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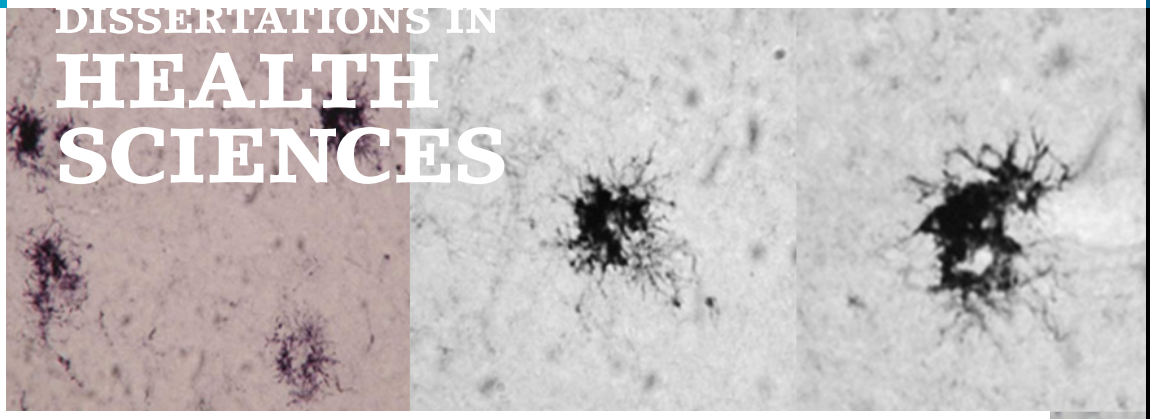
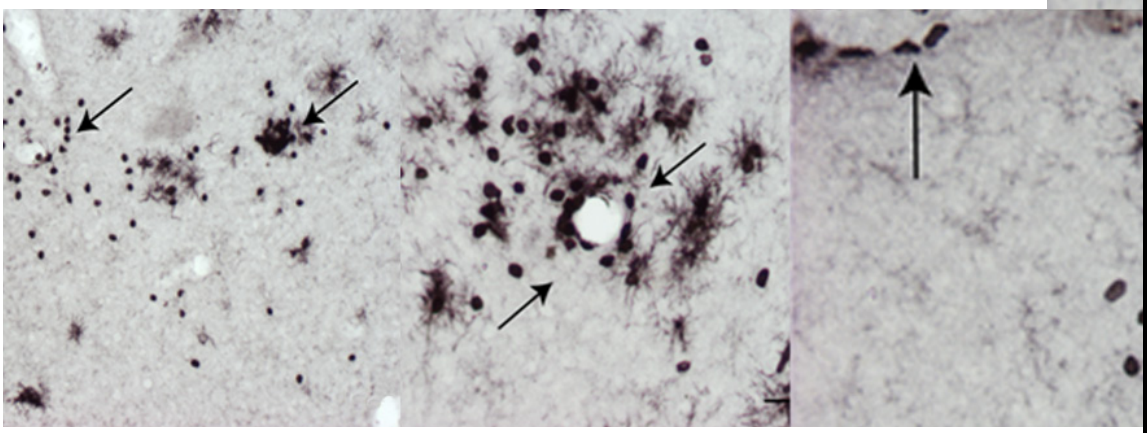


Lakshman Kumar Puli

Experimental Immunomodulation in Alzheimer's Disease



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UNIVERSITY OF
EASTERN FINLAND

LAKSHMAN KUMAR PULI

Experimental Immunomodulation in Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a chronic neurodegenerative disorder that primarily disturbs cognitive and memory functions of elderly people. Amyloid beta (Aβ) protein deposits and neurofibrillary tangles in brain parenchyma with additional brain atrophy as a consequence of massive loss of neurons are key pathological features of this disease. Chronic neuroinflammation also plays a very important role in progression of AD. Microgliosis, astrogliosis, overexpression of cytokines and chemokines are notable features of ongoing neuroinflammatory processes. Innate and cellular arms of immune system play a very decisive role in shaping the progression of AD neuropathology. Such innate or cellular immune responses can either be detrimental or beneficial. Nuclear factor kappa B (NFκB) family transcription factors regulate both immune as well as neuroinflammatory functions in many pathophysiological conditions. Taming detrimental neuroinflammation while harnessing benefits of own immune activation is a promising avenue for current therapeutic strategies. Immunomodulation by genetic and pharmacological agents is a good experimental tool to understand the role of neuroinflammation in AD pathogenesis.

Our present thesis is a combination of two experimental studies that were designed to study the role of neuroinflammation in AD pathogenesis. In both studies we employed APPswe/PS1dE9 transgenic mice as an animal model of AD. In our first study, we used a clinically well-known anti-inflammatory and immunomodulator known as human polyclonal intravenous immunoglobulin (hIVIG). We treated young and aged APP/PS1mice with hIVIG for durations ranging from 3 weeks to 8 months. We assessed treatment effects on behaviour, amyloid pathology, and neuroinflammatory parameters. In our second study, we crossed APP/PS1 mice with mice deficient in *Nfkb1* gene, a key member of NFκB family transcription factors, thereby creating a novel APP/PS1 mouse line under *Nfkb1* knockout background. We assessed the impact of *Nfkb1* gene knockout on behaviour, amyloid pathology and chronic neuroinflammatory processes of mice at three different ages. Acute inflammatory responses of LPS injection in brains of these novel mice were also investigated.

Data from our first study indicate that irrespective of treatment duration, dose or age of mice at treatment onset hIVIG had little effect on preformed Aβ deposits. Furthermore, chronic hIVIG treatment in APP/PS1 mice resulted in elevation of soluble levels of Aβ in the hippocampus. Mechanisms related to disintegration of Aβ plaques, phagocytosis by microglia and peripheral Aβ sink consistently failed to reduce Aβ load significantly in brain and serum of hIVIG treated mice. However, chronic hIVIG treatment brought about significant changes to microglia. Treatment with hIVIG for 8 months resulted in 30% decrease in CD45 expressing activated microglia. Furthermore, confocal analysis revealed significant morphological changes in ionized calcium binding adaptor molecule 1 (Iba-1) positive microglia both near and away from Aβ plaques, indicating a change in activation of specific microglia sub-populations. Chronic hIVIG treatment also suppressed tumor necrosis factor (*Tnf*) gene expression in both wild-type and APP/PS1 mice. Furthermore, a direct correlation between expression of pro-inflammatory cytokine genes and CD45 microglia observed in saline treated mice flipped to an inverse correlation in hIVIG treated

mice, suggesting a change away from pro-inflammatory phenotype of microglia. Chronic hIVIG treatment also resulted in >50 % increase in new born doublecortin positive neurons in dentate gyrus, irrespective of mouse genotype, suggesting a strong neurogenic effect due to hIVIG treatment. Data from the first study clearly indicate that immunomodulation by hIVIG can change microglial phenotype irrespective of amyloid pathology and that anti-inflammatory and neurogenic effects of hIVIG might mediate beneficial effects in AD patients.

In our second study, deletion of *Nfkb1* gene did not cause any overt abnormal behavioural changes in mice, suggesting minimal prospects of adverse effects related to blocking NFκB in the AD brain. Data from our second study consistently showed limited impact of *Nfkb1* gene deletion on progression of amyloid pathology across all the ages tested. Furthermore, also an acute stimulation of microglia by lipopolysaccharide did not result in any changes to amyloid pathology. In aged *Nfkb1* knockout mice under APP/PS1 background, we noticed >50% decrease in CD45 microglia, suggesting suppression of activated microglia. However, we saw a significant increase in pro-inflammatory cytokine gene expression like *Tnf* and interleukin 1 beta in *Nfkb1* knockout mice under APP/PS1 background. Data from this study suggest a change in microglial and inflammatory phenotypes.

Results from both of these studies clearly indicate that immunomodulation in AD may have limited effects on amyloid pathology but can have profound effects on activated microglia and their pro-inflammatory phenotypes.

National Library of Medicine Classification: WT 155, QW 920, QZ 150, WL 102, WL 348

Medical Subject Headings: Alzheimer Disease/pathology; Inflammation; Nervous System; Brain; Disease Models, Animal; Mice, Transgenic; Immunomodulation; Immunoglobulins, Intravenous; Behavior; Amyloid beta-Peptides; Plaque, Amyloid; Gene Knockout Techniques; Transcription Factors; NF-kappa B; Lipopolysaccharides; Microglia; Tumor Necrosis Factors; Interleukin-1beta; Neurogenesis

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TIIVISTELMÄ

Alzheimerin tauti (AT) on krooninen aivojen rappeumasairaus, joka ensisijaisesti ilmenee muistin ja päättelykyvyn heikentymisenä ikääntyneillä ihmisillä. Taudin patologialle tunnusomaisia piirteitä ovat aivokudoksen amyloidi-β (Aβ) proteiinin kertymät ja hermosäievyyhdet sekä aivojen kutistuminen huomattavan hermosolukadon seurauksena. Hermoston kroonisella tulehdusreaktiolla on myös merkittävä osuus AT:n etenemisessä. Käynnissä olevan hermoston tulehdusreaktion tunnusomaisia piirteitä ovat mikrogliasolujen ja astrosyyttien aktivoituminen sekä sytokiinien ja kemokiinien yli-ilmentyminen. Sekä luotainen että soluvälitteinen immuniteetti vaikuttavat ratkaisevasti AT:n tautiprosessin etenemiseen aivoissa, niin että vaikutus voi olla joko haitallinen tai suotuisa. Tumatekijä kappa-B (NFκB) perheen transkriptiotekijät säätelevät sekä immuniteettia että hermokudoksen tulehdusvastetta monissa tautitiloissa. Haitallisen hermoston tulehdusreaktion kesyttäminen ja toisaalta oman immuniteetin aktivoiminen ovat lupaavia lähestymistapoja AT:n hoitoon. Immuunivasteen muuntelu joko geneettisesti tai farmakologisesti on hyvä kokeellinen työkalu selvitettäessä hermoston tulehdusreaktion osuutta AT:n tautimekanismissa.

Tämä väitöskirjatyö yhdistää kaksi kokeellista tutkimusta, joiden tarkoituksena oli selvittää hermoston tulehdusreaktion osuutta AT:n synnyssä. Molemmissa tutkimuksissa käytimme AT:n mallina APPswe/PS1dE9 siirtogeenisiä hiiriä. Ensimmässsä tutkimuksessa hyödynsimme kliinisessä käyttössä hyvin tunnettua tulehdusta hillitsevää ja tulehdusvastetta muuntelevaa hoitoa, polyklonaalista ihmisen laskimoverestä kerättyä immunoglobuliinia (hIVIG). Annoimme nuorille ja vanhoille APP/PS1 hiirille eripituisia hIVIG hoitoja, joiden kesto vaihteli 3 viikosta 8 kuukauteen. Arvioimme hoidon vaikutusta eläinten käyttäytymiseen, amyloidipatologiaan ja hermoston tulehdusreaktioon. Toisessa tutkimuksessa risteytimme APP/PS1 hiiriä hiiriin, joilta puuttui keskeinen NFκB transkriptiotekijän perheen *Nfkb1* geeni, ja loimme näin uudenlaisen *Nfkb1*-tekijän suhteen poistogeenisen APP/PS1 hiirilinjan. Selvitimme *Nfkb1* geenin poiston vaikutuksia hiirten käyttäytymiseen, amyloidipatologiaan ja hermoston tulehdusreaktioon kolmessa eri ikäpisteessä. Lisäksi selvittelimme LPS-injektion aiheuttamaa akuuttia tulehdusvastetta näiden hiirten aivoissa.

