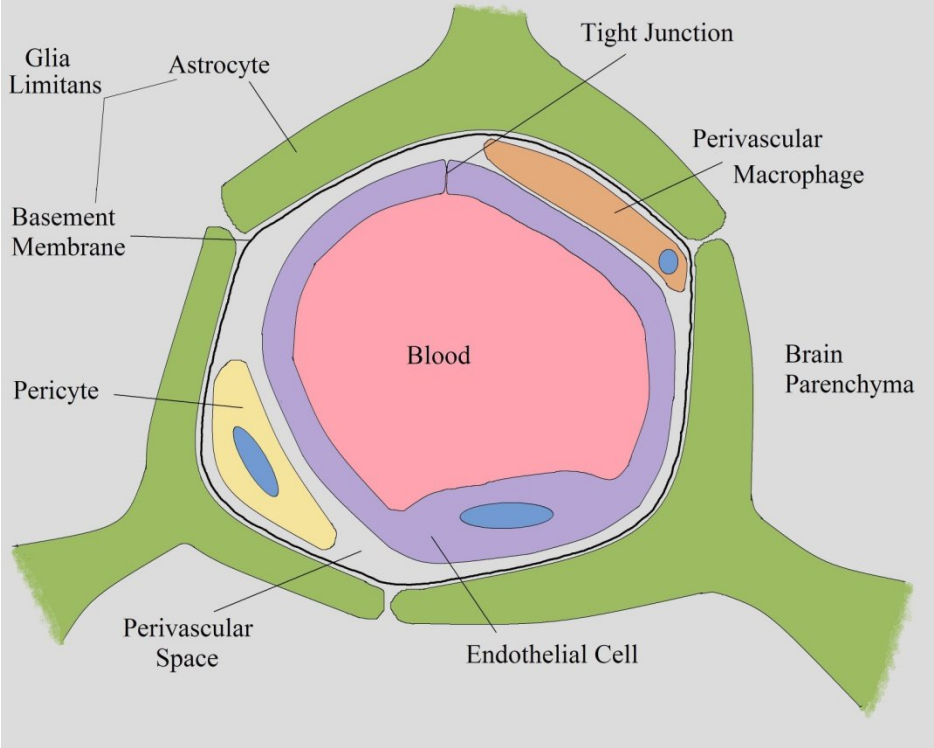
(A) NK cells encounter host blood cells, inhibitory signals are sent from all MHC molecules and the NK cell is not activated. (B) Only half of the MHC of donor cells produce inhibitory signals and the NK cell is activated to kill the donor cell.

Conditions for BMDC accumulation in brain

Blood Brain Barrier

The blood brain barrier (BBB) refers to the physical separation between the circulation and the CNS microenvironment (Fig.3). The BBB denies cells and large molecules access to the parenchyma of the brain and spinal cord, allowing for the proper functioning of neurons, which are very sensitive to changes in their environment (Mizuno et. al. 2003). The BBB is composed of two layers. The first layer consists of endothelial cells (ECs) joined by tight junctions comprising over 40 junctional proteins, which maintain a tight seal between cells. Transport proteins and receptors are present on ECs, to allow passage of necessary molecules, such as glucose, and removal of metabolites (Sa-Pereira et. al. 2012).

Figure 3. Components of the Blood Brain Barrier



Endothelial cells (purple) form tight junctions to make up the first layer of the BBB. The second layer, or glia limitans, is comprised of astrocytic endfeet (green) and a basement membrane (black). Between the two layers is the perivascular space, containing perivascular macrophages and pericytes.

The second layer of the BBB consists of the glia limitans, comprising astrocytic endfeet and an associated basement membrane (labeled as 'astrocyte' in Fig. 3). Astrocytes cover approximately 99% of the brain endothelium and are largely responsible for the maintenance and properties of the BBB through secretion of growth factors and extracellular matrix proteins (Abbott et. al. 2006). TGF-β, glial-derived neurotrophic factor (GDNF), and bFGF promote the survival of ECs and the expression of tight junction proteins, while the extracellular matrix proteins form the basement membrane.

The perivascular space lies between the endothelial cells and the glia limitans. Pericytes and perivascular macrophages are located within the perivascular space. Pericytes surround the brain pre-capillary arterioles, capillaries and post-capillary venules. They have contractile, immunological, phagocytic, migratory and angiogeneic functions, as well as maintaining the properties of the BBB (Sa-Pereira et. al. 2012). Pericytes are essential in the development of tight junctions at the BBB, as evidenced by the fact that mice with reduced numbers of pericytes show increased permeability of the BBB via the accumulation of serum proteins in the brain, as well as a reduction in brain capillary perfusion (Bell et. al. 2010). Perivascular macrophages are the link between the CNS and the peripheral immune system. They have several functions including antigen presentation, sensing neuronal death and causing phagocytosis within the perivascular space (Williams et. al. 2001). The antigen presentation functions of perivascular macrophages (also known as perivascular cells and perivascular microglia) was shown by Hickey and Kimura(1988), using chimeric BN rats reconstituted with BM from F1 Lewis x BN rats. These rats developed experimental autoimmune encephalomyelitis (EAE) when injected with a Lewis rat T cell line reactive to myelin basic protein. Only the donor-derived perivascular macrophages expressed the appropriate MHC II receptor, and thus, this cell type was implicated as an antigen presenting cell in the CNS.

Accumulation of BMDCs in the CNS

Perivascular macrophages are more readily turned over and replaced by myelomonocytic cells from the blood than are parenchymal microglia. Bechmann *et. al.* (2001)used the phagocytic functions of these cells to show the rate of turnover of perivascular cells. Rhodamine-coupled dextran amine was injected, followed by

fluorescein-coupled dextran 4 weeks later. Perivascular macrophages phagocytosed the dextran, and it was still present after 4 weeks. All cells that labeled with rhodamine, also labeled with fluorescein; however, rhodamine negative, fluorescein positive cells were also present, which were interpreted to be macrophages recruited between the two injection time points. This recruitment was estimated at about 6.4% over 4 weeks, suggesting a slow turnover of perivascular macrophages with time. Mildner *et. al.* (2007) saw occasional engraftment of perivascular, Iba-1^{+} macrophages in brain sections of chimeric mice generated through shielding of the head during irradiation followed by BM transplant, showing that BM derived precursors can be recruited to the perivascular space; though, other studies have not seen this effect (Ajami et. al. 2007). The rate and extent of perivascular macrophage turnover under physiological conditions remains unclear.

It has become easier to follow the trafficking of BM derived cells (BMDCs) through the generation of mice that ubiquitously express green fluorescence protein (GFP) under the control of a β-actin promoter to use as BM donors (Okabe et. al. 1997). By using GFP expressing donors, host cells and donor cells can easily be distinguished without a dependence on immunohistochemical techniques. These donor mice have been used in various protocols for the generation of chimeric mice to study the entry of BMDCs into the CNS.

BM Transplantation Following Irradiation

In most BM transplantation experiments, an initial step is generally TBI of the recipient, where the irradiation prevents the division of hematopoetic precursors in BM and creates an open niche for donor cells to engraft. With the TBI protocol, many studies have shown that donor cells do enter the CNS and appear to differentiate into

microglia, expressing the microglial marker Iba-1, and taking on the characteristic stellate morphology of microglia, both in AD model mice, as well as control mice, albeit to a lesser extent in the control mice (Priller et. al. 2001, Hess et. al. 2004, Simard and Rivest, 2004). Engraftment increased in injured or disease model mice, with greater numbers of GFP^+ $Iba\text{-}1^+$ cells at the site of injury or the affected area, such as in the ischemic cortex following transient focal cerebral ischemia by middle cerebral artery occlusion (Priller et. al. 2001) or the cortex and hippocampus of a mouse model of AD (Simard et. al. 2006). From this evidence, it was proposed that circulating BM-derived progenitors contribute to the maintenance of the microglial population and are drawn to an area of injury or disease. However, it was later proposed that the irradiation was affecting the BBB, and that irradiation may affect engraftment of donor cells in the CNS (Mildner et. al. 2007). Many studies have since shown that irradiation increases the expression of pro-inflammatory cytokines, such as IL-1β, TNFα, IL-6 and IL-8, which will in turn increase the expression of cell adhesion molecules (ICAM-1, VCAM-1) by endothelial cells (Linard et. al. 2004, Wilson et. al. 2009). Adhesion molecules bind to integrins on leukocytes, such as LFA-1 and VLA-4, and allow for extravasation of the cells into the CNS.

The specific cell type capable of entering the CNS has not been identified, though certain cell surface proteins are necessary for entry of BMDCs into the CNS. One such protein is chemokine receptor 2 (CCR2). Mildner *et. al.* (2011) showed that when irradiated mice are transplanted with $GFP^+CCR2^{-/-}$ BM cells there is significantly reduced engraftment of GFP^+ cells in the brains of AD mice as compared to mice injected with $GFP^+CCR2^{+/+}$ BM cells, though chimerism of the blood is established to the same degree in both cases.

BM Transplantation without Lethal Irradiation

Using parabiosis and targeted irradiation, it was shown that in the healthy adult, BM-derived precursors do not contribute significantly to the microglial pool. The microglial population is instead believed to be maintained through proliferation of existing microglia (Ajami et. al. 2007, Mildner et. al. 2007). Mildner *et. al.* (2007) explored the possibility that irradiation was permissive for cell entry during the earlier transplantation experiments by localizing irradiation to the body by adjusting the field size to exclude the head using a linear accelerator. These animals kept the fur colour on their head, while losing it on the rest of the body, indicating that the irradiation had in fact been targeted. It was found that GFP^+ cells could enter and engraft in the spinal cord, however, there was no cell entry in the protected brain. Ajami *et. al.* (2007) created parabiotic pairs by surgically attaching a wild type mouse to a partner which ubiquitously expressed GFP, allowing for the identification of any cell in the CNS that arose from circulating progenitors in the adult mouse. This technique eliminates the need for irradiation to repopulate the bone marrow of the wild type mouse, and allows for studying of BM cell trafficking without the confounding effects of irradiation. Ajami *et. al.* (2007) found that there was no engraftment of GFP+ cells in the brain or spinal cord of healthy mice, mice subject to facial nerve axotomy or mice over-expressing mutant SOD-1, a murine model of amyotrophic lateral sclerosis. Chimerism was measured at around 50% in the parabiotic pairs, indicating complete blood sharing between the GFP^+ and GFP^- parabionts. However, while BM derived macrophages were evident in the muscle, they were not detected in the brain (Ajami et. al. 2007). To evaluate the role of irradiation in this model, parabiotic animals were also irradiated, while the GFP^+ partner was shielded. Reconstitution of BM was successful without injection of BM cells, where around 80% of BM were GFP^+. However, no GFP^+ cells were found in the CNS. Together, these

experiments suggest that BM derived cells enter the CNS only under certain conditions. First, the BBB must be disrupted or activated in some way, such as through irradiation. Second, cells that are capable of entering the CNS, presumably some type of progenitor of the monocyte lineage, must be in the circulation. With the injection of BM cells during transplant, these cells are temporarily circulating, which does not occur at significant levels under physiological conditions.

Use of non-irradiative myeloablation with Busulfan

As an alternative method to achieve BM chimerism without TBI, we turned to chemotherapeutic agents that are known to prevent hematopoetic cell division and so create space in the BM for donor reconstitution. Busulfan (BU) (1,4-butanediol dimethanesulfonate) is an alkylating agent which forms G-G bridges, cross-links DNA and induces apoptosis by the production of H_2O_2 (Iwamoto et. al. 2004). The use of BU in humans as a chemotherapeutic agent dates back to the 1950s, where it was found to have a similar effect on BM cells as lethal irradiation (Talbot 1957). It is generally used together with Cyclophosphamide (CY), an immunosuppressant, in human and rodent BM transplants (Tutschka and Santon, 1977; Tutschka et. al. 1987). BU is myeloablative, creating a niche for the donor BM, while CY is an immunosuppressant, preventing rejection of the donor cells by the host immune system (Tutschka et. al. 1987). However, in mice where the donor and recipient have the same MHC haplotype, it is possible to use BU alone, without CY (Barry 2003). Donor BM cells have been seen to engraft in the CNS following Bu treatment and intravenous injection of GFP^+ donor BM, without irradiation (Espejel et. al. 2009, Capotondo et. al. 2012, Lewis et. al. 2013). This suggests that BU must have an effect on the BBB, somehow disrupting it and allowing for the entry of BM cells. Recent work has suggested that resident microglia

must be ablated to some extent in order for donor BMDCs to develop into long lasting microglia-like cells in the CNS, and that BU can accomplish this ablation, since it has the ability to cross the BBB (Capotondo et. al. 2012). However, another study did not see BMDC entry to the CNS with similar doses of BU (Lampron et. al. 2012).

Aims

The aims of this work are as follows:

1. To establish a protocol by which high level chimerism can be maintained in 3x Tg AD mice through non-irradiative means.
2. To analyze the accumulation of BMDCs in the brains of these mice.
3. To qualitatively characterize the histological features of the 3x Tg AD mouse model.

Since our lab has successfully used BU to develop high level chimerism in an ALS mouse model (Lewis et. al. 2013),I aim to use a similar protocol in the AD mice to establish and maintain chimerism after transplantation of GFP^+ BM cells. This will be done using either BU or BU in conjunction with other therapeutics as necessary. If chimerism is successfully established, I will then analyze the localization of BMDCs in the brains of the transplanted mice.

There is literature on the histological characteristics of this mouse model, though some findings are highly debated. Because of the inconsistences in the literature, I expect to confirm the presence of Aβ plaques and hyperphosphorylated tau tangles in the brains of these mice.

Methods

Mice

B6;129-Psen1tm1Mpm Tg(APPSwe,tauP301L) 3x Tg mice were ordered from Jackson Labs. Mice are homozygous for all three transgenes, and no genotyping is necessary. To generate GFP^+ 129/B6 mice, GFP^+C57BL/6 mice were crossed with 129 mice. These mice are phenotyped by viewing an ear punch with an epifluorescence microscope.

Pretreatment and BM transplantation

Mice are given intraperitoneal injections of 20 mg/kg BU per day for 4 (80mg/kg) or 5 (100 mg/kg) consecutive days. 24 hours after the last BU injection, BM is harvested from the femurs and tibia of GFP^+ donor mice. Red blood cells are lysed and removed with the supernatant after spinning the BM cells down at 1500 rpm for 5 min. BM is filtered and resuspended in sterile PBS. 1.5×10^7 BM cells in a total volume of 300 µL are injected into each recipient mouse via the tail vein. For BU + CY treatments mice are treated for 4 days with BU as above, followed by 2 days of 100 mg/kg CY for a total of 200 mg/kg. BM transplant is performed as described above. For NK cell function depletion 30 µL of anti-asialo ganglio-N-tetraosylceramide (ASGM-1) in 300 µL PBS was injected intraperitoneal 2 hours before BM transplant and again on day 14 post-transplant. Irradiated mice were exposed to 10 Gy of ionizing radiation at 2 months of age and transplanted with 5×10^6 BM cells on the same day.

Chimerism monitoring

Blood is drawn weekly from the saphenous vein to test for chimerism. The blood sample is first lysed in 500μL lysis buffer on ice for 8.5 min. 1 ml FACS buffer (2mM EDTA + 2% FBS in PBS) is added and the solution spun down for 5 min at 3000 rpm. Lysis is repeated. After the second lysis, pellets are resuspended in 700 μL FACS buffer and the solution is split into A (labeled for lymphocytes) and B (labeled for myeloid cells) tubes. PE-Cy7 conjugated antibodies used are anti-B220 (1:200) and CD3 (1:200) for lymphoid cells, and anti-Gr-1 (1:2000) and CD11b (1:2000), for myeloid cells. 150 μL of the appropriate Ab solution is added to each tube and placed on ice for 25 min. Cells are then rinsed in 1 mL FACS buffer, spun down for 5 min at 3000 rpm, supernatant removed and rinsed again. Pellets are resuspended in 300 μL FACS buffer and analyzed with a BD Aria FACS machine.

Tissue preparation

Mice are sacrificed with CO2, transcardially perfused with 30 mL PBS followed by 30 mL of 4% paraformaldehyde (PFA). Skull, spine and spleen are removed and fixed overnight in 4% PFA. Brain and spinal cord are dissected out, and left in a 20% sucrose solution, along with the spleen, overnight. Tissue is then blocked in OCT and stored at -80°C. Tissue is sliced at 30 μm and placed in DeOlmos solution to be stored at -20°C.