Ensimmäisen tutkimuksemme tulokset viittaavat siihen, että riippumatta hoidon kestosta, hoitoannoksesta tai hiirten iästä hoidon aloitushetkellä hIVIG vaikuttaa hyvin vähän jo aivoissa oleviin Aβ kasautumiin. Lisäksi krooninen hIVIG hoito APP/PS1 nosti liukoisen Aβ:n tasoja hiirten hippokampuksessa. Tunnetut immuunihoitojen Aβ:n tasoja laskevat mekanismit, Aβ plakkien hajotus, mikorigliavälitteinen fagosytoosi tai perifeerinen Aβ nielu, johdonmukaisesti epäonnistuivat aivojen tai seerumin Aβ määrän vähentämisessä hIVIG:llä hoidetuilla hiirillä. Kuitenkin krooninen hIVIG hoito aiheutti merkitseviä muutoksia mikrogliasoluissa. Kahdeksan kuukauden hIVIG hoito vähensi 30% CD45 pintamolekyylin ilmentymisetä aktivoituneissa mikrogliasoluissa. Lisäksi konfokaalimikroskopiassa tuli esiin merkitseviä rakennemuutoksia ionisoivaa kalsiumia sitovaa sovitinmokelyyli 1:tä (Iba-1) ilmentävissä mikrogliasoluissa sekä amyloidiplakkien läheisyydessä että niistä etäällä, mikä viittaa määrällisiin muutoksiin spefisissä mikroglian

alapopulaatioissa. Krooninen hIVIG hoito myös vaimensi tuumorinekroositeijän (*Tnf*) geenieksрessiota sekä villityyppisillä että APP/PS1 siirtogeenisillä hiirillä. Lisäksi positiivinen tulehdusta edistävien sytokiinien geenieksрession ja CD45-positiivisten mikrogliasolujen määrän korrelaatio kääntyi päinvastaiseksi hIVIG:llä hoidetuilla hiirillä, mikä viittaa mikrogliasolujen ilmiasun muuttumiseen vähemmän tulehdusta suosivaksi. Krooninen hIVIG hoito lisäsi myös >50 % doublecortin-merkkimolekyyliä ilmentävien uudishermosolujen määrää hippokampuksen pykäläpoimussa (gyrus dentatus) molemmilla genotyypeillä, mikä puhuisi sen puolesta, että hIVIG on voimakkaasti hermosolujen uudismuodostusta edistävä hoito. Ensimmäisen tutkimuksen tulokset siis selvästi osoittivat, että hIVIGin välittämä immuunivasteen muuntelu voi muuttaa mikrogliasolujen ilmiasua amyloidipatologiasta riippumatta ja että hIVIG hoitojen suotuisa vaikutus AT potilailla voi osittain selittyä hermosolujen uudismuodostuksen lisääntymisellä.

Toisessa tutkimuksessamme *Nfkb1* geenin poisto ei aiheuttanut hiirillä ilmeistä poikkeavaa käyttäytymistä, mikä viittaa NFκB signaloinnin salpauksella olevan hyvin vähän sivuvaikutuksia. Toisaalta tutkimustuloksemme johdonmukaisesti viittasivat siihen, että *Nfkb1* geenin poistolla on vain vähäisiä vaikutuksia myös amyloidipathologiaan kaikissa tutkituissa ikäpisteissä. Akuutti mikrogliasolujen stimulaatio lipopolysakkaridillakaan ei aiheuttanut sanottavia muutoksia aivojen amyloidipatologiassa. Vanhoilla *Nfkb1* poistogeenisillä, APP/PS1-taustaisilla hiirillä CD45:n ilmeneminen mikrogliasoluissa laski yli 50% *Nfkb1* geenin suhteen villityyppisiin hiiriin verrattuna. Kuitenkin havaitsimme merkitsevän nousun tulehdusta suosivien sytokiinien, kuten *Tnf:n* and interleukiini 1 beetan, ilmaantumisessa APP/PS1-taustaisilla *Nfkb1* poistogeenisilla hiirillä. Tuloksemme viittaavaat siis *Nfkb1* geenipoiston aiheuttavan muutoksia mikrogliasoluissa ja tulehdusvasteen luonteessa.

Kummankin tutkimuksen tulokset viittaavaat selvästi siihen, että aivojen immuunivasteen muuntelu ei merkitsevästi vaikuta aivojen amyloidipatologiaan, mutta voi sen sijaan muuttaa ratkaisevasti mikrogliasolujen ominaisuuksia.

Luokitus: WT 155, QW 920, QZ 150, WL 102, WL 348

Yleinen Suomalainen asiasanasto: Alzheimerin tauti/patologia; tulehdus; hermosto; aivot; tautimallit; eläin; hiiri, siirtogeeninen; immuunisäätely; immunoglobuliinit, suonensisäinen; käyttäytyminen; amyloidi-beeta peptide; plakki, amyloidi; poistogeenitekniikat; transkriptiotekijät; NF-kappa B; lipopolysakkaridit; microgliasolut; tuumorinekroositekijät; interleukiini-1beeta; hermosolujen uudismuodostus

To Raj and his good morning smile ☺

If you win you lead, if you loose you guide….

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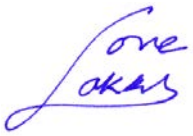

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CONTENTS

Abbreviations

AChEI	Acetylcholinesterase enzyme inhibitor
AD	Alzheimer's disease
ADAS-Cog	Alzheimer 's disease assessment scale – Cognitive subscale
AGE	Advanced glycation end product
AICD	Amyloid precursor protein intracellular domain
AP-1	Activator protein 1
APOE	Apolipoprotein E
APP	Amyloid precursor Protein
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BACE	Beta-site APP cleaving enzyme 1
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CAA	Cerebral amyloid angiopathy
CCL2	Chemokine (C-C motif) ligand 2
CGIC	Clinical global impression of change
CIDP	Chronic inflammatory demyelinating polyneuropathy
CLU	Clusterin
CNS	Central nervous system
CR1	Complement receptor 1
CREB	Cyclic AMP-responsive element-binding protein
CSF	Cerebrospinal fluid
DAB	3, 3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate gyrus
EAE	Experimental autoimmune encephalitis
EP2	Prostaglandin E2 receptor EP2 subtype
ERK	Extracellular signal-regulated kinase
GFAP	Glial fibrillary acidic protein

GSK3-β	Glycogen synthase kinase-3 beta
hIVIG	Human polyclonal intravenous Immunoglobulin
HRP	Horseradish peroxidise
hUCB-MSC	Human umbilical cord blood-derived mesenchymal stem cells
IFN-γ	Interferon gamma
IGF-1	Insulin growth factor 1
IKK	NFκB kinase
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IP-10	Interferon-gamma-inducible protein 10
ITP	Idiopathic thrombocytopenic pupura
LPS	Lipopolysaccride
LTP	Long-term potentiation
LT-α	Lymphotoxin-alpha
MAPK	Mitogen activated protein kinase
MCI	Mild cognitive impairment
MCP-1	Macrophage chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
mGluR4	Metabotropic glutamate receptor 4
MHC	Major histocompatibility complex
MIP-α	C-C motif chemokine 3
MIP-β	C-C motif chemokine 4
MMSE	Mini-mental State Examination
MPL	Monophosphoryl lipid A
NDS-TBS-T	Normal donkey serum in TBS-T
NFT	Neurofibrillary tangle
NFκB	Nuclear Factor kappa B

NLR	Nucleotide-binding domain leucine rich repeat containing receptor
NLS	Nuclear localisation sequence
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered solution
PDTC	Pyrrolidine dithiocarbamate
PET	Positron emission tomograpghy
PGE_2	Prostaglandin E2
PHF	Paired helical filament
PIB	Pittsburg compound B
PIB	Pittsburgh compound B
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PS1	Presenilin 1
PSEN1	Presenilin 1
RA	Rhematoid arthritis
RAGE	Receptor for advanced glycation end products
REL	Proto-oncogene c-Rel
RNA	Ribonucleic acid
STAT	Signal transducers and activators of transcription
TBS	Tris Buffered saline
TBS-T	Tris-buffered saline with triton
TDM	Trehalose dicorynomycolate
TGF-β-1	Transforming growth factor beta-1
TLR	Toll-like receptors
tMCAO	Transient middle cerebral artery occlusion
TNF	Tumor necrosis factor
Tpl2	Tumor progression locus 2
VEGF	Vascular endothelial growth factor

1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects the cognitive functions of elderly and thereby, severely erodes quality of life for patients. By 2010 more than 35.6 million people worldwide were affected by dementia. Nearly 70% of dementia patients suffer from AD (Launer et al., 1999). Currently, little is known about causation of AD, and available drugs can provide only symptomatic relief but cannot stop or delay the disease progression. All along the disease progression, AD brain reacts by fomenting natural self-defensive neuroinflammatory processes that are meticulously enacted by a plethora of inflammatory proteins and immune cells. In the absence of exact knowledge about disease causation, delaying disease progression by understanding the complex spatial and temporal relationships between various components of neuroinflammatory pathways might reveal novel drug targets. Here, we have used two different strategies to tame neuroinflammation in a mouse model of AD. In the first study, we used a clinically established anti-inflammatory drug, and in the second study, we used a novel transgenic AD mouse line, which is deficient in one of the key component of an important signalling pathway responsible for inflammatory gene expression. In both these studies, our objective was to modulate inflammatory processes in the APP/PS1 transgenic AD mouse brain. We used human polyclonal intravenous immunoglobulins (hIVIG) in our first study to assess its acute and chronic effects on neuropathology and cognitive functions of APP/PS1 mouse. A decade long immunotherapy research in AD provided overwhelming evidence for beneficial effects of amyloid beta (Aβ) antibodies. Since hIVIG also contains antibodies against Aβ, we set out to test the hypothesis that treatment with hIVIG would alleviate cognitive deficits and amyloid pathology in the APP/PS1 mouse. In our second study, we targeted NFκB pathway which is a key transcriptional regulator of inflammation. NFκB subunit NFKB1 is one of the active components of protein dimers regulating the canonical pathways of NFκB activation. We crossed APP/PS1 and *Nfkb1* gene knockout mice and created a novel mouse line of *Nfkb1* knockout under APP/PS1 background. This way we hoped to study the impact of *Nfkb1* transcription factor on the behaviour, amyloid and inflammatory pathologies of APP/PS1 mouse. Modulation of inflammatory processes by brain immune cells, microglia, is the key in both studies. Either pharmacological or genetic immunomodulation strategies may reveal novel drug targets, which may be employed to confer long-lasting benefits to chronically inflamed degenerating AD brains.

2 Literature review

2.1 ALZHEIMER'S DISEASE

2.1.1 Alzheimer's disease: Flashback

Alzheimer's disease is a chronic neurodegenerative disorder that primarily disrupts mental and intellectual capacities of elderly. The first documented case report of this debilitating disease was presented in 1906 at the 37th German Psychiatric Assembly by Alois Alzheimer. He described in detail clinical and neuropathological observations of his patient Auguste D, a 51-year-old suffering from severe amnesia. Alzheimer took special note of this particular case as her symptoms and neuropathological findings did not fit into any known class of diseases during that time. English translation of that German report was recently published (Alzheimer, 2006). Briefly, Auguste D's first symptoms were noticed as jealousy towards her husband. Her memory impairment rapidly progressed and this hampered her from executing routine household chores. She had difficulties to navigate around her apartment and moved objects purposelessly and often hid them. She seemed to be extremely delirious and often was disoriented for place and time. She would suddenly scream out loudly with a belief that someone wanted to kill her. She would at times hurl phrases of intense indignation towards the doctor examining her and seemed to have auditory hallucinations. She would scream with a horrible cry for many hours and her behaviour at asylum was described as total helplessness (Maurer , 2006). On memory tests, she correctly recognized and named the objects but failed to recall the last object shown a little while ago. While reading she skipped lines, read phonetically or used senseless intonations. As for writing she repeated single syllables, skipped others and very soon got distracted (amnestic writing disorder). While talking, she often used embarrassingly unusual phrases and chose to use expressions like "milk pourer" instead of cup. Sometimes she did not seem to hear or listen anything at all. She could not recall utility of certain objects that were previously quite familiar to her. Her gait was undisturbed with normal patellar and pupillary reflexes. All these focal signs waxed and waned as dementia progressed. At the time of death in 1906, 4.5 years after admission to asylum, she was totally apathetic, lying on her bed with retracted legs and incontinent. Cause of death was septicaemia due to decubitus (Maurer , 2006).

Autopsy showed that her brain was uniformly atrophied without any visible abnormalities. Among other post-mortem findings were moderate external and internal hydrocephalus, atherosclerosis of blood vessels, pneumonia of both inferior lobes and nephritis. Bielschowsky's silver stain revealed several histopathological abnormalities. A number of tangled bundles of fibrils were found inside neurons. These intracellular neurofibrils remained even after the cell destruction. Roughly, a quarter to a third of neurons in cerebral cortex showed neurofibrillary changes. Alzheimer also noticed complete disappearance of neurons from upper cell layers. Throughout the cortex especially on the upper cell layers he noticed the presence of many miliary foci (amyloid plaques) which were visible even without staining. He also noticed glial reaction but did not see any signs of vessel infiltration. However, there were signs of endothelial proliferation and formation of new blood vessels. For Alzheimer, this was a unique and ''peculiar disease'' with unknown cause. In the following years, four more such cases were described in great detail by Perusini,

Bonfiglio and Sarteschi. All these cases differed from typical senile dementia with respect to early age of onset (around 50's) and were accordingly considered ''*senium praecox*''. These ''peculiar'' cases were later classified under "Alzheimer's disease" by Emil Kraepelin in 1910 in his classic text book of psychiatry and hence the eponym (Ralf Dahm, 2006). Atherosclerotic changes of blood vessels were later omitted by Perusini in his 1909 article describing Auguste D. Furthermore, Graeber and coworkers, after reanalysing original slides from Auguste D in 1998, confirmed the histopathological findings of Alois Alzheimer (Graeber and Mehraein, 1999; Graeber et al., 1998; Graeber et al., 1997; Graeber, 1999) and also ruled out atherosclerosis and metachromatic leukodystrophy as clinical diagnosis for Auguste as hypothesized by some scientists (Amaducci et al., 1991). Alzheimer's autopsy findings from his patient Auguste D became what are known today as hallmarks of Alzheimer's disease: cognitive impairment, neuronal loss, amyloid plaques and neurofibrillary tangles.

Interestingly, Alzheimer was not the first person to discover and describe tangles, plaques, neuronal loss or clinical dementia. In fact, around the same time as Alzheimer, Oskar Fischer in 1907 carried out systematic clinicopathological studies in a number of demented and normal individuals. He used Bielschowsky silver stain and described neuritic plaques (See Figure 2.1) for the first time (Goedert, 2009). He also sorted out eight different types of senile plaques and correlated them to their clinical symptoms which were similar to that of Auguste D. In only 2 out of 35 cases of normal individuals he noticed senile plaques which led him to conclude senile plaques as morphological substrates for the clinical symptoms (Goedert, 2009). Nevertheless, Alzheimer's outstanding contribution was in that he exemplified how careful clinical observations may be linked to elegant neuropathology, in simple words, clinical psychiatry was fused with microscopy and neurochemistry. Alzheimer described brain pathology at the backdrop of detailed clinical picture, but he did not speculate about how to cure it or what causes the disease. Several key milestones that shaped AD field during early times are briefly presented for inquisitive readers who like to know how the story of Alzheimer's unfolded chronologically (Table 2.1a).

Table 2.1a: Highlights of AD research. Important milestones (in grey) offered new directions.

Year	Significance for AD field
1838	Remak; described intracellular neurofibrils.
1883	Paul Böttiger; first synthesized azo dye called Congo red.
1892	Blocq and Marinesco; described senile plaques in epileptics.
1898	Redlich; identified "miliary sclerosis" in senile atrophy.
1898	Binswanger; coined the term presenile dementia.
1902	Bielschowsky; discovered silver staining technique to visualize intracellular neurofibrils.
1906	Alzheimer; presented his first case report at 37th Assembly of Southwest German Psychiatrists in Tubingen. Abstract was published same year.
1907	Fischer O; miliary sclerosis as marker for senile dementia.

1907	Alzheimer; full lecture of this case report was published in “General Journal of Psychiatry and Psycho-forensic Medicine” under the title “Proceedings of Psychiatric Associations.”
1908	Bonfiglio; published a case of a 60-year-old, in Italian with similar symptoms and histopathological findings.(Influenced by Alzheimer)
1909	Perusini; described Alzheimer’s first case in “On histological and clinical findings of some psychiatric diseases of older people”.
1910	Emil Kraepelin first used the eponym Alzheimer’s disease in his text book of psychiatry.
1911	Alzheimer; AD case without neurofibrillary tangles.
1911	T Simchowicz; coined the word “Senile plaque”.
1922	Bennhold; Congo red is a specific stain for amyloid.
1926	E Grunthal; relationship between neurofibrillary degeneration and senile plaques.
1927	Divry; Congo red staining of plaque cores to be amyloid. Tau also stains with congo red. Plaque cores were called ‘‘amorphous material’’ until Divry.
1936	Rothschild and Kasanin; senile dementia is different from pre senile dementia (AD). Rothschild noticed plaques from cognitively normal patients.
1942	Coons; principles of first immunohistochemistry
1948	Alvarez; multiple occlusions of small cortical vessels caused senile dementia.
1948	Newton; Senile dementia, plaques and tangles are related to AD (presenile dementia).
1955	Brody; normal loss of cortical neurons due to ageing.
1959	Margolis; demonstrated significant staining differences between tangles and amyloid with Congo red.
1961	Sjogren and Sourander; relationship between AD and normal ageing.
1963	Terry; Neurofibrillary tangles made of twisted tubules by electron microscopy.
1963	Kidd; Tangles are paired helical filaments.
1964	Terry; electron microscopy studies of AD lesions.
1966	Roth; correlation between dementia and senile plaques of elderly.
1967	Suzuki and Terry; amyloid plaques with dystrophic neuritis and involvement of axioplasmic transport.
1967	Gonatas; abnormal synaptic complex near amyloid plaques.

1968	Blessed; correlation between plaques and cognition implied major role of amyloid in AD.
1968	Blessed, Tomlinson and Roth; Senile dementia and presenile dementia (AD) are pathologically and clinically same.
1971	Gallyas; Silver staining for Alzheimer's NFTs.
1974	Drachman and Leavitt; Scopolamine produced memory loss and confusion in normal patients similar to that of AD patients.
1976	Davies and Moloney; deficiency of choline acetyl transferase in AD cortex.
1976	Bowen; neurotransmitters and indices of hypoxia in senile dementia. Neuropharmacologists entered AD research.
1976	Scheibel; loss of dendritic spines in AD cases.
1976	Katzman; Senile dementia and AD are one single disease. Importance to frequency and mortality due to AD. Aftermath of this paper many clinical neurologists, epidemiologists, radiologists, and psychiatrists got interested in AD.
1977	Perry; necropsy evidence for cholinergic deficits in senile dementia.
1981	Terry; neuron loss quantification in AD and correlation to cognition.
1984	Glenner and Wong; Aβ sequence was discovered.
1985	Masters and Beyreuther; APP protein sequence discovered.
1985	Jean-Pierre Brion found that anti-tau antibodies labelled NFTs and also this was first ever successful immunohistochemistry on neurodegenerative diseases.
1987	Terry; selective loss of cortical neurons due to normal ageing while maintaining the total.
1987	Terry; several cases of typical AD without neurofibrillary tangles.
1987	McGeer; expression of inflammatory markers and involvement of immune cells in AD.
1990	Levy; Dutch mutation E693Q on APP identified.
1991	Terry; synapse loss quantification in AD and correlation to cognitive impairment.
1991	Braak and Braak; staging of Alzheimer's disease.
1991	Goate; London mutation V717I on APP identified.
1991	Murrell; Indiana mutation V717F on APP identified.
1992	Mullan; Swedish mutation K670N/M671L on APP identified.
1992	Hardy and Selkoe; amyloid cascade hypothesis pinpoints Aβ as ''THE TARGET''.

1993	APOE4 as risk factor for sporadic or late onset AD.
1993	Masliah; neocortical synaptic loss due to normal ageing.
1995	Games; first transgenic APP mouse showing Aβ pathology.
1997	Graeber; rediscovery of Alzheimer's second patient (1911 case).
1999	Schenk; immunotherapy for Alzheimer's disease.
2002	Phase 2b trial of active Aβ vaccine AN1792 halted due to aseptic meningitis.
2004	Klunk; *in vivo* amyloid PET Scan imaging using Pittsburgh compound B
2007	Tramiprostate (Alzhmed) fails in phase 3 clinical trial (unexpected improvement in more than 30% placebo treated patients due to concomitant medication).
2008	Ginkgo biloba prevention trial is negative.
2008	Tarenflubril or R-Flurbiprofen (Flurizan) fails in phase 3 clinical trial. (Poor brain permeability together with lack of potency)
2008	Bapineuzumab fails in phase 2 trial with hints of efficacy in post hoc sub-group analysis.
2010	Dimebon (latrepiridine) fails in phase 3 trial –CONNECTION Study (No one knew how it works, but still it reached phase 3 only to fail.)
2010	Semegacestat fails in phase 3 trail (Dose was compromised to prevent toxicity).
2010	Polyunsaturated fatty acid DHA (Docosahexaenoic acid) failed in 18 month trial.
2011	Phase 2b trial with RAGE inhibitor PF-04494700 stopped as it failed interim analysis.
2011	Phase 2 trial of Ponezumab stopped. It is a C-terminal Aβ antibody.
2012	Jonsson; protective APP mutation discovered.
2012	Coenzyme Q, Vitamin C, E and α- lipoic acid failed to improve CSF biomarkers and cognition of AD patients. All drugs except Conzyme Q did reduce brain oxidative stress as a consequence of treatment.
2012	Dimebon yet another phase 3 trial - CONCERT trial fails.
2012	Human intravenous immunboglobulin stops AD progression in 4 mild to moderate AD patients in phase 2 trial for more than 3 years.(may be suppressed inflammation and fostered neurogenesis)
2012	Bapineuzumab and Solaneuzumab fail in phase 3 clinical trials. Pathology in mild to moderate AD too severe to rescue (Too little, too late?).