Immunohistochemistry

Free floating sections are rinsed 3 times for 10s each with PBST (0.3% PBS with Triton-X100), followed by 3, 5 min rinses. Sections are incubated in 0.5 M glycine in PBST for 1 hr and rinsed in PBST. Sections are blocked in 25% NDS + 5% BSA for 1 hr, then primary antibodies are added overnight (Iba-1 1:100; CD31 1:100; 6E10 1:1000;

11A1: 1:100). After rinsing, fluorescence conjugated antibodies are added for 2 hrs at 1:1000 (AF488, AF568 or AF647). Sections are mounted on slides and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI).Congo Red staining was performed by first rinsing free floating brain sections in PBS, followed by 20 min incubation in alkali NaCl, then 25 min in alkali Congo Red solution. Sections were again rinsed in PBS.

Sections were imaged using a Leica DM4000Bepifluorescence microscope, with a 4x, 10x, 20x or 40x lens and Leica DFC350 FX digital camera. A Nikon Eclipse Ti inverted confocal microscope was used for higher power images with 60x objective lens.

Statistics

Two-sided Student's *t*-tests were used to compare chimerism levels in the peripheral blood between NK cell depleted mice and non-NK cell depleted mice using Microsoft Excel.

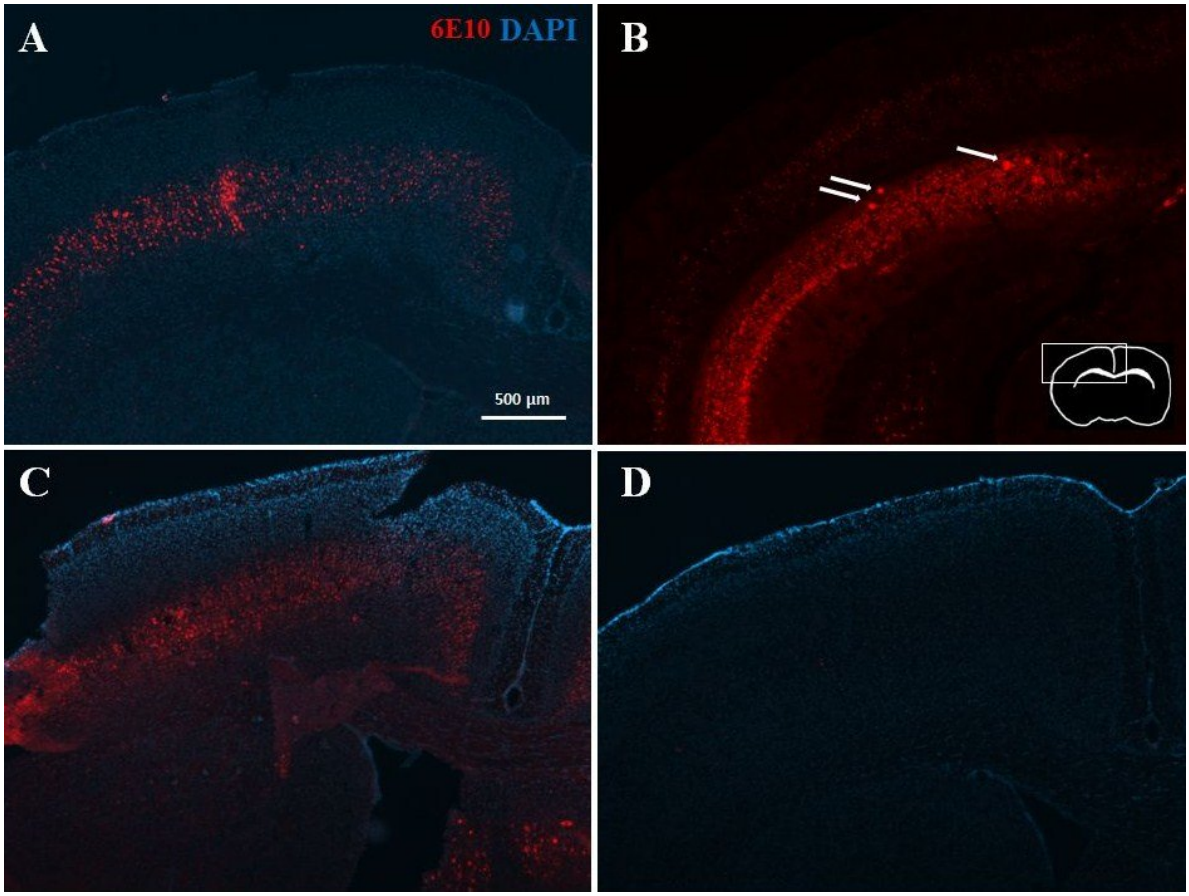
Results

Triple transgenic AD mice express intracellular Aβ, extracellular Aβ plaques and hyperphosphorylated tau

Triple transgenic (3x Tg) mice were sacrificed at 6, 12 or 15 months of age in order to characterize the pathological features of this mouse model of AD. Immunohistochemical labeling was performed on coronal brain sections showing both hippocampus and cortex. Labeling with the 6E10 antibody against amino acids 1-16 of Aβ revealedimmunoreactivityin deep layers of the cerebral cortex at 6, 12 and 15 months of age (Fig. 4). The immunoreactive material has a clearly delineated border and shows nuclear sparing, which suggests that it is intracellular. Immunolabeling can also be visualized within long, thin axons of these cells, which suggests it is intraneuronal (Fig. 5). In the 3x Tg mice, intracellular Aβ immunoreactive material can be seen within the cell bodies and axons of neurons in the hippocampus and cortex of 12 month old 3x Tg mice (Fig. 5). No extracellular Aβ plaques are visible at 6 months of age as revealed by Congo Red staining or immunohistochemistry with the 6E10 antibody (Fig. 4A). Congo Red is a diazo dye, which changes absorbance spectrum upon binding to the β-sheet fibrils of amyloid proteins. Since the cells are not permeabilized during the Congo Red staining protocol,only extracellular Aβ can be seen with an epifluorescence microscope (Klunk et. al. 1999). By 12 months of age, small numbers of plaques become apparent with 3-4 plaques per section (Fig. 4B; arrows); however, not all sections contain plaques as the total number of plaques is quite small. Extracellular Aβ plaques are defined as either Congo Red positive or diffuse 6E10 positive deposits without clearly delineated

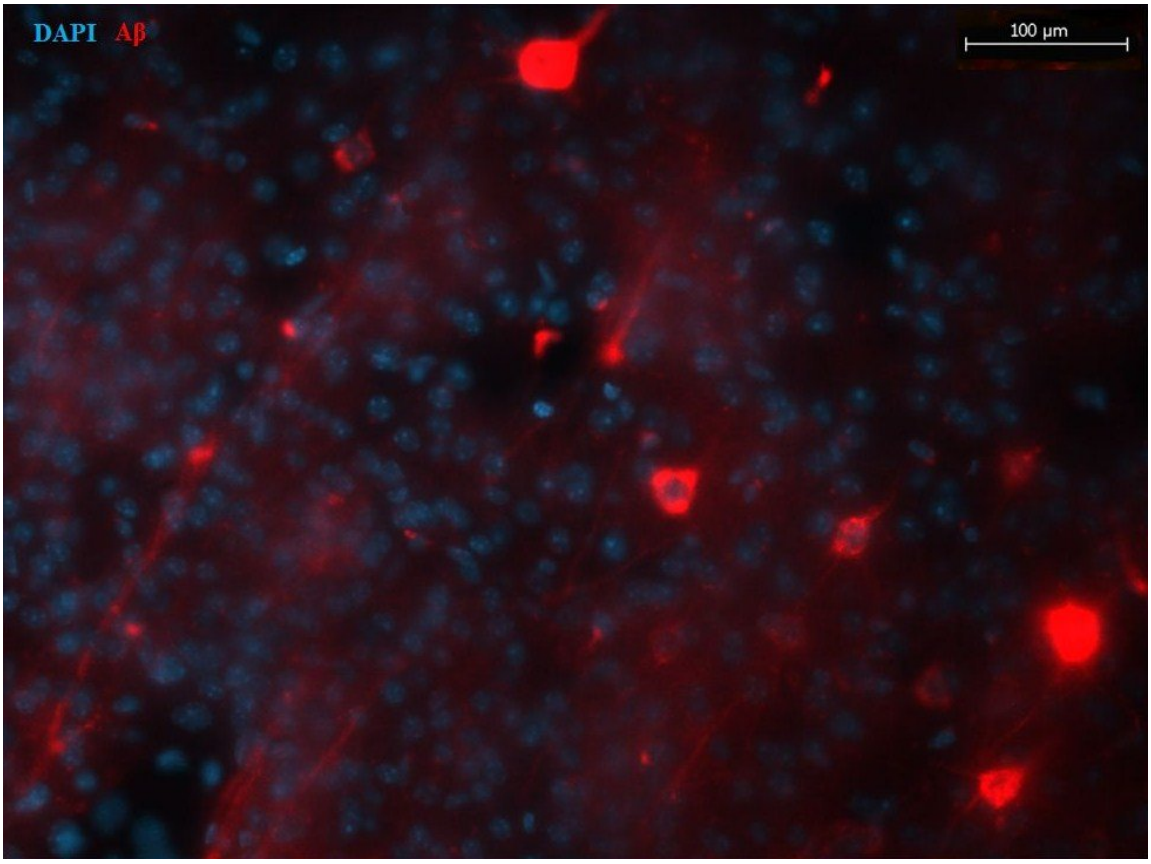
borders or nuclear sparing in sections where Congo Red was not used. At 15 months of age, extracellular Aβ plaques were not seen; but, intracellular Aβ labeling was abundant (Fig. 4). In control B6 tissue, Aβ was notdetected either intracellularly or extracellularly (Fig. 4D).

Figure 4. Amyloid β immunoreactivity with age



(A) 6 month old 3x Tg mice show significant levels of intracellular Aβ with no visible extracellular plaques. (B) 12 month old 3x Tg mice still display high levels of intracellular Aβ and begin to show extracellular plaques (arrows). (C) 15 month old 3x Tg mice again show intracellular Aβ, but extracellular plaques were not visible. (D) control 12 month old B6 mice show no intracellular or extracellular Aβ. Schematic inset in (B) shows area imaged. Immunolabeling of 6E10 is shown in red and DAPI is shown in blue

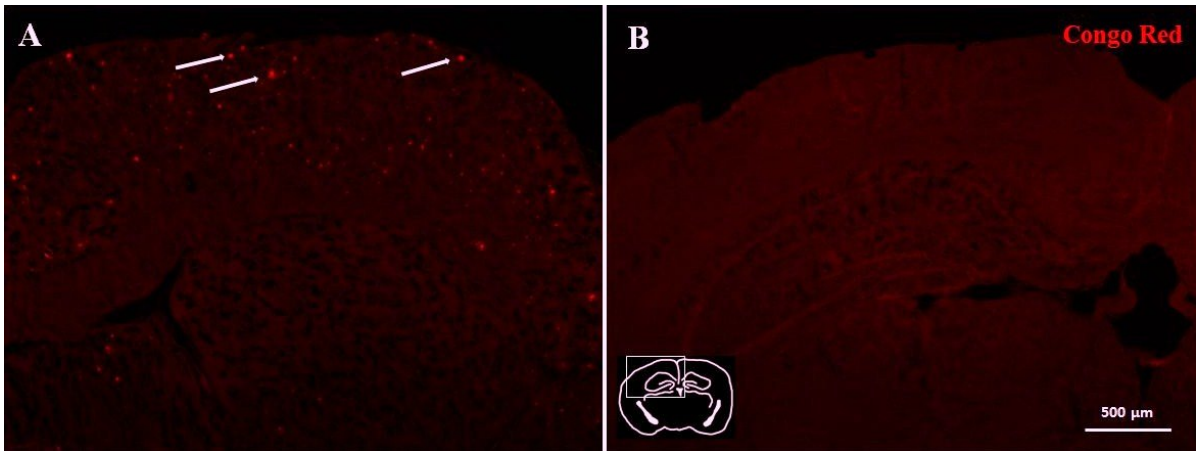
Figure 5. 6E10 immunoreactivity in cerebral cortex



6E10 antibody shows immunoreactivity of neurons and axons in the cortex of 6 month old 3x Tg mice. Immunolabeling of 6E10 is shown in red and DAPI is shown in blue

To further evaluate thelabeling characteristics of extracellular Aβ plaques in aged mice, I used tissue from a different mouse model of AD expressing mutations in APP and PS1 as a positive control and this tissue was prepared in an identical manner as the 3x Tgbrain tissue. These APP/PS1 mutant mice have been previously reported to demonstrate significant levels of extracellular Aβ plaques at 1 year of age (Choi et. al. 2007).Figure 6 shows that Congo Red staining is seen in the APP/PS1 brain tissue (Fig. 6A; Aβ plaques shown by arrows), but that no Congo Red staining is evident in the brains of 3x Tg miceat 15 months of age (Fig. 6B).

Figure 6. Congo Red staining in APP/PS1 and 3x Tg brain

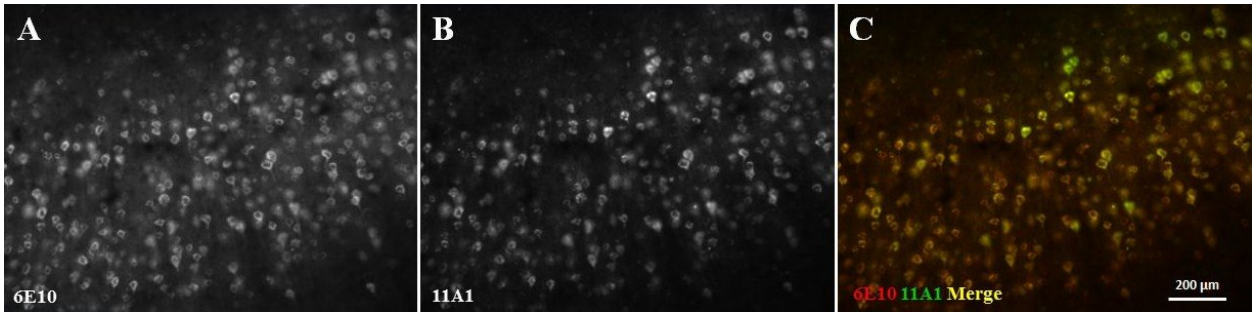


(A) 1 year APP/PS1 brain has significant extracellular Aβ plaque load (arrows) as assessed by Congo Red staining. (B) No extracellular Aβ plaques in 15 month 3x Tg brain. Schematic inset in (B) shows area imaged.