Many perceived AD as a rare "presenile dementia" due to Emil Kraepelin's classification. However, in 1968 a very important study by Blessed, Tomlinson and Roth found clinical and pathological features of senile dementia to be indistinguishable from AD (presenile dementia) (Blessed et al., 1968). Later in 1976, an editorial by Katzman observed that neither clinical, nor neuropathological or ultrastructural features could differentiate these two forms of dementia except for age (Katzman, 1976). He strongly exhorted to combine these two forms of dementia into one single disease, AD. Further, he cautioned clinicians about the impending malignancy of AD in western societies where average life expectancies were on rise (Katzman, 1976). In the aftermath of this editorial, several clinicians, neuropathologists, epidemiologists, radiologists, psychiatrists, biochemists, geneticists joined the race to understand AD etiology. Today, we still do not know exactly what causes AD and whether these hallmarks even qualify as drug targets in AD.

Even though the ultimate cause of AD still remains a mystery, a lot has been understood about its progression. This disease has clinically insidious onset and features progressive loss of memory, language, executive and analytical skills, delirium, hallucinations and depression. Definitive diagnosis of AD is done at *post mortem* with the presence of neurofibrillary tangles, amyloid plaques and neuronal loss in the cortex and hippocampus.

2.1.2 Alzheimer's disease: Today

Roughly 106 years later, genetics and biochemistry has contributed immensely towards our understanding how the disease begins and evolves. Amyloid-β (Aβ) protein was discovered to be the key component of Alzheimer's "miliary foci". Intracellular tau protein was later discovered to be principal component of neurofibrillary tangles (NFT). Detailed clinical correlates of these key lesions have been investigated. Spreading of pathology across various brain regions has been studied in relation to the clinical stage of the disease. Novel imaging techniques, transgenic mouse models, cerebrospinal fluid (CSF) biomarkers all have contributed immensely towards understanding time course and pathogenetic mechanisms for plaques and tangles. Aβ was found to be neurotoxic in various *in vitro* models and was shown to be affecting neuronal function. Abnormal changes to tau protein are now considered as downstream to Aβ pathology (Jin et al., 2011b).

Neuropathological hallmarks of AD can be classified as lesions due to protein accumulation (Aβ plaques and NFT), lesions due to losses (neurons and synapses) or lesions due to body's response (neuroinflammation, glial reactivity) (Duyckaerts et al., 2009). Only the accumulated lesions are currently of diagnostic value. Extracellular Aβ deposits and intracellular aggregation of tau protein are hydrophobic in nature with abnormal misfolded fibrillar structures. Heterogeneity in AD neuropathology was evident right from Alzheimer's time, when his much popularised second case Johann F., lacked neurofibrillary tangles (Klunemann et al., 2002). Thanks to current neuropathological diagnostic criteria strengthened by advances in neuroimaging and CSF biomarkers, the accuracy of clinical diagnosis of AD is more than 90% (Clark et al., 2008).

2.1.3 Amyloid pathology

The Aβ peptide deposits can be detected either by Aβ immunohistochemistry or by Congo red histological stain. The term "senile plaque" for Aβ deposits is not common any more. It

was widely used during early twentieth century to describe extracellular lesions that were characteristically associated with senile dementia (Goedert, 2009). Currently, all extracellular accumulations are called deposits, preceded by a term indicative of technique used to detect them e.g. Aβ deposits are those detected by anti-Aβ antibodies whereas amyloid deposits are those revealed by Congo red or thioflavin S staining. In addition, a term indicative of their shape was also used as prefixes, such as diffuse, focal or stellate Aβ deposit. Diffuse Aβ deposit show very weak immunopositivity (50 μm^2 to > 100μm^2), with loosely defined boundaries and often rely on the quality of immunohistochemistry. Sometimes these diffuse deposits were called "lake-like" (Wisniewski et al., 1998) or "fleecy" (Thal et al., 1999) . Diffuse Aβ deposits were also found in the brains of people with intact cognitive functions indicating that these lesions may not be overtly toxic (Delaere et al., 1990; Dickson et al., 1992). Ability of Pittsburgh compound B (PIB) to bind diffuse Aβ deposits also explains why many cognitively normal subjects showed higher PIB binding (Lockhart et al., 2007). However, it is also believed that most if not all these visibly normal individuals with diffuse Aβ deposits will eventually develop full–blown AD. Anti-Aβ antibodies directed at the N-terminus sometimes fail to detect diffuse Aβ deposits because APOE binds to the N-terminus of the Aβ peptide during early stages of Aβ deposition (Uchihara et al., 1996; Thal et al., 2005).

Focal deposits, on the other hand, are dense spherical accumulations of Aβ peptide. Focal Aβ deposits may or may not be positive for Congo red or thioflavin S stain indicating that not all Aβ deposits are amyloid deposits. Also, not all focal deposits show neuritic corona. In transgenic mouse models of AD, activated microglia are associated with both focal Aβ and amyloid deposits. Amyloid deposits may show a dense core surrounded by a clear halo that separates light and diffuse Aβ immunopositive outer area from the core. The plaque core mainly contains $A\beta_{42}$ peptide. A single Aβ plaque core has been estimated to contain 50-100 fmol of total Aβ peptide (Rufenacht et al., 2005). The halo surrounding the core is occupied by 5-6 microglial cells. The corona of focal Aβ deposit contains neuritic and astrocytic components. The Aβ focal deposit together with neuritic corona constitutes "mature", "classical", "neuritic plaque" (Duyckaerts et al., 2009). The neuritic processes of corona were described in great detail by Oskar Fishcer way back in 1907 (Goedert, 2009). See figure 2.1a for examples of neuritic plaques. These neurites as revealed by silver methods were very abnormally large and "dystrophic". Early neuropathologists used Bielschowsky's technique to visualize neurites, which was soon replaced by Bodian's and Gallyas methods (for better selectivity). Axonal or dendritic nature of these processes was better shown by Golgi's method but this falls short in staining plaques.

Under electron microscope, these degenerating neurites from plaque corona were distended, contained large amounts of lipofuscin, degenerating mitochondria, synaptic vesicles (Luse and Smith, 1964; TERRY et al., 1964) and paired helical filaments (PHF) made of tau protein. Sensitive tau antibody like AT-8 can stain the processes present in plaque corona. Swollen astrocytic processes are also present in the plaque corona, but unlike microglia they do not touch the plaque core but rather occupy the plaque periphery. Advanced AD brains show increased densities of "remnant plaques" that are characterized by mesh-like astroglial fibrils within the entire part of plaque. These possibly indicate a regressive stage of lesions (Oide et al., 2006). Characteristic "cotton wool plaques" were discovered from familial AD patients with deletion of exon 9 of presenilin 1 (*PSEN1*) gene

(Crook et al., 1998). These distinctly large, numerous eosinophilic round plaques resembling cotton wool balls were immunoreative for Aβ but lacked congophilic dense core. Plaque related neuritic pathology and cellular infiltrates were absent. Mostly these are composed of N-truncated forms of $A\beta_{42}$ (Mann et al., 2001). Another characteristic plaque called as "Inflammatory plaque" has also been described recently in AD cases with *PSEN1* mutations. Inflammatory plaque cores were positive for thioflavin-S, cresyl violet and silver stains but were negative for both Aβ and tau or for any other protein that were consistently associated with classic cored plaques (e.g. APOE, IgG, α-synuclein). Some Aβ was evident in the periphery of the core and the core itself was surrounded by numerous HLA-DR positive microglial cells, hence the name Inflammatory Plaque (Shepherd et al., 2005). Most of these plaques were described from AD brains with varying degrees of pathology and is currently not possible to understand chronological progression of these deposits. However, it is believed that initially plaques are of diffuse type, then become Congo-red positive in the core, and finally are surrounded by neuritic corona to form a mature neuritic amyloid cored senile plaque (Metsaars et al., 2003).

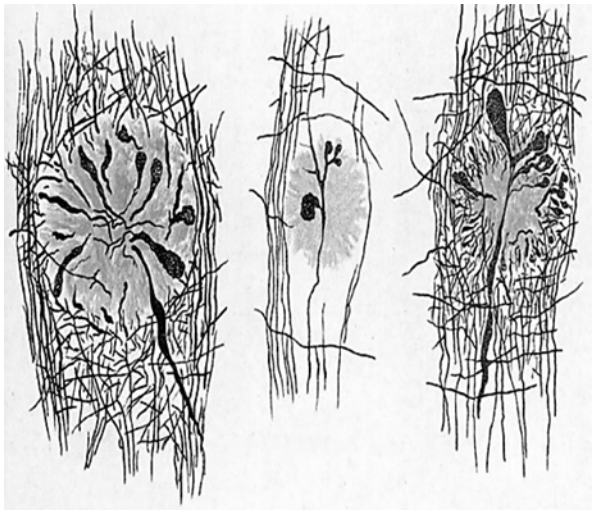


Figure 2.1a. Oscar Fischer's illustrations of neuritic plaques from brains of patients with senile dementia. *Adapted from* Brain (2009) 132: 1102

2.1.4 Amyloid angiopathy

Accumulation of congophilic material, i.e., Aβ protein inside the meningeal and cortical arteries, arterioles, and veins causes cerebral amyloid angiopathy (CAA). Most elderly, whether demented or not, commonly show CAA pathology associated with ageing. Roughly 80% of AD patients show some degree of mild CAA. Familial forms of CAA were associated with Dutch, Flemish and Iowa mutations on the amyloid precursor protein (*APP)* gene. Further, *APP* gene duplication has also been linked to severe CAA. Aβ is first deposited around the basement membrane of blood vessels followed by tunica media. In severe cases

vessels look like a double barrel, and media shows fibrinoid necrosis. Such vascular pathologies result in hemorrhages and cortical infarcts. Moreover, not all cases of AD show CAA; however, some degree of CAA is usually seen in age-matched non-AD brains. Mainly $A\beta_{40}$ is deposited in arteries and capillaries (Thal et al., 2008). Occipital cortex is affected with CAA more often than any other brain regions, and correlates significantly with AD pathology irrespective of Apolipoprotein E (*APOE)* genotype (Attems et al., 2005). Based on capillary involvement sporadic CAA can be either be CAA-Type 1 or CAA-Type2 (Thal et al., 2002). The first type is characterized by immunohistochemically detectable Aβ in cortical capillaries along with leptomeningeal and cortical arteries, arterioles, veins and venules. However, in the second type cortical capillaries are devoid of immunohistochemically detectable Aβ. CAA-Type1 is also linked with presence of *APOE4* genotype and is sometimes also called as dyshoric angiopathy (horos meaning barrier), as amyloid seems to be getting out of blood vessels indicating disruption of blood-brain barrier. CAA-Type2 is more common and is not associated either with *APOE* genotype or AD severity. Capillary CAA can also be detected by antibodies against $A\beta_{42}$ (involves perivascular space and glial limitans), and this correlates with AD pathology. On the other hand, $A\beta_{40}$ immunopositive capillary CAA largely limits itself to the vessel wall, and this type of pathology does not correlate to AD pathology (Thal et al., 2002).

2.1.5 Intraneuronal Amyloid beta pathology

Accumulation of Aβ has also been reported within neurons of humans and many other species. Cell culture experiments indicate subcellular localization of Aβ in endoplasmic reticulum, Golgi complex and endosomal-lysosomal systems (LaFerla et al., 2007). Such sub-cellular Aβ has been proposed to be detrimental for neuronal survival. Pyramidal neurons in the human brain accumulating Aβ intracellularly undergo lysis to form amyloid plaques (D'Andrea et al., 2001). Furthermore, in the APP(SL)/PS1KI AD mouse at 10 months age, more than 50% loss of CA1/2 neurons correlates with intraneuronal Aβ rather than extracellular Aβ (Casas et al., 2004). Moreover, the earliest cognitive impairments in the 3xTg-AD AD mouse model correlate with intraneuronal Aβ accumulation in the hippocampus and amygdala (Billings et al., 2005). Further, in 3xTg-AD AD mouse, passive immunization with Aβ antibodies cleared not only extracellular Aβ but also diminished intraneuronal and tau pathologies, thus further implicating Aβ within neurons to be a key target for alleviating AD pathology (Oddo et al., 2004). However, the role of intraneuronal Aβ in the AD pathogenesis has been subjected to intense scrutiny and skepticism seemingly due to technical reasons. Firstly, the antibodies destined to react with Aβ also cross-react with both APP holoprotein and proteolytic derivatives of APP, thereby the immunoreactive signal might not be specific to Aβ itself. Secondly, pretreatment protocol employed for Aβ immunostaining influenced the quality of intraneuronal signal. Heating protocols enhanced the visualization of intraneuronal Aβ signal whereas formic acid pretreatment did not, which further strengthens the idea that mostly intraneuronal staining might be just a staining artifact. One study (Aho et al., 2010) clearly showed that commonly used 6E10, 4G8 and 82E1 clones of Aβ antibodies fail to distinguish Aβ from APP in human brain tissues irrespective of AD pathology or age. Furthermore, intracellular Aβ staining with C-terminal Aβ neoepitope antibodies is strongly depended on the antibody chosen and pretreatment strategy employed. A very recent study (Winton et al., 2011) using end specific Aβ antibodies (which exclusively recognized Aβ peptides ending in either $A\beta_{40}$ or $A\beta_{42}$ but not Aβ sequences within APP) provided elaborate

and conclusive evidence that indeed the intraneuronal Aβ staining was coming from APP or its derivatives. This evidence was further corroborated in mice obtained by crossing 3xTg-AD mice with beta-site APP cleaving enzyme 1 (*Bace1*) knockout mice (Winton et al., 2011).

Thai and coworkers have proposed staging of parenchymal Aβ plaque pathology in order to delineate the AD progression (Thal et al., 2002). In phase 1, amyloid deposition is restricted to only neocortex (frontal, temporal, parietal or occipital). In phase 2, pathology additionally spreads to allocortical brain regions (entorhinal cortex and hippocampus); while diencephalon, striatum and basal forebrain get involved during phase 3 in addition to neocortex and allocortex. Finally, brainstem and cerebellum get involved during phase 4 and 5, respectively. As for cerebral amyloid angiopathy (Duyckaerts et al., 2009), three stages have been formally recognized. Blood vessels of isocortex are involved in stage 1, followed by vessels of allocortex, midbrain and cerebellum at stage 2. In stage 3, vessels of basal ganglia, thalamus, pons, medulla oblongata get involved. One should note that in cerebellum, amyloid deposition in vessels occurs earlier (stage 2) than corresponding (phase 5) parenchymal deposits (Duyckaerts et al., 2009).

2.1.6 Neurofibrillary tangles and tau pathology

The other hallmark lesion of AD is neurofibrillary tangle (NFT). See figure 2.1b for some examples of NFTs from brains of senile dementia patients. Neurofibrils are normal cytoskeletal components of neurons that can be visualized by silver staining. Alzheimer in his first case report of Auguste D reported neurofibrils to be abnormally thickened, bundled and persistent even after the death of the cell. Dyes that do not stain normal healthy neurofibrils stained NFTs, thus hinting towards its pathological nature. Around same time in 1907, S. C. Fuller also described neurofibrils in pathological conditions including senile dementia. Alzheimer also described similarities of neurofibrillary pathology in cases referred to him by Dr. Pick that were different from Auguste's NFTs in terms of their distinct round shape and total lack of plaque pathology (this disease is nowadays called Pick's disease, although Alzheimer first described the changes). Subsequently, amyloid nature of NFTs was established by thioflavin S staining (Divry, 1934) hinting that these were composed of highly organized structures. Later in 1960s, electron microscopy revealed paired helical filament (PHF) structures to be components of abnormal cytoskeletal filaments (KIDD, 1963; TERRY, 1963). PHF filaments are fibrils of 10 nm diameter, and they pair in a helical conformation at a regular periodicity of 65 nm. Filaments are made of tau in cross β-sheet configuration. Several polyclonal and monoclonal antibodies were raised either to purified PHF or to normal cytoskeletal associated proteins. Immunocytochemistry and biochemical studies revealed that these PHF composed neither of neurofilament proteins nor tubulins but contained microtubule associated tau protein (Brion et al., 1985). This microtubule associated tau protein was later found to be abnormally phosphorylated (Grundke-Iqbal et al., 1986). Abnormal phosphorylation fuelled research for identifying relevant kinases and phosphatases, which ultimately resulted in identification of glycogen synthase kinase-3 beta (GSK3-β) enzyme which currently is one pharmacological drug target for AD.

Figure 2.1b. Oscar Fischer's illustrations of neurofibrillary tangles from brains of patients with senile dementia. *Adapted from* Brain (2009) 132: 1102

Such tau deposits were also detected in many other conditions like progressive supranucleur palsy, corticobasal degeneration and Pick's disease that lack Aβ plaque pathology. Tau mediated neurodegeneration may result from toxic gain of function acquired by aggregated conformations of tau and loss of normal functions of tau in diseased conditions. Tau has many important cellular functions in healthy neurons which precludes it from being a viable drug target during diseased conditions. Tau is microtubule-associated-protein, abundant in axons and primarily helps to stabilize microtubules. There are six isoforms of tau based on alternate splicing of mRNA. Structurally, towards the carboxy-terminal portion of protein, microtubule binding domain is present which is composed of repeats of highly conserved tubulin binding motif, followed by basic proline rich region and a projection domain comprising acidic amino terminal region. The six isoforms differ from each other in the number of tubulin binding repeats (either three or four, hence called 3R or 4R isoforms respectively) and in the presence or absence of 29 amino acid long inserts on the N-terminal region. During development, expression of various tau isoforms is variable; however, in the adult expression in many brain regions of 3R and 4R isoforms follows 1:1 ratio, and any deviation from this ratio is characteristic of neurodegenerative FTD taupathies (Hong et al., 1998). In a widely accepted model, tubulin binding repeats of tau bind to specific pockets in β-tubulin protein on the inner surface of microtubules whereas proline rich positive region binds tightly to negatively charged microtubule surface and negatively charged projection domain branches away from microtubule surface possibly due to electrostatic repulsion. Further, tubulin binding pockets on adjacent protofilaments may be occupied by different repeats of same microtubule binding domain, thereby causing cross-linking of three or four dimers (Amos, 2004). Apart from microtubule stabilization function of tau, other functions like interaction of projection domain with plasma membrane, binding of proline rich domain with the SH3 domains of src-family tyrosine kinases such as FYN, RNA, and presenilin 1 involves tau as promiscuous partner to heterologous interactions (Ballatore et al., 2007).

Accumulation of tau deposits in human brain takes place exclusively in neurons. Tau accumulations can be seen in both axons and dendrites. Tangles and pretangles are associated with accumulation of tau in soma. Abnormal accumulation of phosphorylated tau that fills

the entire soma but without aggregation constitutes "pretangle" (Bancher et al., 1989). Tau positivity in neuropil threads are mostly dendritic processess while in neuritic plaque corona are mainly composed of tau positive axonal processes (Duyckaerts et al., 2009). Their presence in astrocytes (Thorn-shaped astrocytes) is mostly associated with age rather than AD pathology. Double labeling with monoclonal antibodies like AT-8 and GFAP reveal abnormal tau accumulation in distal and proximal processes of astrocytes (Schultz et al., 2004). Extracellular NFTs retain cell shape but lack plasma membrane, and is the common end point for neurofibrillary pathologies. Intracellular NFTs show identifiable nucleus and plasma membrane. In addition, some advanced cases of AD show ghost tangles characterized by absence of intracellular neurofibrillary pathology and absence of C-terminus of abnormally phosphorylated tau (Endoh et al., 1993). NFTs can be located mainly to medium-sized pyramidal neurons of the hippocampus, entorhinal cortex and of layers III and V of the isocortex. Neurons of the transentorhinal area (layer III +V) and of layer II of the entorhinal cortex are the first to show NFT pathology (Braak and Braak, 1990). Olfactory regions are also reported to be involved during early stages of AD (Kovacs et al., 2001). Alz50 and AT8 are two popular monoclonal antibodies that are in use to visualize NFTs. Alz50 is a conformational antibody that recognizes and requires two discontinuous epitopes, one on N-terminus and other at microtubule binding domain for efficient binding (Carmel et al., 1996). AT8 antibody recognizes an epitope double phosphorylated at serine 202 and threonine 205 or serine 199 and 202 or serine 205 and 208 (Porzig et al., 2007). NFTs can also be detected by antibodies against ubiqutin (p62), prothrombin, thrombin, heparan sulfate proteoglycan, Fe65, flotillin, CDK5 and GSK-3β (Duyckaerts et al., 2009). Neurofibrillary pathology is not cleared from the brain and it can remain in the extracellular space without causing an inflammatory reaction. This feature allows researchers to relate density of NFTs to the severity of disease stage and to track progression of lesions at each stage. Progression of NFTs seemed to be related to anatomical tracts. Such extension of tau pathology through anatomical connections demonstrates hierarchical distribution of tau pathology that can be linked to clinical symptoms. This is the basis for Braak neurofibrillary stages. Braak and Braak characterized the progression of AD in the cerebral cortex based distribution of NFTs and neuropil threads into six distinct stages. Stages I and II involve trans-entorhinal and entorhinal regions followed by involvement of hippocampus in stages III and IV. Isocortex gets finally involved in stages V and VI (Braak and Braak, 1991). Braak staging of neurofibrillary pathology is currently being used routinely in neuropathology. Subcortical nuclei are not considered in Braak staging; however, the involvement of for instance *nucleus basalis of Meynert* and *locus coeruleus* is seen quite early in the disease progression (Duyckaerts et al., 2009).