The 6E10 Aβantibody binds to amino acids 1-16 of Aβ, which is also present in APP. Since APP is overexpressed within neurons of these mice, it is possible that the staining pattern is labeling APP and/or Aβ. To clarify whether the 6E10 immunoreactivity is directed against APP or Aβ, I employed a second Aβ antibody (11A1), which is specific for a β-turn at positions 22 and 23 of Aβ (Murakami et. al. 2010). This 11A1 antibody binds specifically to Aβ oligomers as the Aβ species with this β-turn rapidly form oligomers and has been identified as a more toxic form of Aβ (Murakami et. al. 2010). Figure 7 shows immunolabeling with both the 6E10 and 11A1 antibodies in neurons of the cerebral cortex in 12 month old 3x Tg mice. At this magnification, in general, there was a similar pattern of immunolabeling using both antibodies, with many cortical neurons demonstrating immunoreactivity against 6E10 and 11A1 (Fig. 7C; merge, yellow). Using confocal microscopy, and higher power magnification the 11A1 antibody demonstrates punctuate labeling in the cell body. The immunolabeling extends into the proximal dendrites of the labeled neurons. Immunoreactivity against 6E10 was

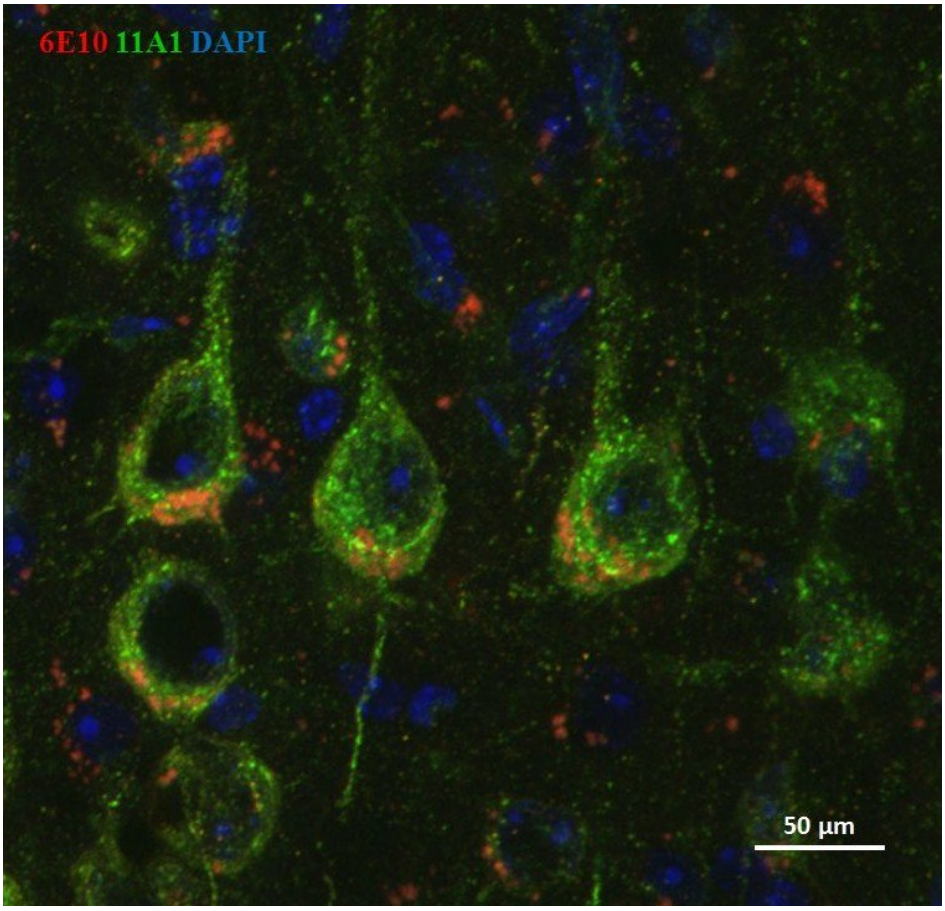
also evident within the cell body, but often more limited in extent than for 11A1 immunolabeling (Fig. 8).

Figure 7. Similar staining pattern of 6E10 and 11A1 at low power magnification



(A) 6E10 binds amino acids 1-16 of Aβ and shows intracellular Aβ in cortex of 12 month 3x Tg mouse brain.(B) 11A1 immunolabeling in cortex reveals cellular labeling similar to A. (C) Merge of immunoreactivity to the two antibodies shows similar labeling, with many cells showing co-labeling (yellow) at 20x magnification.

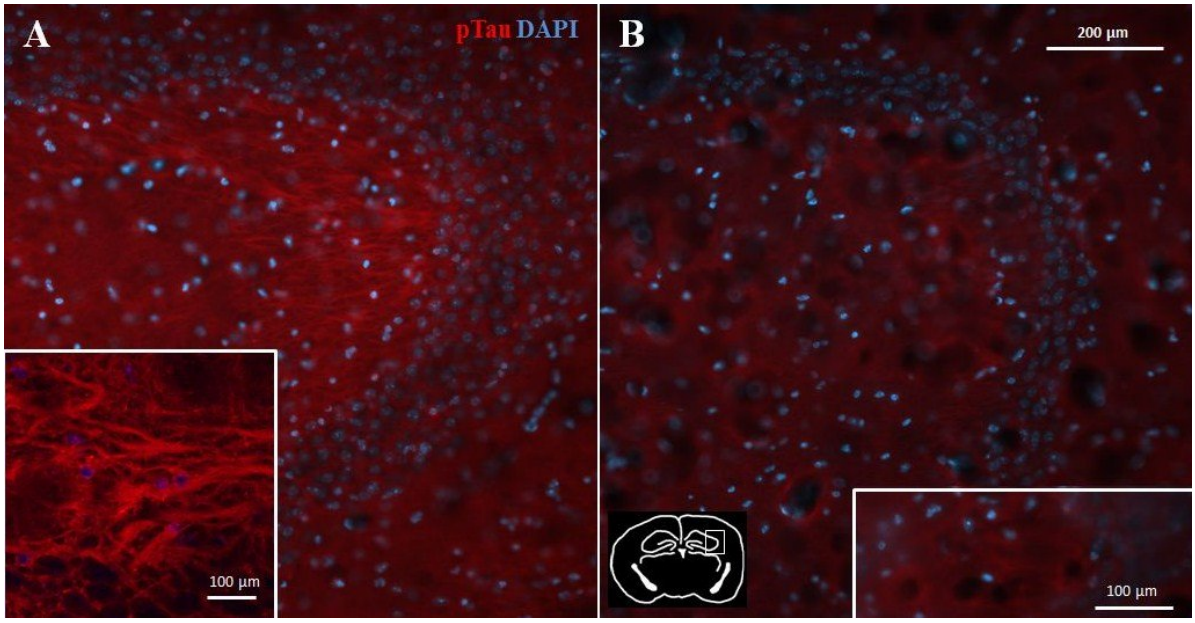
Figure 8. High power image of intracellular Aβ



Oligomeric Aβ antibody (11A1; green) shows diffuse labeling of the cell body and axons. 6E10 antibody (red) labels more distinct regions in the neuronal cell body. Cell nuclei are labeled with DAPI (blue)

An antibody specific to tau phosphorylated at serine 396 (pS396) shows that phosphorylated tau is apparent in the 3x Tg mice at 12 months of agebut not in wild-type B6 mice (Fig 9). Phosphorylated tau can be seen mainly in neurons of the hippocampus but not at significant levels in the cortex at 12 months of age, which is consistent with data reported in the literature (Oddo et. al. 2003).

Figure 9. Phosphorylated tau in neurons of the hippocampus

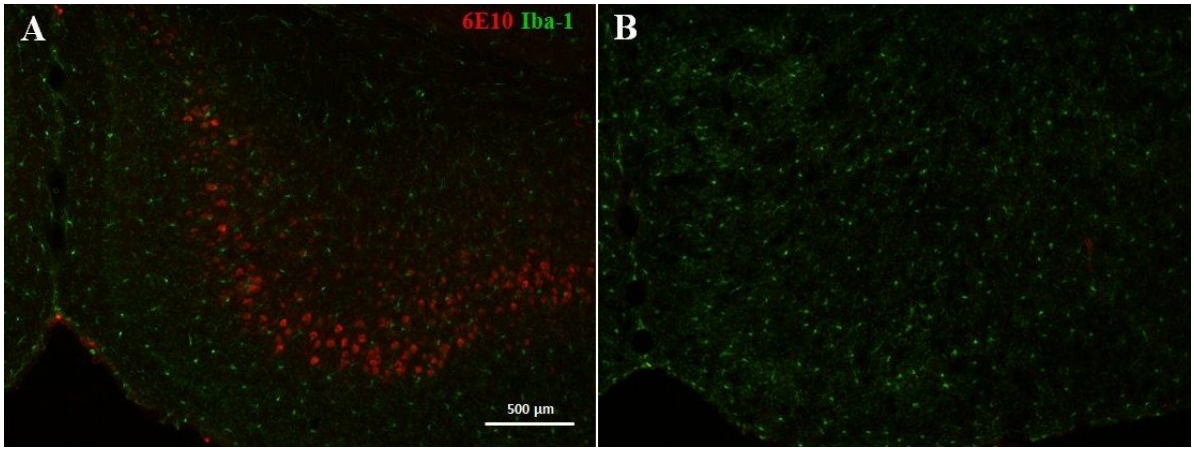


(A) 1 year old 3x Tg mouse hippocampus showing phosphorylated tau. (B) 1 year old B6 mouse hippocampus without phosphorylated tau. Schematic inset in (B) shows area imaged.

Microglia accumulate around Aβ plaques

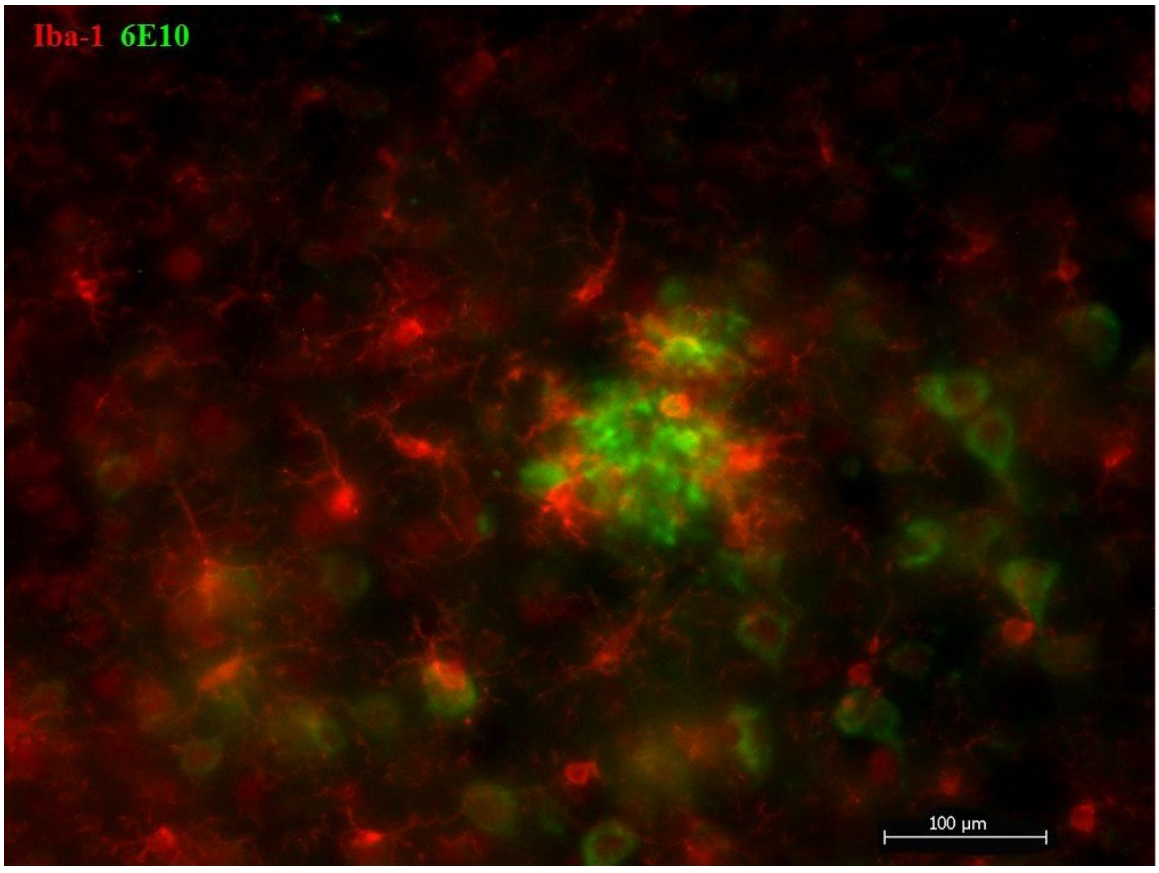
Endogenous microglia in the cortex of 3x Tg mice do not show overt signs of activation in relation to intracellular Aβ (Fig. 10). The microglia maintain a normal morphology with small cell bodies and branching processes, with a regular distribution throughout the brain. However, microglia closely associated with extracellular Aβ plaques differ slightly in appearance, having larger cell bodies and shorter processes (Fig. 11). There is also a higher density of microglia surrounding Aβ plaques, indicating that there is likely some signal attracting them to the plaques.

Figure 10. Microglia associated with Aβ



(A) 1 year old 3x Tg shows no morphological signs of activation of microglia associated with intracellular Aβ load. (B) 1 year old B6 has no intracellular Aβ and no microgliosis.

Figure 11. Microglia associated with Aβ plaque



Microglia (red) closely associate with Aβ deposits (green) in brain of 1 year old 3x Tg mice.

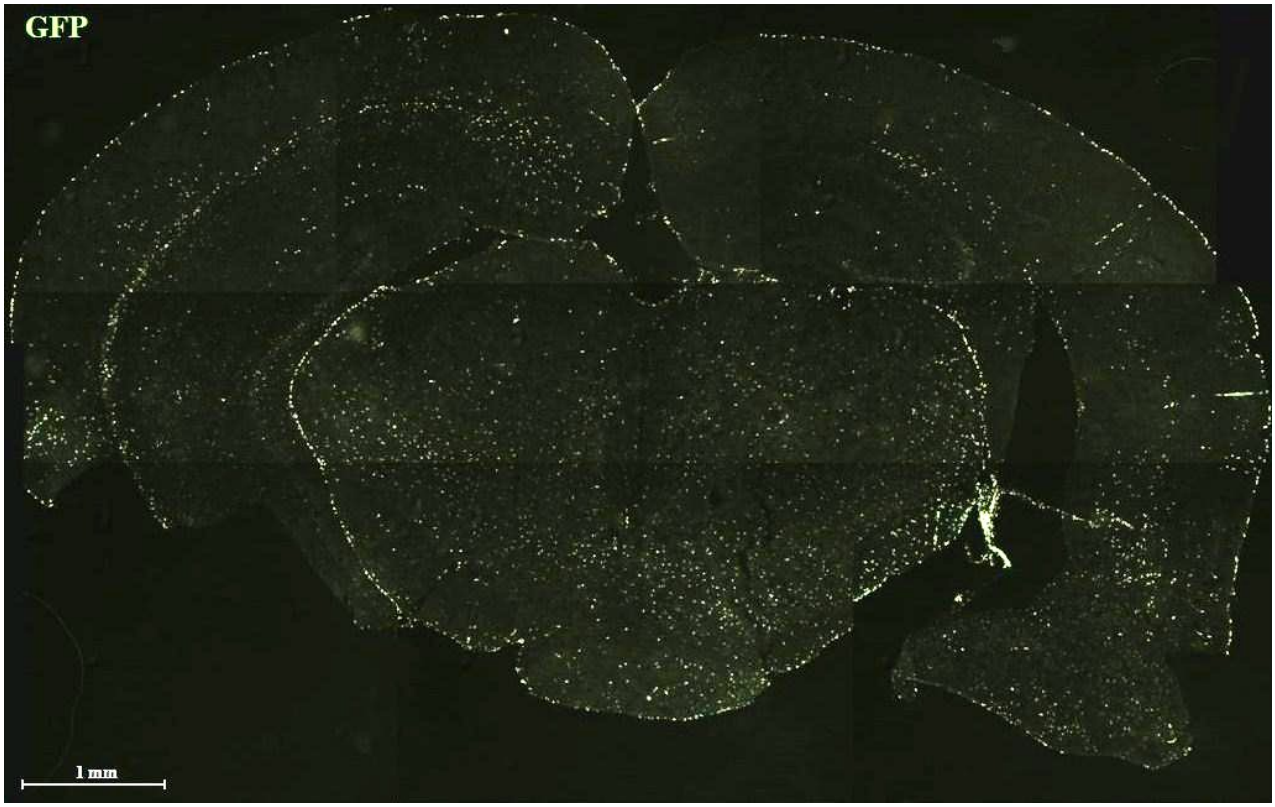
Bone marrow-derived cells accumulate in brain of AD mouse following irradiation

3x Tg mice (n=3) were exposed to 10 Gy radiation at 2 months of age and transplanted with $5x10^6GFP^+$ bone marrow cells from a C57BL/6 mouse ubiquitously expressing GFP on a β-actin promotor. A C57BL/6 mouse was transplanted in the same way to be used as a control; however, the transplant was not successful and it did not survive. Levels of GFP^+ cells in the blood were monitored by FACS until high level chimerism (>80%) persisted. This was established by 3 weeks post-transplant. These mice were sacrificed at 12 months of age and brain sections examined for distribution of GFP^+ cells. BMDCs can be seen throughout the brain with significant accumulation in the meninges and brainstem. BMDCs are also found in the cortex and hippocampus, which are areas of pathology in AD (Fig. 12).

Since the C57BL/6 irradiated control did not survive, I used C57BL/6 mice that were treated with 80 mg/kg BU and 200 mg/kg CY before transplanting with 1.5 x 10^7GFP^+ BM cells, as controls for the irradiated 3x Tg mice. Irradiated mice were not used as controls as there was evidence that irradiation might modify the properties of microglia independently of disease and as we believed that the BU treatment would be less stressful for the mice. However, we ran into difficulties with rejection of donor BM in BU treated 3x Tg mice and so have nothing to compare directly to these BU treated C57BL/6 mice. Wildtype transplanted mice did not have a large number of GFP^+ cells in the brain 8 months after transplant. As with the irradiated 3x Tg mice, the majority of GFP^+ cells in the BU + CY treated 3x Tg mice were in the meninges. There were a few GFP^+ cells in the cortex and hippocampus, but not to the extend as in the irradiated 3x Tg mice. The C57BL/6 BU treated mice are not ideal controls since irradiated mice have higher numbers of GFP^+ cells infiltrating the CNS than mice that are myeloablated with

BU (Lewis et. al. 2013, Kierdorf et. al. 2013); however these BU treated C57BL/6 mice were also used as controlsfor experiments employing BU treated 3x Tg mice.

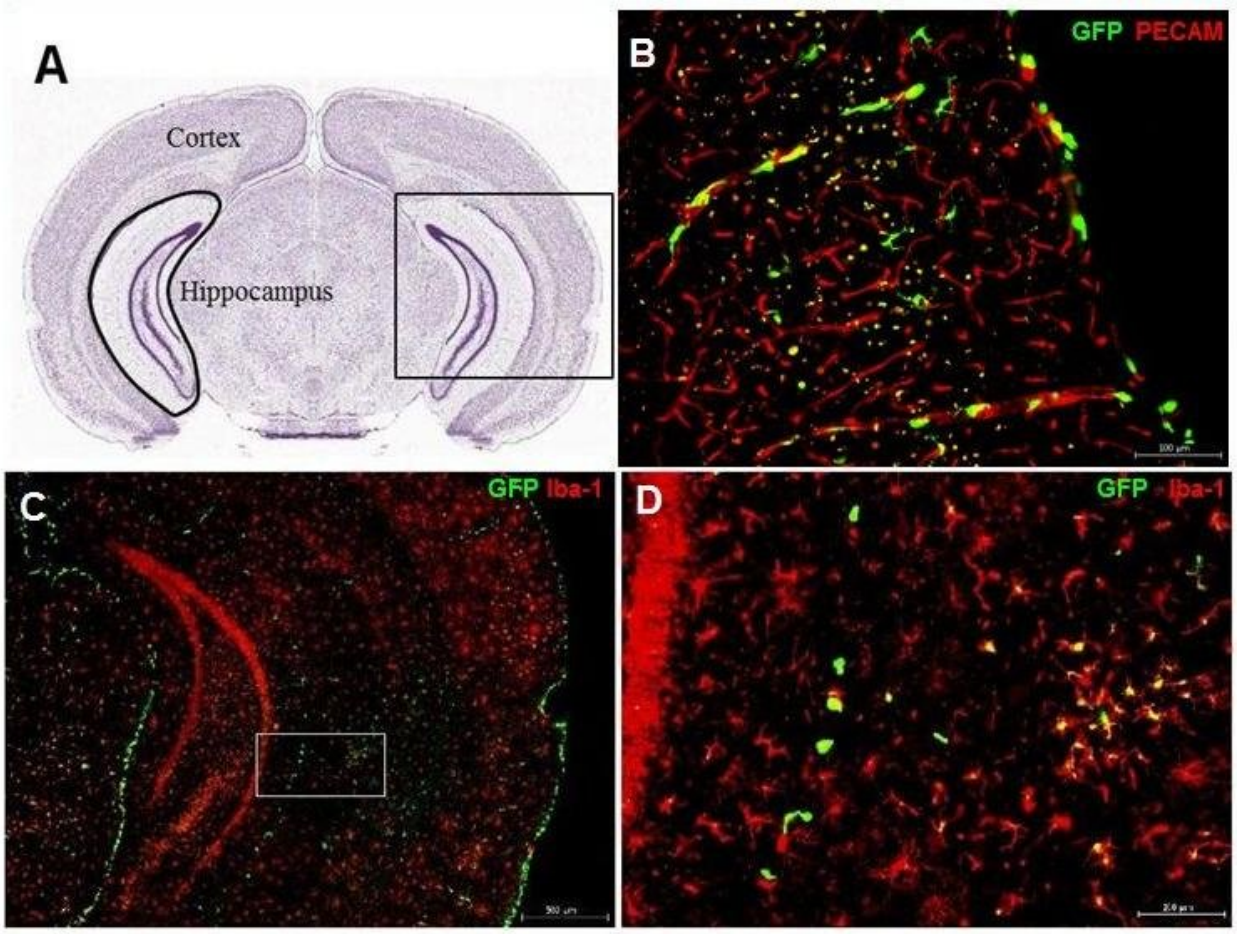
Figure 12. Bone marrow derived cell accumulation after irradiation



Bone marrow derived cell (GFP^+) accumulation in 1 year old irradiated 3x Tg mouse. GFP^+ cells are seen throughout the brain including the cortex and hippocampus.

In the 3x Tg mice, many of the donor derived cells are closely associated with platelet endothelial cell adhesion molecule-1 (PECAM-1) labeled blood vessels, indicating they are likely perivascular cells instead of cells that have crossed the BBB to penetrate the parenchyma. However, GFP^+ cells can also be observed further removed from blood vessels, presumably within the parenchyma (Fig. 13b). Both the blood vessel-associated GFP^+ cells and the parenchymal GFP^+ cells co-label with Iba-1, a microglia/macrophage marker (Fig. 13c,d).

Figure 13. GFP$^+$ cell engraftment in brain of irradiated and transplanted AD mice at 1 year of age



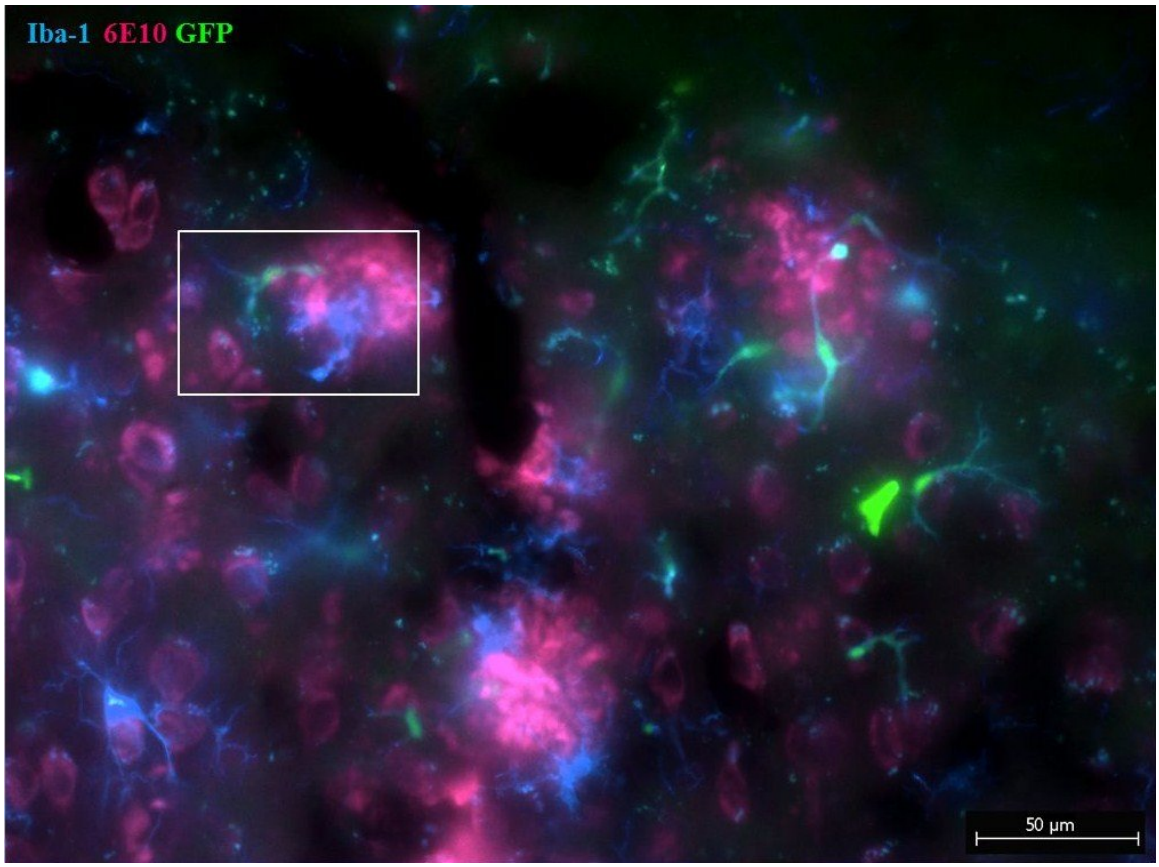
(A) Schematic of mouse brain outlining hippocampus, cortex and imaging field for B.(B) BMDCs (green) largely accumulate in the meninges or associate with CD31$^+$ blood vessels (red) (C) Representative section showing Iba-1 (red) and GFP (green). (D) Higher power image of boxed area in (C).

Bone marrow-derived cells associate with extracellular Aβ plaques

It was observed that endogenous microglia accumulate around Aβ plaques. BMDCs can also been seen aggregated around extracellular Aβ plaques along with endogenous microglia (Fig. 14). The BMDCs co-label with Iba-1 and display a ramified morphology with processes extending into the Aβ plaques. BMDCs had a less 'activated' appearance than host microglia associated with Aβ plaques, which had larger

cell bodies and retracted processes (Fig. 14 box). This suggests that they may be better able to degrade extracellular Aβ as they have not converted to a more inflammatory phenotype, which can be linked to CNS damage in neurological diseases.

Figure 14. GFP cells associated with Aβ plaques



GFP+ cells, along with endogenous microglia (blue) associate with extracellular Aβ deposits (pink) and co-label with the microglial marker Iba-1. Donor cells display a less activated phenotype with smaller cell bodies and longer, more ramified processes (box).

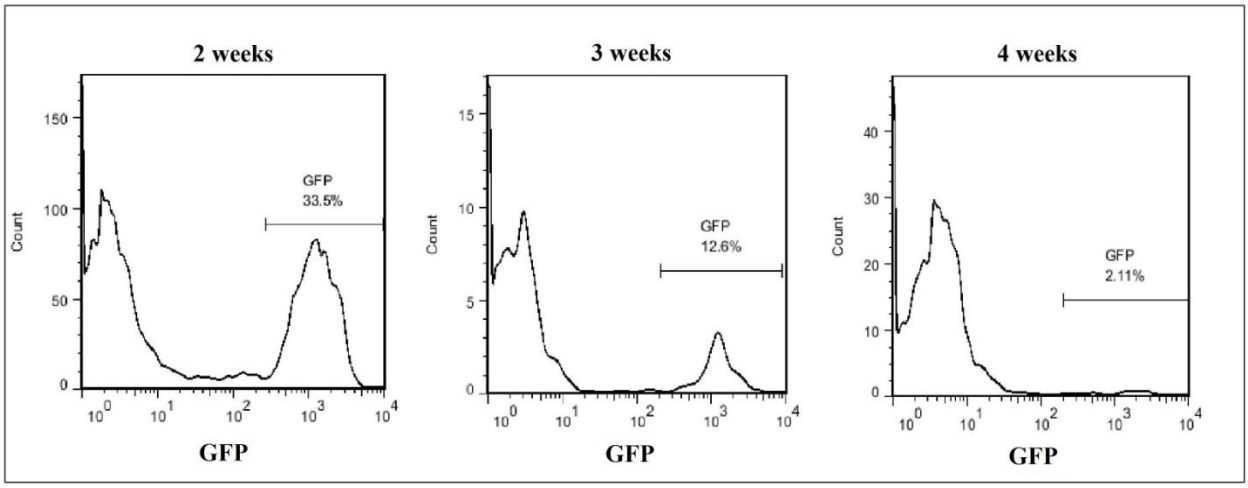
Busulfan alone is not sufficient to promote sustained chimerism in 129/B6 hybrid AD mice

Having observed that pretreatment of BM transplant recipient mice with 80 mg/kg BU successfully established >90% chimerism in C57BL/6 mice transplanted with GFP^+ C57BL/6 BM, we attempted the same protocol in the 3x TgAD mice. This resulted in the

initial presence of GFP^+ cells in the peripheral blood of the 3x TgAD mice, which quickly declined by 3 weeks post-transplant (n=2).

A second set of mice (n=3), were pretreated with 100 mg/kg BU followed by tail vein injection of C57BL/6 BM with the rationale that increasing the degree of myeloablation with the higher BU dose would enhance engraftment. Under these conditions, there was the presence of GFP^+ cells in peripheral blood which comprised around 33% of total cells at 2 weeks post-transplant. Chimerism declined steadily until 4 weeks post-transplant, when there was essentially no GFP^+ cells detected in blood (Fig. 15).

Figure 15. Chimerism after 100 mg/kg BU pretreatment

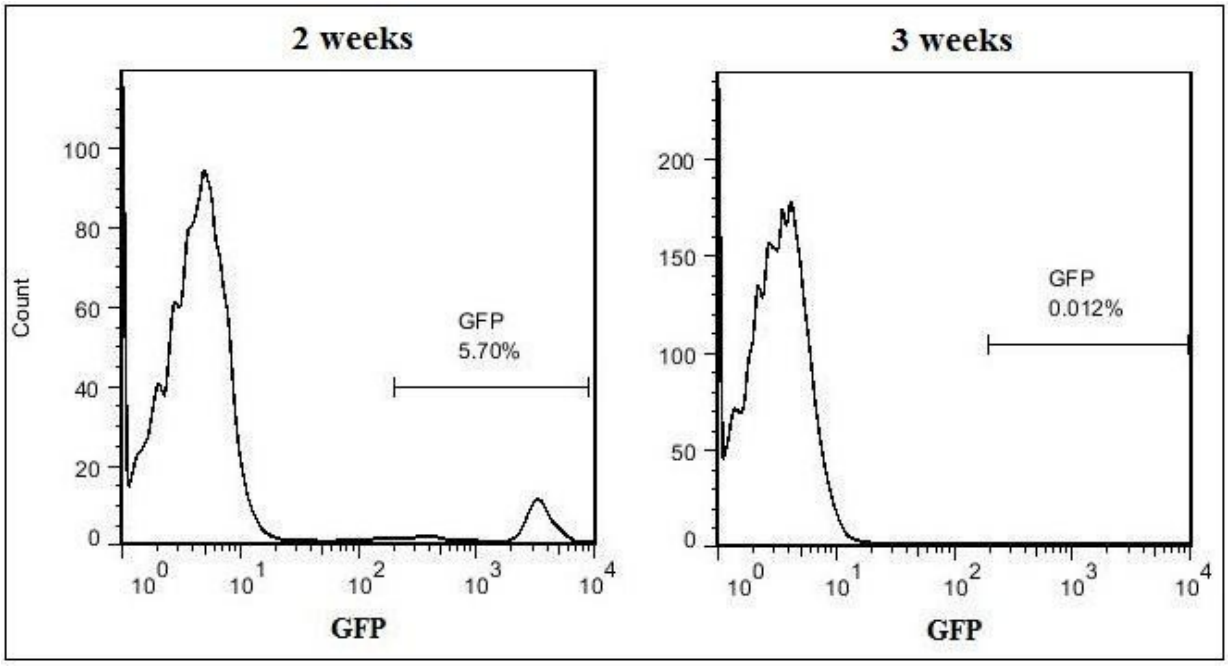


Representative histograms from 3x TgAD mice transplanted with B6 BM after 100 mg/kg BU pretreatment. At 2 weeks post-transplant peripheral blood is 33% GFP^+; at 3 weeks it is 12% GFP^+ and at 4 weeks has declined to 2% GFP^+.

With the hypothesis that the unsustained chimerism was due to rejection of donor BM, I next treated the 3x TgAD mice (n=3) with 80 mg/kg BU and transplanted with GFP^+ BM from the F1 generation of a C57BL/6 x 129 cross to improve histocompatibility.

These mice had limited amounts of GFP$^+$ cells in peripheral blood at 2 weeks post-transplant, and no GFP$^+$ cells by 3 weeks (Fig. 16).