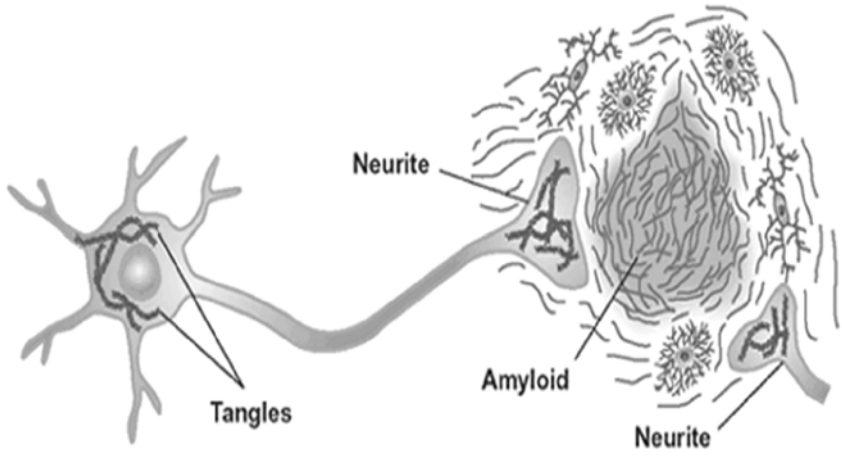


Figure 2.1c. Illustration of amyloid core of neuritic plaque surrounded by glia. Also, a neuron bearing tangle pathology is also seen near the plaque microenvironment. *Adapted from* Nature Neuroscience (2002)5:633

The relationship between Aβ and tau pathologies (Figure 2.1c) is very controversial. Familial APP and PS1 mutations cause abundant Aβ as well as tau pathologies, whereas tau mutations result only in NFT pathology without Aβ deposition. Hence it appears that APP processing or abnormal Aβ accumulation triggers a disease mechanism that ultimately results in neuronal death and NFTs. Furthermore, *in vitro* demonstration that Aβ oligomers can phosphorylate tau and evidence from 3xTg-AD mice (Oddo et al., 2004) suggest that tau hyperphosphorylation is a secondary event to or consequence of Aβ pathology. However, if this is true, one should find NFTs only near Aβ deposits, which is not the case. Spatially, NFTs location is not determined by Aβ deposits. Moreover, tau pathology has been reported to start many decades before Aβ pathology initiates (Duyckaerts and Hauw, 1997).

2.1.7 Neuronal loss and synaptic pathology

Another hallmark of AD brain is massive neuronal loss in focal brain regions. Estimation of neuronal loss needs to apply a correction for potential bias in counting neurons. Large neurons have a higher probability than small ones to be incorporated in a microscopic section thereby leading to overrepresention of large neurons. Moreover, atrophy of large neurons may lead to a wrong conclusion that neurons have been lost when in fact they have only become smaller (Duyckaerts et al., 1989). Counting nucleoli (much smaller in size) might decrease the bias. Unbiased dissector technique (Sterio, 1984) or Dirichlet tessellation methods (Duyckaerts et al., 1994) defined neuron loss in AD to be focal rather than global in nature (Regeur et al., 1994). Up to 90% neuronal loss has been documented in layer II of entorhinal cortex, CA1 region (three-fold reduction), superior temporal gyrus and supramarginal gyrus (Duyckaerts et al., 2009). A correction factor needs to be incorporated while reporting neuronal densities because of a potential bias. In the isocortex, large and medium sized neurons are most vulnerable while large Betz cells are relatively spared.

Olfactory bulb, amygdala, *nucleus basalis of Meynert*, *locus coeruleus*, substantia nigra and serotoninergic raphe nuclei are the major brain structures with well-established neuronal loss in AD. Loss of neurons in AD may not be primarily due to neurofibrillary degeneration. Typically, it would take 15-25 years for tau pathology to evolve from tau hyperphosphorylation to formation of NFTs but neuronal loss by apoptosis takes just a day (Buee et al., 2010). Apoptosis due to Aβ toxicity has been suggested to be the one of the reason for neuronal loss.

Synaptic pathology in AD patients has been appreciated by electron microscopical studies on biopsy and autopsy tissues (Scheff and Price, 1993; DeKosky and Scheff, 1990). Synaptic pathology in AD is characterized by alteration of synapses, presence of synapses near and within Aβ deposits, and loss of pre- and post-synaptic markers (Duyckaerts et al., 2009). These changes correlated with cognitive decline in mild AD patients (Scheff et al., 2007). Changes to number and morphological alterations in pre-synaptic, post-synaptic endings, axons, dendrites, in and around Aβ deposits are evident also with light microscopy (Gonatas et al., 1967). Presynaptic vesicular markers like synaptophysin are decreased in AD, while presynaptic membrane markers like SNAP25 remain stable (Shimohama et al., 1997) (Masliah et al., 2001). Loss of synaptophysin appears to be the best correlate for intellectual deficit (Terry et al., 1991). Degenerative synapses found in Aβ deposits and clusters of synapses along the neuropil may be non-functional. Loss of dendritic spines has also been reported in AD mice (Grutzendler et al., 2007).

Macroscopically, AD brains exhibit massive atrophy. Inferior temporal and superior and mid-frontal gyri are affected severely. Microscopic lesions contribute in part to macroscopic changes. Areas commonly involved in tau pathology (Whitwell et al., 2008) and those exhibiting neuronal loss (entorhinal, hippocamous and amygdale) also show atrophy (Halliday et al., 2003)(Duyckaerts et al., 2009)(Duyckaerts et al., 2009)(Duyckaerts et al., 2009)(Duyckaerts et al., 2009)(Duyckaerts et al., 2009)(Duyckaerts et al., 2009)(Duyckaerts et al., 2009)(Duyckaerts et al., 2009). Atrophy does not correlate with amyloid deposits but shows strong correlation to neuronal loss (Kril et al., 2004). MRI measurements show both decrease in thickness and length of cortical ribbon. One visible consequence of cortical thinning is prominent dilatation of lateral ventricles. MRI scans can detect this pattern of cortical thinning quite early in the clinical course of AD (Dickerson et al., 2011). Atrophy of grey matter is accompanied by atrophy of white matter (Lerch et al., 2008).

2.1.8 Pathomechanisms for Alzheimer's disease

Genetic, biochemical, and ultra-structural studies have contributed to our understanding of complexities involved in AD. Alzheimer's disease has become a major social and economic burden on developed as well as developing countries around the world (Qiu et al., 2009). It is estimated that worldwide by 2030 there will be 65.7 million people suffering from dementia, most of which will be suffering from AD (70%) and this figure is projected to double every 20 years, to 115.4 million by 2050. In Europe, there will be 16.2 million people suffering from dementia by 2050 (Wancata et al., 2003). Worldwide estimated costs of dementia were approximately US$ 604 billion in 2010, which is bound to increase quickly. With 7.7 million new cases of dementia each year worldwide (implies one case every 4 seconds); it is going to throw a serious challenge to our efforts and resources (WHO report, 2012). Incidence of AD

in people > 65 years age increases exponentially with age and mostly this rise is noted in seventh and eighth decades of life.

Age is the biggest risk factor of AD. Roughly, less than 5% of the AD cases are early-onset (familial) and more than 95% of the cases are sporadic or late-onset. Mutations in amyloid precursor protein (*APP*) and presenilin genes (*PSEN1* and *PSEN2*) cause early-onset autosomal dominant form of AD (onset age < 60 years). *APP* and *PSEN1* gene mutations predispose to familial onset AD, whereas *APOE4* genotype is the strongest risk factor for sporadic AD. APOE has been to shown to be involved in Aβ clearance, and alleles *APOE2*, *APOE3* and *APOE4* are increasingly less effective in clearing Aβ from brains of PDAPP mice (Holtzman et al., 2000). Even though many risk and protective factors have been shown to be linked to AD it should be noted that these factors merely tell us about the probability of acquiring the disease. These factors signal their involvement in disease mechanisms and may provide us a probabilistic clue about the outcome of the disease. However, disease risk indicated by these factors falls short of sensitivity and/or specificity to be used as diagnostic marker (Reitz et al., 2011).

Human *APP*, *PSEN1* and *PSEN2* genes are all involved in the production and processing of Aβ protein, the principal component of senile amyloid plaques found in the brains of AD patients. Mutations on these three genes increase Aβ production and ratios of $A\beta_{42}/A\beta_{40}$, thus implicating the role of Aβ in the AD pathogenesis (Bertram and Tanzi, 2008). Duplication of the *APP* gene has also been reported to cause early-onset AD (Rovelet-Lecrux et al., 2006). Furthermore, Down syndrome patients with an extra copy of chromosome 21, on which *APP* gene resides, develop symptoms similar to early-onset AD (Millan Sanchez et al., 2012). Clinically, early- and late-onset AD are indistinguishable and remain undetectable at the desease onset, while by the time cognitive deficits appear, the patient's brain is already engrossed with full-blown irreversible pathology. Owing to massive loss of neurons and synaptic connections during late stages of disease, it is now believed that there might be a therapeutic window during the early phases of disease progression when neurons, synapses as well as cognitive functions are still intact.

Mechanisms related to processing, formation, aggregation and deposition of Aβ peptide offer many opportunities for drug discovery programs to intervene in disease pathogenesis. Aβ peptide is formed by the sequential proteolytic cleavages of the 695 amino acid APP protein. The *APP* gene is located on chromosome 21, and alternative splicing of the transcript from this gene results in various isoforms like APP695 (common in neurons), APP751 and APP770. APP protein is a type 1 integral membrane protein (Kang et al., 1987), with a large extracellular region (Kunitz protease inhibitor, E1 and E2 domains), hydrophobic transmembrane domain and amyloid precursor protein intracellular domain (AICD) containing C-terminal. *APP* knockout mice are viable and fertile; however, they show age-related cognitive decline, reactive gliosis and deficits in neuronal and muscular functions (Zheng et al., 1995; Dawson et al., 1999).

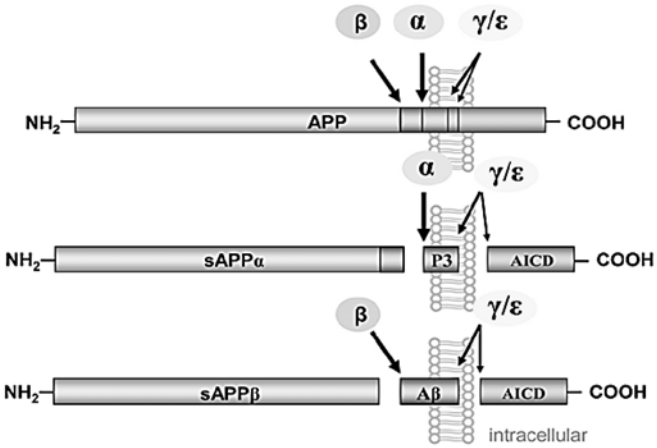


Figure 2.1d. APP processing by α-, β- and γ secretases and their major proteolytic products. *Adapted from* Neuron (2010) 66:631

APP protein is processed in two distinct pathways (Zhang et al., 2012). First, in non-amyloidogenic pathway APP is sequentially cut by two processes, α- secretase and γ-secretase. Cleavage by α-secretase results in release of large extracellular soluble sAPP-α and membrane bound C-terminal fragment containing 83 amino acids (C83). C83 is then cleaved by γ-secretase to release p3 peptide and AICD. sAPP-α has neurotrophic and neuroprotective effects. In the second, amyloidogenic pathway, APP is first cleaved by β-secretase, thus releasing sAPP-β and membrane associated C93 fragment. Further, C93 is cleaved by γ-secretase to release AICD and Aβ peptide (Zhang et al., 2012). The released extracellular Aβ peptide aggregates to form amyloidogenic Aβ assemblies and eventually gets deposited in amyloid plaques. See figure 2.1d for illustration of APP processing.