Figure 16. Chimerism after 80 mg/kg BU pretreatment and 129/B6 BM transplant



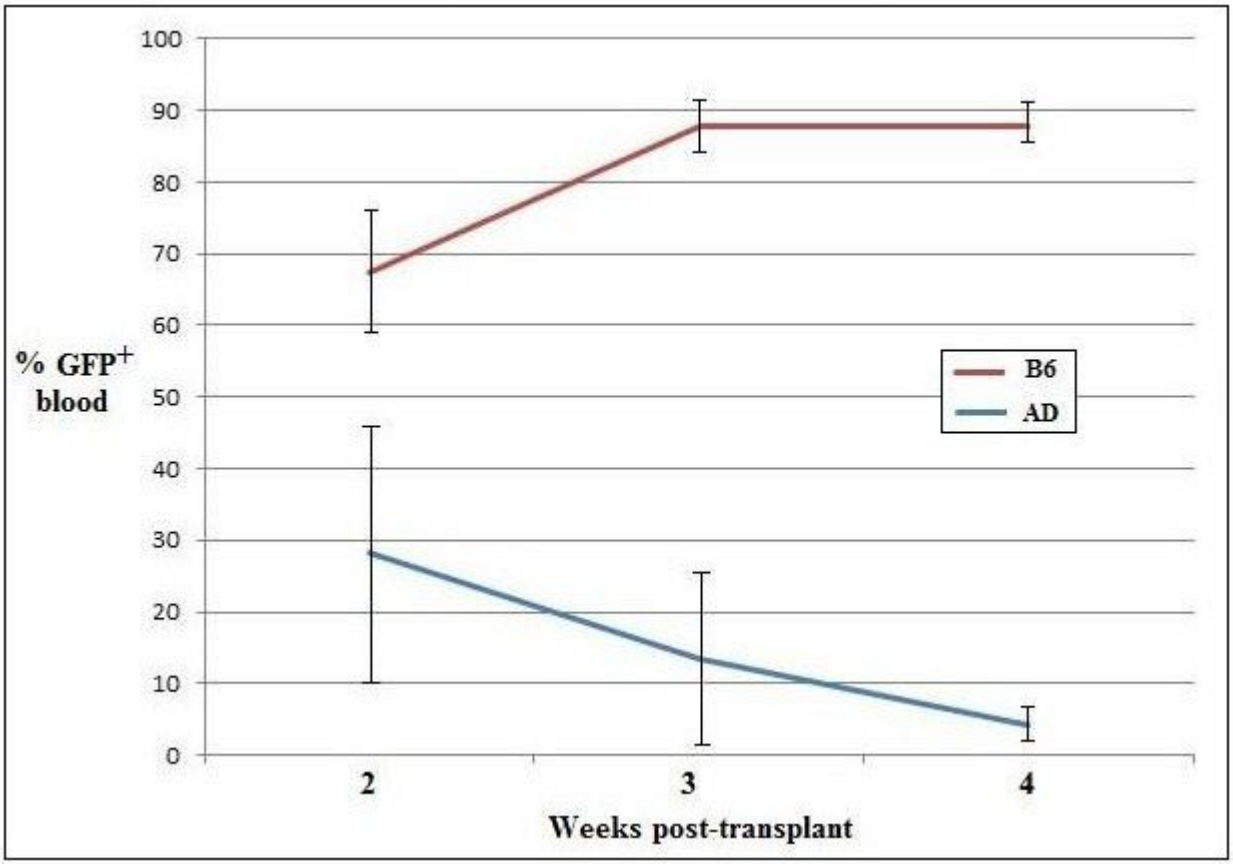
Representative FACS histograms from 3x TgAD mice transplanted with 129/B6 BM after 80 mg/kg BU pretreatment. 5% of peripheral blood is GFP$^+$ at 2 weeks post-transplant and 0% at 3 weeks.

Busulfan and Cyclophosphamide together give initial but temporary engraftment of GFP$^+$ BM

In attempts to prevent rejection of the bone marrow, 3x Tg AD and control B6 mice were treated with the immunosuppressant CY along with myeloablative BU (n=5 per group). Mice treated with 80mg/kg BU and 200 mg/kg CY were transplanted with B6 BM and peripheral blood was monitored for GFP$^+$ cells. Two weeks post-transplant 3x Tg AD mice averaged 28% ± 18% GFP+ peripheral blood, while B6 mice averaged 67% ± 8% chimerism. In the 3x Tg AD mice, the chimerism dropped to 13% ± 11% by week

3 and to 4% ± 2% by week 4. Chimerism in the B6 mice increased to 87% ± 4% in week 3 and 88% ± 3% in week 4 (Fig. 17).

Figure 17. Average chimerism following BU and CY pretreatment in 3x Tg AD and B6 mice

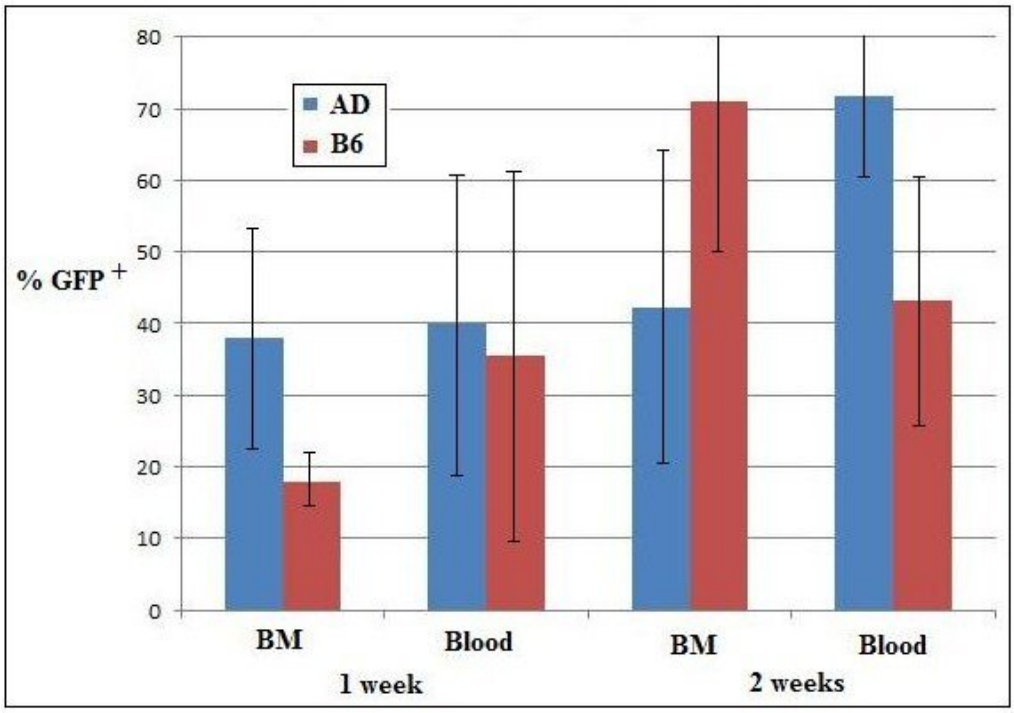


Graph of average percentage of GFP^+ cells in peripheral blood of 3x TgAD and B6 mice following BU and CY pretreatment and injection of GFP+ B6 BM. Chimerism in B6 started higher than in 3x Tg AD mice and increased to week 4 post-transplant. In 3x TgAD mice, chimerism was initially lower and dropped quickly through 4 weeks post-transplant.

To ensure that GFP^+ cells were populating the recipients BM and not just seen in the blood early after transplant, I treated 3x Tg AD and B6 mice with 80 mg/kg BU and 200 mg/kg CY and analyzed peripheral blood and BM at 1 and 2 weeks post-transplant (n=3 per group). There was a great deal of variation in percentage of GFP^+ cells in the BM and blood; however, both 3x Tg AD and B6 mice showed levels of GFP^+ cells in the

BM ranging from 8-58% with no difference between the AD and B6 mice. A similar effect was seen in the blood, with values ranging from 4-87% and no difference between 3x TgAD and B6 mice. This was seen again at 2 weeks, large variation in BM (17-93%) and blood (18-88%) with no difference between 3x TgAD and B6 groups in either BM or blood (Fig. 18).

Figure 18. Average chimerism in blood and BM of AD and B6 mice following BU and CY pretreatment



GFP^+ cells in BM and blood of 3x TgAD and B6 mice 1 and 2 weeks post-transplant. Values were highly variable, with no significant differences seen between AD and B6 mice in either BM or blood.

Depletion of NK cell activity in the host allows for sustained peripheral blood chimerism

Having observed that donor GFP^+ cells are present in the bone marrow following BU pretreatment and transplantation, it would appear that the donor cells are able to migrate to the BM, but are later destroyed by the host's cells. Under the hypothesis that

the rejection of the donor BM is due to hybrid resistance, I treated the 3x Tg mice with 80 mg/kg BU and an antibody against ASGM-1, to inhibit host NK cell function (n=11). Of the 11 mice treated with BU and anti-ASGM-1, 7 were seen to have chimerism over 60% at 2 weeks post-transplant. Of these 7 mice, chimerism dropped below 20% in 2 and remained above 70% in the other 5 at 3 weeks post-transplant. Chimerism in one of the 5 mice dropped to 21% at week 4; however the remaining 4 mice have shown sustained chimerism above 80% up to week 11 post-transplant (Fig. 19). Measurements have not been taken at later time points, but at this time chimerism is usually stable and essentially complete (Lewis et al. 2013).

Figure 19. Average chimerism following BU and NK cell activity depletion

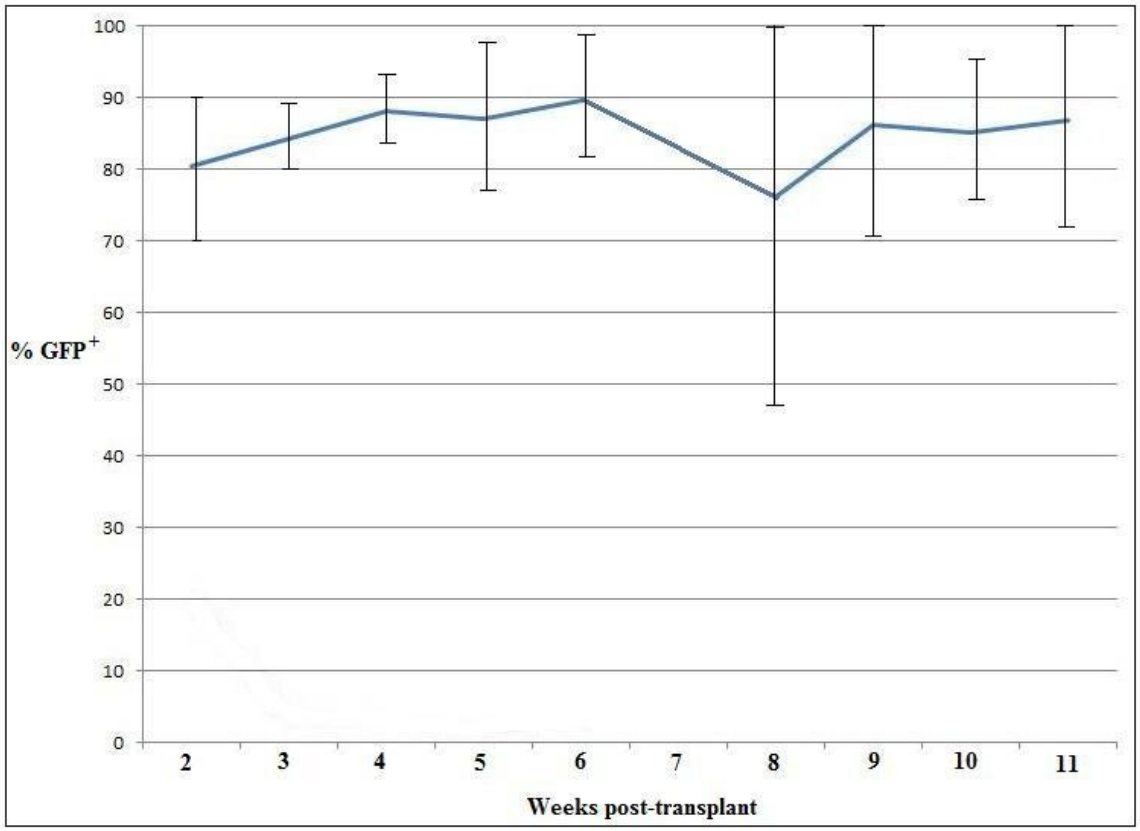


Fig. 19. Average %GFP$^+$ cells in peripheral bloodof3x Tg mice with sustained chimerism (n=4) following BU and anti-ASGM-1 treatment and GFP$^+$ BM transplant. Chimerism reached 80% within 2 weeks after injection and maintained this high level of chimerism.

Chimerism was measured for all mice treated with BU alone or with BU and CY in combination, and this was compared to mice treated with BU and anti-ASGM-1 to determine if there was a significant difference in levels of GFP^+ cells in the peripheral blood (Fig. 20).The mice in the non-NK cell depleted group were not treated in an identical manner as mice from different trials were pooled. This non- NK cell depleted group comprised mice treated with 80 mg/kg BU andC57BL/6 GFP^+ BM (n = 2), 100 mg/kg BU and C57BL/6 GFP^+ BM (n = 2), 80 mg/kg BU and C57BL/6x129 GFP^+ BM (n = 4) and 80 mg/kg BU + 200 mg/kg CY and C57BL/6 GFP^+ BM (n = 5). The NK cell depleted group was treated with 80 mg/kg BU and anti-ASGM-1 antibody and transplanted with C57BL/6 GFP^+ BM (n = 11). At 2 weeks post-transplant, no significant difference was found between the mean chimerism of the two groups, with the non-NK cell depleted group having a mean chimerism of 35%±8% compared to the NK cell depleted group at 56%±9% ($p > 05$). At 3 and 4 weeks post-transplant, a significant difference was observed between the non-NK cell depleted group and the NK cell depleted group at 13%±4% vs. 42%±12% (3 weeks; $p < 0.05$) and 5%±1% vs 36%±13% (4 weeks; $p < 0.05$; Fig. 20).

Figure 20. Chimerism in NK cell depleted mice vs. non-NK cell depleted mice

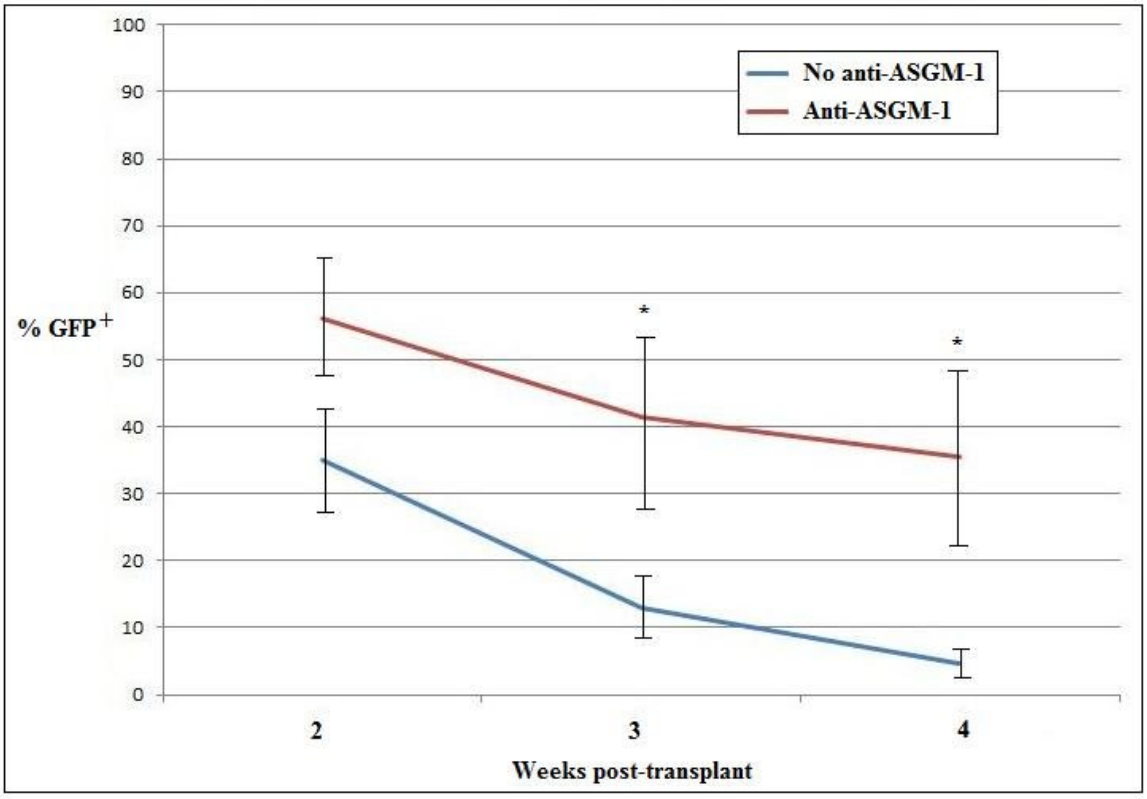


Fig. 20. Average % GFP^+ cells in peripheral blood of 3x Tg mice myeloablated with BU or BU and CY (blue line) compared to 3x Tg mice myeloablated with BU and treated with NK cell activity depleting anti-ASGM-1 antibody (red line). At 2 weeks post-transplant, there was no significant difference in mean chimerism. At 3 and 4 weeks post-transplant, chimerism was significantly higher in the NK cell depleted mice (Anti-ASGM-1; $p>0.05$).

Discussion

Use of the 3x Tg Model of AD

The aim of this work was to evaluate the distribution of BMDC in the CNS in a murine model of AD. As indicated above, there are a number of murine models of AD. From my preliminary reading, it appeared that the 3xTg mouse would be a good animal model of AD. The strengths of this model are that it develops both Aβ plaques and hyperphosphorylated tau NFTs, the mutant genes are co-integrated at the same site so that all offspring express all three transgenes, and genotyping of offspring is not necessary, as well as similar disease in both genders (Oddo et al. 2003). However, in my results I did not observe many Aβ plaques, even at 15 months of age. Based on the literature, we were expecting to see more plaques (Oddo et. al. 2003, Billings et. al. 2005), which would then be used to quantify BMDCs accumulation around the plaques. However, this was not the case, and experiments would need to be extended to later time points, or performed in a different mouse model with more rapid disease progression to study the relation between BMDC migrationwith disease. From my work, there appears to be a limited amount of extracellular Aβ in the 3x Tg mouse in the first year of disease. There does seem to be intracellular Aβ, likely intraneuronal in distribution, by 6 months of age, though whether this is intracellular Aβ or APP is not entirely clear.

The literature has been slow to accept the presence of intracellular Aβ in AD even though there have been reports of intracellular Aβ identificationusing immunohistochemistry as early as the 1980s (Grundke-Iqbal et. al. 1989). One factorthat

contributes to the lack of acceptance of intracellular Aβ in these murine models of AD is the technical issue of whether Aβ antibodies would cross-react with APP or fragments of APP, such as sAPP, or the C83 and C99 fragments of APP, since they share the same amino acid sequence and many of the same epitopes. In order to unambiguously classify intracellular Aβ-immunoreactive material as Aβ, it is necessary to use multiple antibodies to both N and C terminal epitopes exposed through cleavage of APP (Takahashi et. al. 2002).

There are many different antibodies for Aβ in use, specific to different regions of the Aβ protein. One popular antibody is the 6E10 antibody which has been reported to be specific to residues 1-16 of Aβ (McLean et. al. 2012). However, this antigenic sequence is present in APP as well and it has been claimed that cleavage of APP is not required to expose the antigenic site, in which case this antibody would label both Aβ and APP (Winton et. al. 2011). There are some antibodies that have been reported to be specific to neo-epitopes, which are not present in APP, but only in Aβ after being cleaved by γ-secretase and a conformational change having occurred due to the cleavage. Some examples of these antibodies are BA27, specific to Aβ ending at residue 40, and BC05, specific to Aβ ending at residue 42 (Iwatsubo et. al. 1994). Using these antibodies, along with others specific to Aβ sequences accessible in APP or to full-length APP, Winton *et. al.* (2011) attempted to determinewhether intracellular Aβ accumulates in the 3x Tg mice. Using this panel of antibodies and others, this group claimed that immunoreactivity is associated with APP accumulation rather than the presence of intracellular Aβ. This observation would be consistent with the APP overexpression in these mice (Oddo et. al. 2003). While the experiments are seemingly straightforward, there has been controversy over this issue. Some points of debate

around the experiments conducted by Winton *et. al.*(2011) concern the antigen retrieval methods, fixation and the lack of a positive control for the Aβ specific antibodies. Intracellular Aβ can be difficult to detect, requiring optimization of antigen retrieval techniques. Fixation methods are also important as paraffin embedded sections also seem to be less sensitive than fixed frozen sections for detection of intracellular Aβ. Winton *et. al*. (2011) used either paraffin embedded sections, or 10% PFA-fixed tissue, a much higher concentration of PFA than is usually used, which also reduces antigen sensitivity (D'Amico et. al. 2009). Finally, Winton *et. al.*(2011) did not employ positive controls showing that the supposedly Aβ-specific antibodies are in fact specific to Aβ. Given that this group presented largely negative results to prove that there is no intracellular Aβ accumulation in the 3x Tg mice, it would be necessary to present a positive control showing that the lack of detection of Aβis not a methodological problem.

I have used two different antibodies against different regions of the Aβ protein, to show that there is intracellular Aβ accumulation in neurons in AD. The 6E10 antibody binds to amino acids 1-16 of Aβ, which is present in APP as well, and seems to be in the same conformation. Because of this, 6E10 immunoreactivity occurs both with Aβ and APP. The second antibody I used was the 11A1 antibody, which binds to a neoepitope exposed with cleavage of APP. This neoepitope is a β-turn at amino acids 22 and 23 and Aβ proteins with this turn have been shown to quickly form oligomers (Murakami et. al. 2010). Immunoreactivty with the 11A1 antibody, combined with clear delineation of immunoreactive material and nuclear sparing is evidence that the labeled material is intracellular Aβ. This immunoreactivity could be shown to be specifically within neurons by co-staining with the neuronal nuclear antibody NeuN.

This putative intracellular Aβ has been documented in the brains of AD patients post-mortem; however, intracellular Aβ has also been seen in normal age-matched controls, as well as in children and infants using several different antibodies including 6E10, 4G8 (residues17-24 of Aβ) among others (Wegiel et. al. 2007). This intracellular Aβ is not localized to structures affected in AD, but is instead distributed diffusely throughout the brain, and varies between individuals. Intracellular Aβ is more readily seen in mouse models of AD than in humans. This observation may be due to mutations in APP and PS1 in these mice, which alters the metabolism of Aβ leading to more intracellular accumulation than is usual in mice or humans (Martin et. al. 1995). It has been hypothesized that intracellular Aβ accumulation might precede extracellular Aβ plaque formation, and that intracellular Aβ load decreases as plaque load increases (Oddo et. al. 2006). Since human brains are generally not available for analysis until late in disease, it is possible that the intracellular Aβ has already decreased and that Aβ is observed as extracellular plaques during histological analysis.

There are two possible routes by which Aβ could accumulate within neurons. First, Aβ could be cleaved from APP at the cell membrane and released extracellularly. From there Aβ could be endocytosed by neurons through a number of receptors including LRP and RAGE receptors or via internalization of other receptors after Aβ binding. Evidence for this route is seen *in vitro* as well as *in vivo*. Aβ binds to the α7 nicotinic acetylcholine receptor (α7nAChR), which results in internalization of the receptor as well as the bound Aβ (Nagele et. al. 2002). In the same study, it was seen that 3xTg AD mice had lower levels of α7nAChR on neurons in areas of intracellular Aβ accumulation. Decreased membrane expression of Aβ binding receptors LRP and

RAGE has also been seen in areas of intracellular Aβ accumulation (Bu et. al. 2006, Iribarren et. al. 2005).

A second possibility is that Aβ is produced intracellularly and does not need to be taken up from the extracellular space. APP for the most part localizes to the plasma membrane; however, it has also been detected in the membranes of the endoplasmic reticulum, Golgi network, mitochondria, endosomes and lysosomes (Mizuguchi et. al. 1992). If cleavage of APP occurs at these other locations, Aβ can be liberated into the cytosol instead of being secreted to the extracellular space. Evidence for this possible mechanism comes from *in vitro* studies using wild-type APP or mutant APP (APP_{swe}) expressing NT2N neuron cells (Martin et. al. 1995). Martin *et. al*. found that mutant neurons produced intracellular Aβ whereas wild-type neurons did not. The mutation in APP may lead to differential localization of APP or altered processing of APP, which gives rise to intracellular Aβ. Intracellular accumulation of Aβ can also be seen with duplication of the APP gene (Rovelet-Lecrux et. al. 2006). Together these studies suggest that altered expression, or mutation of APP may cause it to be localized to membranes within the cell leading to intracellular accumulation of Aβ. This may be one explanation as to why intracellular Aβ is seen more in AD mice than in humans with sporadic AD. Many mouse models of AD have mutations in APP or overexpress APP, whereas humans who develop sporadic AD do not have alterations in the APP gene and so localization may be maintained at the plasma membrane decreasing the amount of Aβ released within neurons.

While it is generally accepted that extracellular Aβ plaques play a role in the pathology of AD, the effect of intracellular Aβ is not as clearly understood or accepted. Intracellular Aβ has been shown to lead to muscle cell death in inclusion body myositis

(IBM), a muscle degeneration disease (Sugarman et. al. 2002). IBM patients display Aβ aggregation within muscle fibres leading to degeneration of muscle. Mice which overexpress the APPswe mutation under the control of the muscle creatine kinase gene promoter develop intracellular Aβ and muscle degeneration similar to IBM patients (Sugarman et. al. 2002). While there are several theories as to how intracellular Aβ accumulation can lead to neurodegeneration or synaptic loss, it is evident that clearance of intracellular Aβ can improve cognitive function in 3xTg AD mice (Billings et. al. 2005). Billings *et. al.* injected an anti-Aβ antibody into the third ventricle of 3xTg AD mice at 4 months of age, after intracellular Aβ begins to accumulate, but before plaque formation. The treated mice showed decreased levels of intracellular Aβ as well as improved cognitive function through water maze testing compared to untreated AD mice.

There is a directrelationship between intracellular and extracellular Aβ. From mouse models, it has been seen that intracellular Aβ precedes extracellular Aβ plaques and that by removing Aβ through immunotherapy, extracellular Aβ is cleared first followed by intracellular Aβ. When Aβ is allowed to build back up, intracellular Aβ accumulation again precedes formation of extracellular Aβ plaques (Oddo et.al. 2006). Whether it is intracellular or extracellular Aβ that is causing the damage in AD, the relationship between the two is promising for my work. BMDCs in the AD brain could potentially clear extracellular Aβ, as seen in the study by Oddo *et. al.* This could then lead to decreases in intracellular Aβ as well, and its potentially detrimental effects on neurons.

Quantification of Aβ and BMDC Association

Quantification of Aβ is an important measure of effectiveness of treatments of AD in mouse models. This quantification can be done in a few ways depending on whether

intracellular or extracellular Aβ is of interest. Intracellular Aβ can be quantified using imaging software, such as Scion Image system or Image Pro Plus, by maintaining a constant threshold intensity, and counting the number of pixels stained with an Aβ antibody over a constant area of the brain sections (Oddo et. al. 2006, Nagele et. al. 2002). This is assuming that the antibody is labeling intracellular Aβ, which may or may not be the case. Extracellular Aβ plaques can be counted manually from IHC labeled brain sections (Oddo et. al. 2006) or can be expressed as a proportion of the total area of the region of interest using imaging software and labeling technique that does not show intracellular Aβ (Krabbe et. al. 2013). Extracellular plaques have also been classified according to size and manually counted within different size categories (Mildner et. al. 2011).

I have not quantified intracellular Aβas it does not play a role in the main aim of this thesis; but is more an interesting, and somewhat unexpected observation. If BMDCs are seen to accumulate around intracellular Aβ, implicating intracellular Aβ as a signal for BMDCs, quantification may be necessary in the future. Extracellular Aβ plaques were not quantified simply because of the lack of plaques in the 3x Tg mice. Small numbers of plaques (3-4 per section) were observed in some 12 month old mice; however, plaques have not been observed in the 15 month old mice, which we had expected to see by this age.Hyperphosphorylated tau could be quantified in the same way as intracellular Aβ. I have not done this since NFT load does not play a pivotal role in this work.

One aim of this thesis was to quantify BMDC accumulation around extracellular Aβ plaques. BMDC association with Aβ plaques has been presented in several different ways. It can be expressed as a percentage of Aβ plaques with GFP^+ cells touching

them (Mildner et. al. 2011), differentiating morphology of GFP^+ cells associated with Aβ plaques (Stadler et. al. 2005), or expressing number of GFP^+ cells associated with each plaque, with plaques being categorized based on size (Mildner et. al. 2011) to give a few examples. The exact criteria for a GFP+ cell to be considered associated with an Aβ plaque is not always clear; however, has been defined as the cell body of the BMDC is in contact with the core of a Congo Red plaque (Simard et. al. 2006). Whether this definition is used by other groups is unclear.

Given the difficulty in establishing persistent chimerism in the 3x Tg mice, as well as the small numbers of extracellular plaques present, this quantification has not been performed. Now that we can establish chimerism in the mice using BU and NK cell depletion, these studies can be carried out, and time frames may need to be extended to ensure significant levels of extracellular Aβ plaques.

Busulfan versus Irradiation

BM transplantation often employs total body irradiation (TBI) as an initial step to open the BM niche for donor cells to repopulate. However, there are side effects from irradiation preconditioning treatment, which come with a risk of mortality in patients due to non-disease or transplant-related complications. In people undergoing BM transplantation, non-infectious transplant-related complications account for 50% of mortality (Andersen et. al. 2006). The high mortality rate is likely due to a cytokine storm induced by irradiation, which can then lead to veno-occlusive disease, idiopathic pneumonia, or multi-organ dysfunction (Hill et. al. 1997). Andersen *et. al.* (2009) showed that patients treated with TBI before transplantation showed higher levels of soluble TNF-α receptor 1 (sTNFR1), as well as other inflammatory cytokines, in blood compared to those treated with BU and CY, indicating a greater damaging effect of

irradiation. BU is not free of side effects however; as high dose BU is associated with seizures and veno-occlusive disease (Jones et. al. 1987). However, veno-occlusive disease can be milder with BU than with TBI, especially with the lower doses of BU used in reduced-intensity conditioning (RIC) regimens, which are not completely myeloablative and most often indicated in very young or old populations (Fadilah and Agilah, 2012). The reason RIC is not used in all BM transplants is that there is a higher occurrence of relapse when treating hematopoietic malignancies, as there is incomplete ablation of the malignancy (Fadilah and Agilah, 2012). In the case where a mixed chimerism is sufficient, and there is no malignancy to ablate, RIC should be sufficient in any population, not just in the more at risk populations.

Irradiation also has an effect on the BBB, increasing permeability and activating endothelial cells to increase expression of adhesion molecules. This has been deemed at least partially responsible for the entry and engraftment of BMDCs in the CNS of mice following BM transplantation, as shown through previously discussed parabiosis and head shielded irradiation experiments (Ajami et. al. 2007, Mildner et. al. 2007). Given that the BBB can already be affected in AD, it would not be ideal to incorporate a treatment strategy that causes more damage to the BBB. BU could be a less toxic pretreatment option than TBI as it seems to have less of an effect on BBB permeability than irradiation (Kierdorf et. al. 2013). Experiments by Kierdorf *et. al.* (2013) showed a decreased presence of albumin in the brains of mice pre-treated with BU as opposed to those pre-treated with irradiation, indicating decreased permeability of the BBB following BU.

BU does have some effect on the BBB and has been shown to promote engraftment of BMDCs in the CNS, though this occurrence is much more debated than

with the use of irradiation as preconditioning (Capotondo et. al. 2012, Lampron et. al. 2012, Lewis et. al. 2013, Kierdorf et. al. 2013). BU has the ability to cross the BBB (Hassan et. al. 1992) and so could, like irradiation, affect the CNS microenvironment. Capotondo *et. al.* (2013) have recently suggested that both irradiation and BU cause apoptosis of endogenous microglia in the brain. This partial clearing of the niche is necessary for donor-derived cells to accumulate and expand within the CNS, repopulating the space left by the death of the host microglia. The problem with the study by Capotondo *et. al.* is that all analysis of cell counts was done through FACS, and there is no way to be certain that the cells were in the brain parenchyma, as opposed to perivascular spaces or meninges. Further, data from both diseased mice as well as wild type controls were pooled together, even though there was a significant difference in numbers of cells in the brains in each case.