Gene mutations related to *APP*, *PSEN1* and *PSEN2* genes have been implicated in familial AD. Table 2.1b summarizes some popular *APP* mutations. These mutations cause aberrant APP processing resulting in overproduction of the more amyloidogenic $A\beta_{42}$. Interestingly, Osaka mutation of *APP* gene results in enhanced formation of Aβ variant lacking glutamate-22 (E22Δ) causing excessive formation of Aβ oligomers but not Aβ fibrils. Dementia caused by this mutation reinforces the idea that synaptic and cognitive impairments in AD are caused by Aβ oligomers (Tomiyama et al., 2008). Nearly all mutations of the *APP* gene lead to early onset AD except for one recently reported protective *APP* mutation which not only reduces risk for sporadic AD but also reduces cognitive decline in the elderly (Jonsson et al., 2012).

Table 2.1b: Common *APP* mutations associated with familial Alzheimer's disease

NAME	MUTATION	REFERENCE
Swedish	K670N/M671L	(Mullan et al., 1992)
Dutch	E693Q	(Levy et al., 1990)
London	V717I	(Goate et al., 1991)
Indiana	V717F	(Murrell et al., 1991)
Florida	I716V	(Eckman et al., 1997)
Iowa	D694N	(Grabowski et al., 2001)
Arctic	E693G	(Nilsberth et al., 2001)
Osaka	E693Δ	(Tomiyama et al., 2008)
Iceland	A673T	(Jonsson et al., 2012)

2.1.9 Amyloid cascade hypothesis

Unfortunately, causation of AD has not been established fully. According to the amyloid cascade hypothesis, Aβ protein gets deposited in the brains of AD patients due to some unknown reason which triggers a cascade of events downstream resulting in formation of neurofibrillary tangles and neuronal death (Hardy and Higgins, 1992; Hardy and Selkoe, 2002). Aβ isolated from AD patients was sufficient to affect neuronal function and caused neurotoxicity (Mucke and Selkoe, 2012). Autosomal mutations in *APP*, *PSEN1* and *PSEN2* genes resulting in overproduction of Aβ cause familial forms of AD. The same mutations when expressed in mice results in AD-like pathological features. Also, the presence of *APOE4* allele, which is strongest risk factor for sporadic AD, is associated with reduced clearance of Aβ from brains of PDAPP mice (Holtzman et al., 2000). Increased gene dosage of *APP* in Down's syndrome patients also causes AD like pathology (Hardy and Selkoe, 2002). This biochemical, genetic and histopathological evidence shows that Aβ deposition in brain is the primary event that initiates a sequence of downstream events that ultimately lead to AD dementia (Hardy and Allsop, 1991). Therefore, irrespective of the trigger continuous presence of Aβ levels inside brain is toxic for the normal functioning of neurons. More recently, Aβ oligomers are considered to be the most toxic species responsible for loss of synapses. In addition, microglia and astrocyte release many pro-inflammatory chemokines and cytokines that are neurotoxic. Also, it has been shown that Aβ dimers isolated from cortex of AD patients at subnanomolar concentrations induced tau hyperphosphorylation and neuritic degeneration; this was reversed by Aβ targeting antibodies (Jin et al., 2011b). These findings suggest that accumulation of toxic Aβ species lies upstream to tau pathology and neuronal dysfunction in AD. Altered neuronal homeostasis and oxidative injury further deteriorates causing widespread neuronal dysfunction ultimately leading neurodegeneration and dementia. See figure 2.1e for graphic illustration.

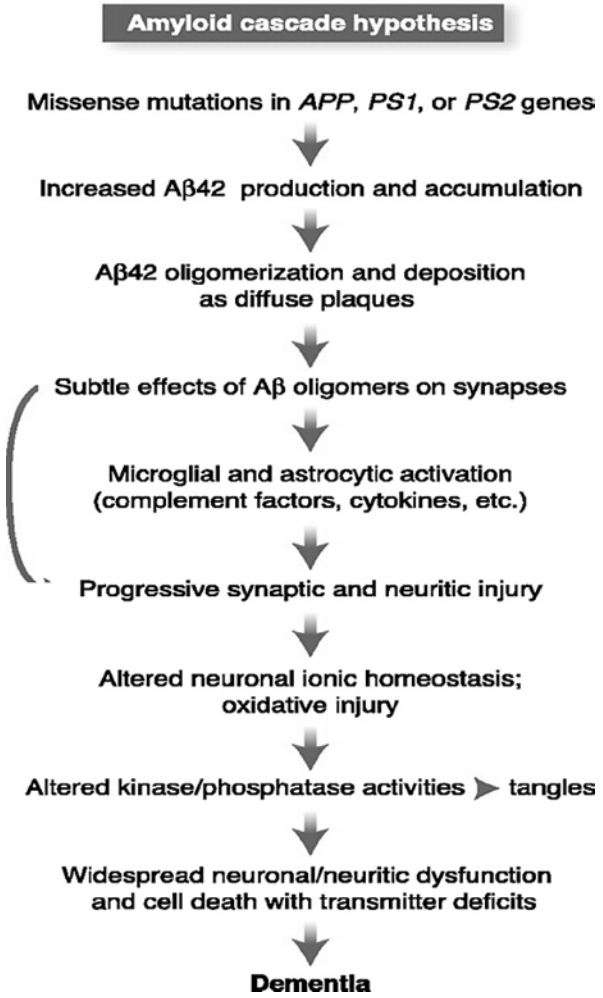


Figure 2.1e. Amyloid cascade hypothesis. *Adapted from* Science (2002) 297:353

An important role for Aβ peptide in the AD pathomechanism is undoubtedly accepted but it is still not clear if Aβ is initiating the disease, driving the disease, or a mere end product of disease. Mere presence of Aβ in brain does not precipitate AD symptoms, thus it is unlikely that it causes sporadic AD. A host of direct and indirect Aβ mediated neurotoxicity studies *in vitro* and *in vivo*, indicate that Aβ might be an active driver of disease. Therefore,Aβ targetting antibodies (Bapineuzumab, Solaneuzumab, Ponezumab) considered as disease modifying agents were presumed to influence AD progression. These Aβ antibodies should have at least slowed deterioration of cognitive and daily functions in patients, but unfortunately this did not happen (Strobel, 2012b)(Fagan, 2012) and all these antibodies failed in clinical trials.

Beginning the treatments at early stages of AD might be a good avenue. For mild-moderate stage AD patients, neuronal loss and concominant pathogies are too severe for drugs to confer clinically visible benefits. Nevertheless, all the drugs so far targeting Aβ production or clearance have failed. So, there might be factors other than Aβ which actively drive the disease. This gap between the basic concepts and facts from clinical trials are the key reasons that Aβ hypothesis has been challenged by some researchers. Also, some clinically diagnosed AD patients do not show amyloid deposits in brain whereas some other cognitively normal people show abundant amyl(Jin et al., 2011a)(Jin et al., 2011a)oid in the PET scans (Edison et al., 2007) (Jin et al., 2011a).

Initial deposition of neurotoxic Aβ is necessary for subsequent tangle pathology, neurodegeneration and cognitive decline (Jin et al., 2011b). This implies a direct correlation between neuritic plaques and memory decline but such clinicopathological correlations are however very weak (Giannakopoulos et al., 2009). Furthermore, in a small number of AD patients harbouring *PSEN1* mutations amyloid pathology is diffuse and resembles cotton wool plaques. Neuritic plaques and inflammatory pathologies of AD are absent, suggesting that Aβ plaques may not be necessary for AD (Hardy et al., 1998). More perplexingly, even very high expression of Aβ in mouse brains results in very scanty loss of neurons (Zahs and Ashe, 2010). Interestingly, familial AD mutations of *APP* and *PSEN1* genes might also result in defective endolysosomal trafficking and proteolysis independent of Aβ. Furthermore, synaptic dysfunction and neuronal degeneration can be also caused by defective PS1 (Pimplikar et al., 2010). Moreover, if Aβ pathology lies upstream to tau pathology as noted in amyloid cascade hypothesis, one would expect to find NFTs around Aβ deposits, but in reality spatial location of NFTs and Aβ lesions do not correspond and appear independent of each other. Finally, as tau pathology starts decades before of Aβ deposition, it does not seem to be a consequence of initial Aβ deposition (Karran et al., 2011). Mutations in tau gene can also result in neuronal pathology similar to that of AD patients (Hardy et al., 1998). However, due to absence of genetic, biochemical and clinical data challenging the mechanistic role of Aβ in AD pathogenesis many researchers are hesitant to pursue alternatives for amyloid cascade hypothesis (Karran et al., 2011; Selkoe, 2011). However, absence of proof about causation of AD by Aβ is by no means itself evidence that Aβ is a wrong AD target. In the same tone, scanty evidence for Aβ as clinically validated target is by no means itself evidence that it is the end of road for AD therapeutics. AD is a complex and challenging disease.

2.1.10 Transgenic mouse models of Alzheimer's disease

Several early attempts to mimic and reproduce AD pathology in laboratory animals failed. Initially, direct Aβ injections into rat brains were used. Injections of Aβ in combination with chaperones like perlecan resulted in extracellular congophilic, thioflavin S positive amyloid deposits (Snow et al., 1994). Even though such extracellular amyloid fibrils caused astrocyte activation and massive phagocyte infiltration, they did not cause neurotoxicity and neurodegeneration similar to that seen in AD patients (Holcomb et al., 2000). Discovery of *APP* gene mutations in familial cases of AD greatly fuelled the race to create the first transgenic mouse model. Various mutations discovered in human *APP* gene were overexpressed in the mouse genome with the aid of a variety of promoters for expression in cortical and hippocampal neurons. Table 2.1c provides a brief summary of popular transgenic models of AD. These mice developed both diffuse and classic fibrillar Aβ plaques in the

cortex and hippocampus in an age-dependent manner, similar to human AD. Moreover, these mice also showed progressive cognitive deficits in a number of behavioural tests for memory and cognition (Lee et al., 2004; Arendash et al., 2001; Kelly et al., 2003). However, there was no linear relationship between levels of Aβ and memory impairments (Westerman et al., 2002b). Overproduction of Aβ by these mice was found to be responsible for cognitive impairments, because the *Bace1* $^{-/-}$ *APP* mouse was protected from memory impairment (Luo et al., 2001). When presenilin gene mutations were introduced in addition to *APP* mutations, the plaque pathology became much more aggravated (Borchelt et al., 1997). The Aβ present in diffuse deposits was predominantly $A\beta_{42,}$ whereas $A\beta_{40}$ was mainly deposited in cerebral blood vessels (Savage et al., 1995).