Recent work in our lab has shown that donor derived cells in the CNS following BM transplantation, using BU as the preconditioning treatment, are more ramified in morphology, with less engraftment of ameboidshaped cells in the spinal cord of ALS mouse models (Lewis et. al. 2013). The ameboidshaped cells that predominate in irradiated mice show immunophenotype and morphology consistent with activated microglial cells (Vallieres and Sawchenko, 2003) and may be more damaging than beneficial in the case of CNS disease compared to stellate-shaped cells, which closely resemble resident microglia.

As might be expected from the effect of irradiation on the body, irradiation also has lasting effects on the brain (Mildner et. al. 2011). In mice transplanted with GFP^+ BM cells following either TBI (unprotected) or with head shielding (protected), endogenous microglia show altered morphology even 7 months post-transplant as a

result of the irradiation. In the unprotected animals, endogenous microglia were not as closely associated with Aβ plaques and showed signs of activation, namely retraction of cellular processes and rounding of somata, as well as increased expression of CXCL10, CCL2 and TNFα. In the protected animals, microglia were much more tightly associated with Aβ plaques, with microglial cellular processes reaching the core of Aβ plaques (Mildner et. al. 2011). Whether the activating effect of irradiation can be ameliorated with the use of BU instead of irradiation is an important question requiring further exploration. As inflammation is a major player in AD pathology, it would be beneficial to limit the induction of further inflammation in the CNS. Furthermore, the more stellate shaped cells accumulating in the CNS following BU pretreatment (Lewis et. al. 2013) may be better able to phagocytose Aβ than the activated ameboid-shaped cells accumulating following irradiation.

Another advantage of using BU over irradiation is that while irradiation is immunosuppressive, BU is only myeloablative, leaving the host's adaptive immune system intact (Tutschka and Santos, 1977). Immunosuppression can be critical in the case of allogeneic BM transplant in preventing rejection of the donor cells. However, with autologous transplant, immunosuppression becomes unnecessary and puts the host at risk of opportunistic infection. For the use of BMDCs as gene delivery vehicles, autologous transplant of transfected cells would be ideal since a supply of cells would be readily available through mobilization of the hosts BM and the risks of transplant complications decrease because transplanted cells are immunologically identical to the host.

Hybrid Resistance

Given the above benefits of a less intensive chemotherapeutic pre-treatment regimen, I examined donor cell engraftment in the brains of AD mice using a BU conditioning treatment instead of irradiation. There were substantial difficulties in employing this treatment, which appeared to be related to the hybrid background of the 3x Tg mice used. The rejection observed in the 3x Tg mice could be due to the phenomenon of hybrid resistance. One potential approach to minimize hybrid resistance is by treating mice with anti-ASGM-1 antibodies on the day of BM transplant and again at 2 weeks post-transplant. This treatment neutralizes NK cell function in the host preventing the killing of donor cells and allowing them a chance to engraft and establish persistent high-level chimerism.Using this modification I was able to achieve a sustained, high level of chimerism in many of the 3 xTg mice. These levels of chimerism were comparable to those seen with irradiation.

It has been shown that B6 mice and 129 mice express different Ly49 genes with differing specificities and affinities for MHC I alleles (Makrigiannis et. al. 2001). These differences could explain the rejection of B6 BM by the 129/B6 3x Tg mice. There may be a difference in the activating and inhibitory signals received by host NK cells, in this case favouring activation of these cells and resulting in the lysis of donor BM cells by host NK cells. Though very similar, there are differences between the MHC haplotype of B6 and 129 mice as well. B6 mice are H-2^{b} haplotypes whereas 129 mice are H-2^{bc} haplotypes, indicating a difference in the T region of the MHC I locus (Table 1). Specifically, the difference is seen at TL, an MHC class Ib locus, where B6 mice have a *b* allele and 129 mice have an *f* allele (Fischer-Lindahl 1997). This slight difference

could have accounted for the rejection of BM transplantation; however, this is unlikely as the BM was from a parental strain, which the recipient would presumably be tolerant of.

Table 1. MHC haplotype of 129 and C57BL/6 mice

Mouse Strain	MHC Haplotype	H-2K	H-2D	H-2L	I-A	I-E	Qa-2	Qa-1	CD8a (Ly-2)	CD8b (Ly-3)	CD45 (Ly-5)	Thy-1 (CD90)	Tla
129/-	b	b	b	null	b	k	a	b	2	2	2	2	f
C57BL/6	b	b	b	null	b	null	a	b	2	2	2	2	b

Table 1.MHC haplotype of 129 and C57BL/6 mice, differing only at Tla locus

Some of the 3x Tg mice that I treated with BU and anti-ASGM-1 have developed sustained, high-level chimerism whereas all of the 3 xTg mice that were treated with BU or BU and CY, did not develop chimerism. There was a significant difference in chimersim between the NK cell depleted mice and the non-NK cell depleted mice when the mice from the different treatment protocols were pooled together. All C57BL/6 mice transplanted with syngeneic BM developed high level chimerism even with BU alone. This is evidence that the rejection was due to hybrid resistance. With NK cell depletion, experiments looking at accumulation of BMDCs in the brain of the 3x Tg AD mice can be performed with BU instead of irradiation. We can use this to explore association of BMDCs with Aβ as well as morphology and activation of BMDCs accumulating around extracellular Aβ plaques with BU versus irradiation pretreatment.

BBB in AD

It has been reported that the BBB is compromised in AD. Most studies showing increased BBB permeability in AD patients use a CSF/serum albumin ratio as an

indication of leakage of blood proteins to the CNS (Farrall and Wardlaw, 2009). Farrall and Wardlaw (2009) analyzed data from a large number of studies of AD patients compared to age-matched, healthy controls as well as in young health people compared to older, healthy people. Overall, they found that the majority of studies reported an increase in CSF/serum albumin ratio with age in the healthy population, along with a greater increase in AD patients compared to healthy age-matched controls (Farrall and Wardlaw, 2009). Similar results have been seen in mouse models of AD, both in single transgenic APP mutants as well as APP/PS1 double transgenic mice, using Evans Blue as an indicator of BBB permeability (Donahue and Johanson, 2008).

The mechanism behind the increased permeability is not clearly defined; however, several theories exist. Aβ may be toxic to tight junction proteins of endothelial cells, as seen with decreased expression of zona occluden-2 and occludin in rat brain microvessels exposed to Aβ (Donahue and Johanson, 2008). Donahue and Johanson (2008) also saw that in AD brain tissue, compared to healthy age-matched controls, there was thinning and fragmentation of the extracellular matrix protein agrin, which is important in the maintenance of the basement membranes forming part of the BBB. The thinning and fragmentation was often closely associated with areas of Aβ deposition suggesting a negative effect of Aβ on agrin.

While there is an apparent increase in BBB permeability in AD according to these studies, an important question is whether there is an accompanying increase in the immune cell infiltration of the brain. The BM cells that might engraft in the CNS are much larger than the proteins that have been previously shown to pass through the BBB in AD; so, infiltration of cells will not necessarily increase as well as proteins. It seems that activation of the BBB is required to increase immune cell infiltration and not just an

increase in BBB permeability (Larochelle et. al. 2011). Li *et. al.* (2009) showed that Aβ can increase expression of CCR5 on endothelial cells of the BBB. CCR5 can bind MIP-1α and increase extravasation of T cells across the BBB in vitro (Li et. al. 2009). This could explain why more T cells are found in the post mortem brains of AD patients when compared to age matched controls (Togo et. al. 2002). Monocytes, as well as most hematopoietic cells express MIP-1α, a ligand for CCR5, so there may also be increased migration of other hematopoietic cells into the brain of AD patients (Menten et. al. 2002). MIP-1α expression is enhanced in monocytes in the presence of inflammatory cytokines IFN-γ and IL-1β as well as LPS (Menten et. al. 2002). If there is an increased influx of a microglial precursor cell from circulation there would be increased engraftment of donor cells in the brain following BM transplantation, which might be beneficial for clearance of Aβ or potentially for delivery of therapeutics to diseased areas of the brain.

References

Abbott NJ, Rönnbäck L, Hansson E. 2006. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7(1):41-53.

Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FMV. 2007. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nat Neurosci 10:1538-1543.

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, et. al. 2000.Inflammation and Alzheimer's disease. Neurobiol Aging 21:383-421.

Al-Adra D, Chan WFN, Anderson CC. 2011. Nonobese diabetic natural killer cells: a barrier to allogeneic chimerism that can be reduced by rapamycin. Transplantation 92:977-984.

Andersen J, Heilmann C, Jacobsen N, Nielsen C, Bendtzen K, Müller K. 2006. Differential effect of conditioning regimens on cytokine responses during allogeneic stem cell transplantation. Bone Marrow Transplant 37:635-640.

Bakker AB, Wu J, Phillips JH, Lanier LL. 2000. NK cell activation: distinct stimulatory pathways counterbalancing inhibitory signals. Hum Immunol 61:18-27.

Barao I, Murphy WJ. 2003. The immunobiology of natural killer cells and bone marrow allograft rejection. Biology of Blood and Marrow Transplantation 9:727-741.

Barry C. 2003. Phenotypically curing β-thalassemic mice in a non-myeloablative setting.Honours thesis.UBC.

Bechmann I, Priller J, Kovac A, Böntert M, Wehner T, Klett FF, Bohsung J, Stuschke M, Dirnagl U, Nitsch R. 2001. Immune surveillance of mouse brain perivascular spaces by blood-borne macrophages. Eur J Neurosci 14:1651-1658.

Beers DR, Henkel JS, Xiao Q, Zhao W, Wang J, Yen AA, Siklos L, McKercher SR, Appel SH. 2006. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences 103:16021-16026.

Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, Zlokovic BV. 2010. Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. Neuron 68:409-427.

Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM. 2005. Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. Neuron 45:675-688.

Boles NC, Lin KK, Lukov GL, Bowman TV, Baldridge MT, Goodell MA. 2011. CD48 on hematopoietic progenitors regulates stem cells and suppresses tumor formation. Blood 118:80-87.

Bu G, Cam J, Zerbinatti C. 2006.LRP in amyloid-beta production and metabolism. Ann N Y Acad Sci 1086:35-53.

Capotondo A, Milazzo R, Politi LS, Quattrini A, Palini A, Plati T, Merella S, Nonis A, di Serio C, Montini E, Naldini L, Biffi A. 2012. Brain conditioning is instrumental for successful microglia reconstitution following hematopoietic stem cell transplantation. Proceedings of the National Academy of Sciences 109:15018-15023.

Chakrabarty P, Jansen-West K, Beccard A, Ceballos-Diaz C, Levites Y, Verbeeck C, Zubair AC, Dickson D, Golde TE, Das P. 2010. Massive gliosis induced by interleukin-6 suppresses Aβ deposition in vivo: evidence against inflammation as a driving force for amyloid deposition. The FASEB Journal 24:548-559.

Chen X, Walker DG, Schmidt AM, Arancio O, Lue L, Yan SD. 2007. RAGE: a potential target for Abeta-mediated cellular perturbation in Alzheimer's disease. Curr Mol Med 7:735-742.

Chiang PK, Lam MA, Luo Y. 2008. The many faces of amyloid beta in Alzheimer's disease. Curr Mol Med 8:580-584.

Choi SH, Leight SN, Lee VM-, Li T, Wong PC, Johnson JA, Saraiva MJ, Sisodia SS. 2007. Accelerated Abeta deposition in APPswe/PS1deltaE9 mice with hemizygous deletions of TTR (transthyretin). J Neurosci 27:7006-7010.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ. 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672-674.

Cudkowicz G, Bennett M. 1971. Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F 1 hybrid mice. J Exp Med 134:1513-1528.

D'Amico F, Skarmoutsou E, Stivala F. 2009. State of the art in antigen retrieval for immunohistochemistry. J Immunol Methods 341:1-18.

De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, Klein WL. 2007. Aβ Oligomers Induce Neuronal Oxidative Stress through an N-Methyl-D-aspartate Receptor-dependent Mechanism That Is Blocked by the Alzheimer Drug Memantine. Journal of Biological Chemistry 282:11590-11601.

Deane R, Wu Z, Zlokovic BV. 2004. RAGE (Yin) Versus LRP (Yang) Balance Regulates Alzheimer Amyloid β-Peptide Clearance Through Transport Across the Blood–Brain Barrier. Stroke 35:2628-2631.

Decker H, Lo KY, Unger SM, Ferreira ST, Silverman MA. 2010. Amyloid-β Peptide Oligomers Disrupt Axonal Transport through an NMDA Receptor-Dependent Mechanism That Is Mediated by Glycogen Synthase Kinase 3β in Primary Cultured Hippocampal Neurons. The Journal of Neuroscience 30:9166-9171.

Donahue JE, Johanson CE. 2008. Apolipoprotein E, amyloid-beta, and blood-brain barrier permeability in Alzheimer disease. J Neuropathol Exp Neurol 67:261-270.

Duran-Struuck R, Dysko RC. 2009. Principles of bone marrow transplantation (BMT): providing optimal veterinary and husbandry care to irradiated mice in BMT studies. J Am Assoc Lab Anim Sci 48:11-22.

Espejel S, Romero R, Alvarez-Buylla A. 2009. Radiation damage increases Purkinje neuron heterokaryons in neonatal cerebellum. Ann Neurol 66:100-109.

Fadilah SA, Aqilah MP. 2012. Promising role of reduced-toxicity hematopoietic stem cell transplantation (PART-I). Stem Cell Rev 8:1254-1264.

Farrall AJ, Wardlaw JM. 2009. Blood–brain barrier: Ageing and microvascular disease – systematic review and meta-analysis. Neurobiol Aging 30:337-352.

Fiala M, Lin J, Ringman J, Kermani-Arab V, Tsao G, Patel A, Lossinsky AS, Graves MC, Gustavson A, Sayre J, Sofroni E, Suarez T, Chiappelli F, Bernard G. 2005. Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients. J Alzheimers Dis 7:221-232.

Fischer Lindahl K. 1997. On naming H2 haplotypes: functional significance of MHC class Ib alleles. Immunogenetics 46:53-62.

Galambrun C, Pondarré C, Bertrand Y, Loundou A, Bordigoni P, Frange P, Lutz P, Mialou V, Rubie H, Socié G, Schneider P, Bernaudin F, Paillard C, Michel G, Badens C, Thuret I. 2013. French Multicenter 22-Year Experience in Stem Cell Transplantation for Beta-Thalassemia Major: Lessons and Future Directions. Biology of Blood and Marrow Transplantation 19:62-68.

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M. 2010. Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. Science 330:841-845.

Goedert M, Jakes R. 2005. Mutations causing neurodegenerative tauopathies. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1739:240-250.

Graeber MB. 2010. Changing Face of Microglia. Science 330:783-788.

Grundke-Iqbal I, Iqbal K, George L, Tung YC, Kim KS, Wisniewski HM. 1989. Amyloid protein and neurofibrillary tangles coexist in the same neuron in Alzheimer disease. Proc Natl Acad Sci U S A 86:2853-2857.

Hardy JA, Higgins GA. 1992. Alzheimer's disease: the amyloid cascade hypothesis. Science 256:184-185.

Herberman RB, Nunn ME, Holden HT, Lavrin DH. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer 16:230-239.

Herzig MC, Winkler DT, Burgermeister P, Pfeifer M, Kohler E, Schmidt SD, Danner S, Abramowski D, Stürchler-Pierrat C, Bürki K, van Duinen S,G., Maat-Schieman M, Staufenbiel M, Mathews PM, Jucker M. 2004. Abeta is targeted to the vasculature in a mouse model of hereditary cerebral hemorrhage with amyloidosis. Nat Neurosci 7:954-960.

Hess DC, Abe T, Hill WD, Studdard AM, Carothers J, Masuya M, Fleming PA, Drake CJ, Ogawa M. 2004. Hematopoietic origin of microglial and perivascular cells in brain. Exp Neurol 186:134-144.

Hickey WF, Kimura H. 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science 239:290-292.

Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. 1997. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood 90:3204-3213.

Hoozemans JJM, O'Banion MK. 2005. The role of COX-1 and COX-2 in Alzheimer's disease pathology and the therapeutic potentials of non-steroidal anti-inflammatory drugs. Curr Drug Targets CNS Neurol Disord 4:307-315.

Ihl R, Frölich L, Winblad B, Schneider L, Burns A, Möller H. 2011. World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for the biological treatment of Alzheimer's disease and other dementias. World J Biol Psychiatry 12:2-32.

Ingelsson M, Fukumoto H, Newell KL, Growdon JH, Hedley-Whyte E, Frosch MP, Albert MS, Hyman BT, Irizarry MC. 2004. Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain. Neurology 62:925-931.

Iribarren P, Zhou Y, Hu J, Le Y, Wang JM. 2005. Role of formyl peptide receptor-like 1 (FPRL1/FPR2) in mononuclear phagocyte responses in Alzheimer disease. Immunol Res 31:165-176.

Iwamoto T, Hiraku Y, Oikawa S, Mizutani H, Kojima M, Kawanishi S. 2004. DNA intrastrand cross-link at the 5'-GA-3' sequence formed by busulfan and its role in the cytotoxic effect. Cancer Sci 95:454-458.

Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. 1994. Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta

monoclonals: evidence that an initially deposited species is A beta 42(43). Neuron 13:45-53.

Jarrett JT, Berger EP, Lansbury PT, J. 1993. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 32:4693-4697.

Jones RJ, Lee KS, Beschorner WE, Vogel VG, Grochow LB, Braine HG, Vogelsang GB, Sensenbrenner LL, Santos GW, Saral R. 1987.Venoocclusive disease of the liver following bone marrow transplantation. Transplantation 44:778-783.

Kaku M, Tsutsui K, Motokawa M, Kawata T, Fujita T, Kohno S, Tohma Y, Ohtani J, Tenjoh K, Tanne K. 2003. Amyloid beta protein deposition and neuron loss in osteopetrotic (op/op) mice. Brain Res Brain Res Protoc 12:104-108.

Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, Sisodia S, Malinow R. 2003. APP Processing and Synaptic Function. Neuron 37:925-937.

Kierdorf K, Katzmarski N, Haas CA, Prinz M. 2013. Bone marrow cell recruitment to the brain in the absence of irradiation or parabiosis bias. PLoS One 8:e58544-e58544.

Klunk WE, Jacob RF, Mason RP. 1999. Quantifying Amyloid β-Peptide (Aβ) Aggregation Using the Congo Red-Aβ (CR–Aβ) Spectrophotometric Assay. Anal Biochem 266:66-76.

Krabbe G, Halle A, Matyash V, Rinnenthal JL, Eom GD, Bernhardt U, Miller KR, Prokop S, Kettenmann H, Heppner FL. 2013. Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. PLoS One 8:e60921-e60921.

Kreutzberg GW. 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312-318.

Kumar-Singh S, Theuns J, Van Broeck B, Pirici D, Vennekens K, Corsmit E, Cruts M, Dermaut B, Wang R, Van Broeckhoven C. 2006. Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. Hum Mutat 27:686-695.

Lampron A, Lessard M, Rivest S. 2012. Effects of Myeloablation, Peripheral Chimerism, and Whole-Body Irradiation on the Entry of Bone Marrow-Derived Cells Into the Brain. Cell Transplantation, Cell Transplant 21:1149-1159.

Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature 391:703-707.

Larochelle C, Alvarez JI, Prat A. 2011. How do immune cells overcome the blood–brain barrier in multiple sclerosis?. FEBS Lett 585:3770-3780.

Lewis CB, Manning J, Barr C, Peake K, Humphries RK, Rossi F, Krieger C. 2013. Myelosuppressive conditioning using busulfan enables bone marrow cell accumulation in the spinal cord of a mouse model of amyotrophic lateral sclerosis. PLoS One 8:e60661-e60661.

Li M, Shang D, Zhao W, Tian L, Li B, Fang W, Zhu L, Man S, Chen Y. 2009. Amyloid beta interaction with receptor for advanced glycation end products up-regulates brain endothelial CCR5 expression and promotes T cells crossing the blood-brain barrier. J Immunol 182:5778-5788.

Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, Cole GM. 2000. Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. J Neurosci 20:5709-5714.

Linard C, Marquette C, Mathieu J, Pennequin A, Clarençon D, Mathé D. 2004. Acute induction of inflammatory cytokine expression after γ-irradiation in the rat: Effect of an NF-κB inhibitor. International Journal of Radiation Oncology*Biology*Physics 58:427-434.

Ljunggren HG, Kärre K. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J Exp Med 162:1745-1759.

Makrigiannis AP, Pau AT, Saleh A, Winkler-Pickett R, Ortaldo JR, Anderson SK. 2001.Class I MHC-binding characteristics of the 129/J Ly49 repertoire. J Immunol 166:5034-5043.

Martin BL, Schrader-Fischer G, Busciglio J, Duke M, Paganetti P, Yankner BA. 1995. Intracellular accumulation of beta-amyloid in cells expressing the Swedish mutant amyloid precursor protein. J Biol Chem 270:26727-26730.

Mason LH, Gosselin P, Anderson SK, Fogler WE, Ortaldo JR, McVicar DW. 1997. Differential tyrosine phosphorylation of inhibitory versus activating Ly-49 receptor proteins and their recruitment of SHP-1 phosphatase. J Immunol 159:4187-4196.

McLean D, Cooke MJ, Wang Y, Fraser P, George-Hyslop PS, Shoichet MS. 2012.Targeting the amyloid-β antibody in the brain tissue of a mouse model of Alzheimer's disease. J Controlled Release 159:302-308.

Meda L, Cassatella MA, Szendrei GI, Otvos L, J., Baron P, Villalba M, Ferrari D, Rossi F. 1995. Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature 374:647-650.

Menten P, Wuyts A, Van Damme J. 2002. Macrophage inflammatory protein-1. Cytokine Growth Factor Rev 13:455-481.

Meyer-Luehmann M, Coomaraswamy J, Bolmont T, Kaeser S, Schaefer C, Kilger E, Neuenschwander A, Abramowski D, Frey P, Jaton AL, Vigouret J, Paganetti P, Walsh DM, Mathews PM, Ghiso J, Staufenbiel M, Walker LC, Jucker M. 2006.

Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. Science 313:1781-1784.

Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch U, Mack M, Heikenwalder M, Brück W, Priller J, Prinz M. 2007. Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. Nat Neurosci 10:1544-1553.

Mildner A, Schlevogt B, Kierdorf K, Böttcher C, Erny D, Kummer MP, Quinn M, Brück W, Bechmann I, Heneka MT, Priller J, Prinz M. 2011. Distinct and non-redundant roles of microglia and myeloid subsets in mouse models of Alzheimer's disease. J Neurosci 31:11159-11171.

Mizuguchi M, Ikeda K, Kim SU. 1992. Differential distribution of cellular forms of beta-amyloid precursor protein in murine glial cell cultures. Brain Res 584:219-225.

Mizuno T, Kawanokuchi J, Numata K, Suzumura A. 2003. Production and neuroprotective functions of fractalkine in the central nervous system. Brain Res 979:65-70.

Morgan D, Gordon MN, Tan J, Wilcock D, Rojiani AM. 2005. Dynamic complexity of the microglial activation response in transgenic models of amyloid deposition: implications for Alzheimer therapeutics. J Neuropathol Exp Neurol 64:743-753.

Murakami K, Horikoshi-Sakuraba Y, Murata N, Noda Y, Masuda Y, Kinoshita N, Hatsuta H, Murayama S, Shirasawa T, Shimizu T, Irie K. 2010. Monoclonal antibody against the turn of the 42-residue amyloid ß-protein at positions 22 and 23. ACS Chem Neurosci 1:747-756.

Murphy WJ, Kumar V, Bennett M. 1987. Rejection of bone marrow allografts by mice with severe combined immune deficiency (SCID).Evidence that natural killer cells can mediate the specificity of marrow graft rejection. J Exp Med 165:1212-1217.

Nagele RG, D'Andrea ,M.R., Anderson WJ, Wang H. 2002. Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease. Neuroscience 110:199-211.

Nakajima K, Tsuzaki N, Shimojo M, Hamanoue M, Kohsaka S. 1992. Microglia isolated from rat brain secrete a urokinase-type plasminogen activator. Brain Res 577:285-292.

Nelson PT, Alafuzoff I, Bigio EH, Bouras C, Braak H, Cairns NJ, Castellani RJ, Crain BJ, Davies P, Del Tredici K, Duyckaerts C, Frosch MP, Haroutunian V, Hof PR, Hulette CM, Hyman BT, Iwatsubo T, Jellinger KA, Jicha GA, Kövari E, Kukull WA, Leverenz JB, Love S, Mackenzie IR, Mann DM, Masliah E, McKee AC, Montine TJ, Morris JC, Schneider JA, Sonnen JA, Thal DR, Trojanowski JQ, Troncoso JC, Wisniewski T, Woltjer RL, Beach TG. 2012. Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. J Neuropathol Exp Neurol 71:362-381.

Neugroschl J, Sano M. 2010. Current treatment and recent clinical research in Alzheimer's disease. Mt Sinai J Med 77:3-16.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. 2003. Triple-Transgenic Model of Alzheimer's Disease with Plaques and Tangles: Intracellular Aβ and Synaptic Dysfunction. Neuron 39:409-421.

Oddo S, Caccamo A, Smith IF, Green KN, LaFerla FM. 2006.A dynamic relationship between intracellular and extracellular pools of Abeta. Am J Pathol 168:184-194.

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 1997. `Green mice' as a source of ubiquitous green cells. FEBS Lett 407:313-319.

Priller C, Bauer T, Mitteregger G, Krebs B, Kretzschmar HA, Herms J. 2006. Synapse formation and function is modulated by the amyloid precursor protein. J Neurosci 26:7212-7221.

Priller J, Flügel A, Wehner T, Boentert M, Haas CA, Prinz M, Fernández-Klett F, Prass K, Bechmann I, de Boer ,B.A., Frotscher M, Kreutzberg GW, Persons DA, Dirnagl U. 2001. Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. Nat Med 7:1356-1361.

Querfurth HW, LaFerla FM. 2010. Alzheimer's Disease. N Engl J Med 362:329-344.

Radde R, Bolmont T, Kaeser SA, Coomaraswamy J, Lindau D, Stoltze L, Calhoun ME, Jäggi F, Wolburg H, Gengler S, Haass C, Ghetti B, Czech C, Hölscher C, Mathews PM, Jucker M. 2006. Abeta42-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology. EMBO Rep 7:940-946.

Rodríguez-Martín T, Cuchillo-Ibáñez I, Noble W, Nyenya F, Anderton BH, Hanger DP. 2013. Tau phosphorylation affects its axonal transport and degradation. Neurobiol Aging 34:2146-2157.

Rogers J, Lue L. 2001. Microglial chemotaxis, activation, and phagocytosis of amyloid β-peptide as linked phenomena in Alzheimer's disease. Neurochem Int 39:333-340.

Rovelet-Lecrux A, Hannequin D, Raux G, Le Meur N, Laquerrière A, Vital A, Dumanchin C, Feuillette S, Brice A, Vercelletto M, Dubas F, Frebourg T, Campion D. 2006. APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. Nat Genet 38:24-26.

Santacruz K, Lewis J, Spires T, Paulson J, Kotilinek L, Ingelsson M, Guimaraes A, DeTure M, Ramsden M, McGowan E, Forster C, Yue M, Orne J, Janus C, Mariash A, Kuskowski M, Hyman B, Hutton M, Ashe KH. 2005. Tau suppression in a neurodegenerative mouse model improves memory function. Science 309:476-481.

Sá-Pereira I, Brites D, Brito MA. 2012. Neurovascular unit: a focus on pericytes. Mol Neurobiol 45:327-347.

Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu B, Jacobsen SEW, Pollard JW, Frampton J, Liu KJ, Geissmann F. 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science 336:86-90.

Serrano F, Klann E. 2004. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Ageing Research Reviews 3:431-443.

Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. 2011.Neuropathological Alterations in Alzheimer Disease. Cold Spring Harbor Perspect Biol 3:a006189.

Simard AR, Rivest S. 2004. Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. FASEB J 18:998-1000.

Simard AR, Soulet D, Gowing G, Julien J, Rivest S. 2006. Bone Marrow-Derived Microglia Play a Critical Role in Restricting Senile Plaque Formation in Alzheimer's Disease. Neuron 49:489-502.

Stalder M, Deller T, Staufenbiel M, Jucker M. 2001. 3D-Reconstruction of microglia and amyloid in APP23 transgenic mice: no evidence of intracellular amyloid. Neurobiol Aging 22:427-434.

Sugarman MC, Yamasaki TR, Oddo S, Echegoyen JC, Murphy MP, Golde TE, Jannatipour M, Leissring MA, LaFerla FM. 2002. Inclusion body myositis-like phenotype induced by transgenic overexpression of beta APP in skeletal muscle. Proc Natl Acad Sci U S A 99:6334-6339.

Takahashi RH, Milner TA, Li F, Nam EE, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK. 2002. Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. Am J Pathol 161:1869-1879.

TALBOT TR, J. 1957. Some aspects of the mechanism of action of derivatives of myleran and nitrogen mustard. Trans Am Clin Climatol Assoc 69:113-117.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R. 1991. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 30:572-580.

Thrash JC, Torbett BE, Carson MJ. 2009. Developmental regulation of TREM2 and DAP12 expression in the murine CNS: implications for Nasu-Hakola disease. Neurochem Res 34:38-45.

Togo T, Akiyama H, Iseki E, Kondo H, Ikeda K, Kato M, Oda T, Tsuchiya K, Kosaka K. 2002. Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. J Neuroimmunol 124:83-92.

Tutschka PJ, Copelan EA, Klein JP. 1987. Bone marrow transplantation for leukemia following a new busulfan and cyclophosphamide regimen. Blood 70:1382-1388.

Tutschka PJ, Santon GW. 1977. Bone marrow transplantation in the busulfin-treated rat. III. Relationship between myelosuppression and immunosuppression for conditioning bone marrow recipients. Transplantation 24:52-62.

Vallières L, Sawchenko PE. 2003. Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. J Neurosci 23:5197-5207.

Vom Berg J, Prokop S, Miller KR, Obst J, Kälin R,E., Lopategui-Cabezas I, Wegner A, Mair F, Schipke CG, Peters O, Winter Y, Becher B, Heppner FL. 2012. Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. Nat Med 18:1812-1819.

Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. 2009. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29:3974-3980.

Wallin AK, Blennow K, Andreasen N, Minthon L. 2006. CSF biomarkers for Alzheimer's Disease: levels of beta-amyloid, tau, phosphorylated tau relate to clinical symptoms and survival. Dement Geriatr Cogn Disord 21:131-138.

Wegiel J, Kuchna I, Nowicki K, Frackowiak J, Mazur-Kolecka B, Imaki H, Wegiel J, Mehta PD, Silverman WP, Reisberg B, Deleon M, Wisniewski T, Pirttilla T, Frey H, Lehtimäki T, Kivimäki T, Visser FE, Kamphorst W, Potempska A, Bolton D, Currie JR, Miller DL. 2007. Intraneuronal Abeta immunoreactivity is not a predictor of brain amyloidosis-beta or neurofibrillary degeneration. Acta Neuropathol 113:389-402.

Weitz TM, Town T. 2012. Microglia in Alzheimer's Disease: It's All About Context. Int J Alzheimers Dis 2012:314185-314185.

Williams K, Alvarez X, Lackner AA. 2001. Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. Glia 36:156-164.

Wilson CM, Gaber MW, Sabek OM, Zawaski JA, Merchant TE. 2009. Radiation-induced astrogliosis and blood-brain barrier damage can be abrogated using anti-TNF treatment. Int J Radiat Oncol Biol Phys 74:934-941.

Winton MJ, Lee EB, Sun E, Wong MM, Leight S, Zhang B, Trojanowski JQ, Lee VM-. 2011. Intraneuronal APP, not free Aß peptides in 3xTg-AD mice: implications for tau versus Aß-mediated Alzheimer neurodegeneration. J Neurosci 31:7691-7699.

Wyss-Coray T. 2006. Inflammation in Alzheimer disease: driving force, bystander or beneficial response?. Nat Med 12:1005-1015.

Yokoyama WM, Ryan JC, Hunter JJ, Smith HR, Stark M, Seaman WE. 1991. cDNA cloning of mouse NKR-P1 and genetic linkage with LY-49. Identification of a natural killer cell gene complex on mouse chromosome 6. The Journal of Immunology 147:3229-3236.

Zaghi J, Goldenson B, Inayathullah M, Lossinsky AS, Masoumi A, Avagyan H, Mahanian M, Bernas M, Weinand M, Rosenthal MJ, Espinosa-Jeffrey A, de Vellis J, Teplow DB, Fiala M. 2009. Alzheimer disease macrophages shuttle amyloid-beta from neurons to vessels, contributing to amyloid angiopathy. Acta Neuropathol 117:111-124.