The mouse model used in our studies, APP/PS1dE9, harbours both *APP* and *PSEN1* gene mutations, which aggravates the severity of plaque and inflammatory pathology. At 15 months of age, APP/PS1dE9 mice display diffuse and fibrillar Aβ plaques, activated microglia and astrocytes, elevated brain levels of pro-inflammatory cytokine, greatly diminished neurogenesis and impaired cognitive functions (Garcia-Alloza et al., 2006).

Table 2.1c: Some commonly used AD transgenic mouse models.

Mouse	Genetic make-up	Pathology	Reference
PDAPP	695, 751and 771 hAPP isoforms with Indiana mutation; PDGF promoter.	Aβ deposits from 6 months; hyperphosphorylated tau and synapse loss.	(Games et al., 1995)
Tg2576	695 hAPP isoform with Swedish double mutation (K670N/M671L); hamster prion protein promoter.	Aβ deposits from 9-11 months; Intraneuronal Aβ	(Hsiao et al., 1996)
APP23 (Novartis mouse)	751 hAPP isoform with Swedish double mutation (K670N/M671L); mouse Thy-1.2 promoter.	Aβ deposits from 6 months; profound CAA; Little neuronal loss	(Sturchler-Pierrat et al., 1997)
TgCRND8	hAPP with both Indiana and Swedish double mutation; prion protein promoter	Aβ deposits and learning impairment from 3 months	(Chishti et al., 2001)
J20	hAPP with both Swedish and Indiana mutations; PDGF promoter	Aβ plaques from 8-10 months; Loss of presynaptic synaptopyhsin immunoreactivity; Aβ plaque independent toxicity.	(Mucke et al., 2000)
LineC3-3	Chimeric mouse/human APP with swedish double mutation & containing humanized Aβ domain; mouse prion	No plaques until 18 months	(Borchelt et al., 1997)

	promoter.		
APPswe/PS1A 246E	Coexpress huPS1-A246E and Chimeric mouse/human APP with swedish double mutation & containing humanized Aβ domain; mouse prion promoter.	Aβ plaques by 12 months	(Borchelt et al., 1997)
3xTg-AD	hAPP with Swedish mutation, Tau with P301L mutation and PS1 with M146V	Progressively develop intracellular Aβ, plaques, Neurofibrillary tangles, synaptic dysfunction	(Oddo et al., 2003)

Partial recapitulation of human AD pathology in a transgenic mouse was considered, as vindication for the amyloid cascade hypothesis. Mechanisms downstream of Aβ deposition are nicely modelled by these transgenic mice. An ideal mouse model for any disease should have construct validity, face validity and predictive validity. Construct validity ensures that the causation of pathology in the mouse model is similar to humans. Face validity ensures that different pathological manifestations, symptoms and phenotypes that appear in the model are similar to that in humans. Lastly, predictive validity ensures translation of drug efficacy in mice to humans. So far these transgenic *APP* mice have incomplete construct validity for both familial and sporadic AD. As for face validity, it is also partial as only amyloid, synaptic, inflammatory and cognitive pathologies are seen while key NFT pathology and neuronal loss are absent. Lastly, so far they have extremely low predictive validity (ability to translate efficacy in mouse models to humans). Furthermore, in the absence of cause-effect relationship for Aβ in AD, one needs to be aware of the caveats associated with these models. . Further, in many transgenic mouse models of AD (e.g. APP23 (Van Dam, 2003) and Tg2576,(Westerman et al., 2002a)) , cognitive decline occurs well before the initiation of Aβ deposition. Even with massive Aβ deposition in these mice very little neurodegeneration is seen. In the absence of any noticeable neurodegeneration in these mice, they may at best model asymptomatic AD patients (Zahs and Ashe, 2010).

2.1.11 Drugs for Alzheimer's disease

Currently available drugs can provide only symptomatic relief to AD patients and they cannot stop, delay or reverse the disease pathogenesis. Donepezil, rivastigmine and galantamine are acetylcholinesterase enzyme inhibitors (AChEIs), whereas memantine is uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist (Reisberg et al., 2003; Birks, 2006). AChEIs are indicated for mild to moderate AD, whereas memantine is approved for moderate to severe AD either as monotherapy or in combination with AChEIs. yet another AChEI huperzine is currently approved in China for mild to moderate AD (Wang et al., 2009). Drug discovery for AD has been a daunting exercise for researchers at academia and industry. As prevention rather treatment paradigm is becoming increasingly favored,

early diagnostic markers of AD progression are needed to identify patients with mild cognitive impairement (MCI). Moreover, 30-40% of MCI patients do not develop AD and are negative on amyloid positron emission tomography (PET) scanning. Predictors of progression like *APOE* genotype, clinical and cognitive scores, CSF markers, amyloid imaging may greatly help decreasing the heterogeneity of AD patient population (Sperling et al., 2011b). Presence of amyloid signal in PET scan together with low CSF $A\beta_{42}$ and high CSF tau in prospective AD subjects will minimize diagnostic innacuracies. Eventhough, patient stratification based on markers like *APOE4* genotype is good, however might necessitate separate trials for carriers and noncarriers as 30-50% of AD patients are *APOE4* negative (Aisen, 2011). Amyloid cascade hypothesis promulgates two signature lesions of AD as mostly sought drug targets for AD. Many of the investigational drugs act by targeting either Aβ production or enhancing Aβ clearance. Inhibitors for BACE and γ-secretase enzymes, Aβ aggregation blockers work by reducing Aβ production or aggregation, while active and passive vaccination strategies aim to enhance clearance of Aβ from brain. Other strategies like targeting tau phosphorylation, mitochondrial function, neurotransmission, balance of growth factors and anti-inflammatory drugs are in various stages of clinical development (Mangialasche et al., 2010).

Figure 2.1f provides a brief summary of the current drug trials in humans. However, the first wave of drugs failed to bring any meaningful clinical improvements to subjects in clinical trials and thus were abandoned. Tramiprostate (Alzhmed), tarenflubril (Flurizan), semagacestat, latrepiridine (Dimebon), antioxidants and latest bapineuzumab have failed in phase 3 clinical trials. Many excuses like drug not entering the brain in sufficient concentrations, patients on placebo not deteriorating as much as they should, patients with severe pathologies, drugs not engaging target Aβ efficiently, wrong and compromised dosage regimens, unacceptable adverse effects were put forward to account for these failures. Also, it is not clear how much reduction of brain amyloid is necessary to attain meaningful clinical outcomes. Lately, researchers have also been stressing for initiating the treatments early on before the onset of symptoms because there will be not much remaining to rescue by these drugs when given at advanced stages of AD. However one should cautiously note that rate of Aβ deposition is much more steeper during early stages than later stages, where it relatively plateaus off (Karran et al., 2011). Thus targeting Aβ can be even more challenging during early stages of AD. Apparently, in AD drug development program, right target, right drug and right disease stage seem to be extremely critical for favourable outcomes (Sperling et al., 2011b).

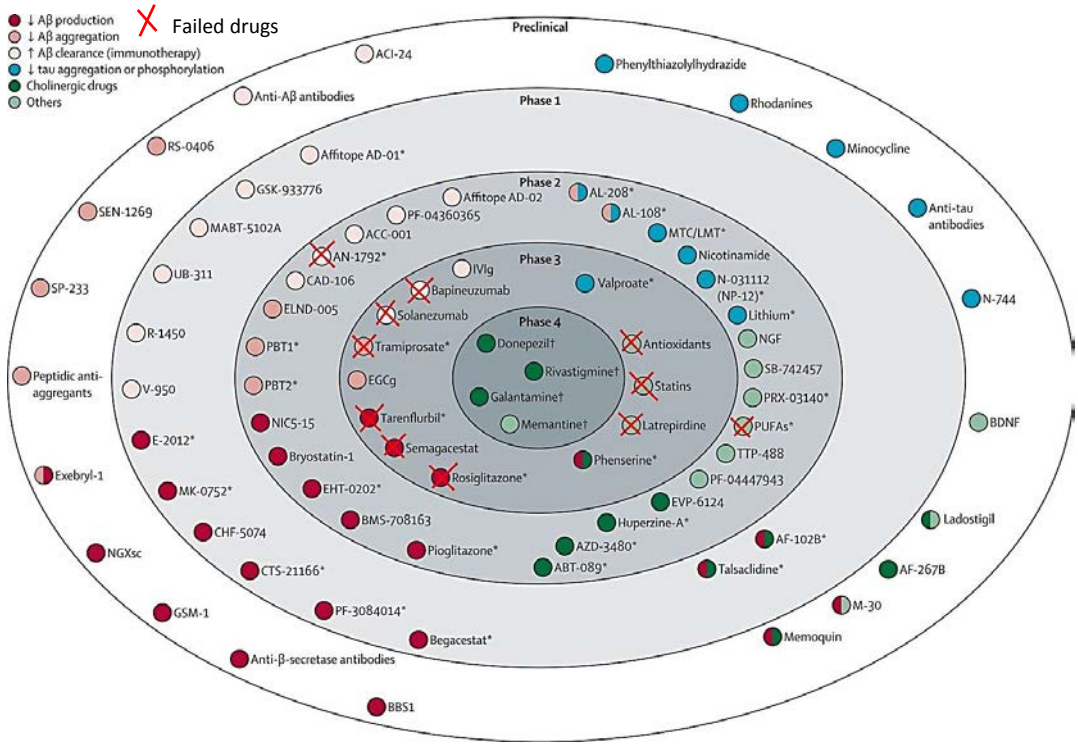


Figure 2.1f. Current status of AD drugs in various clinical phases of drug development. *Adapted from* Lancet Neurology (2011) 10:501

Aβ as drug target is very elusive in that different researchers see it in many different forms and quantify it differently. Aβ protein with 40 or 42 amino acids on C-terminal are distributed differentially in the brain. $A\beta_{40}$ is predominantly present in vasculature, whereas $A\beta_{42}$ present in neuritic plaques is more hydrophobic and prone to aggregation. It is not clear which one is a better target. Also, neuropathologists detect and quantify fibrillar Aβ deposits of brain parenchyma and vasculature with Congo red or thioflavin S staining, whereas diffuse Aβ is detected only by Aβ immunohistochemistry. Biochemists detect and measure Aβ from the perspective of solubility in different reagents like water-soluble fractions, detergent-soluble fractions, and water-insoluble fractions (solubilized in formic acid or guanidine solution). Physical chemists view Aβ from the standpoint of aggregation status like oligomers (monomers, dimers, trimers etc), protofibrils (Aβ aggregated in β pleated structure) and 6-9 nm fibrils that form Aβ deposits. It is still not clear which pool of Aβ is responsible for the loss of cognition in AD patients. Recently, it was found that Aβ oligomers interfere with normal functioning of neurons and are also synaptotoxic in nature (Wilcox et al., 2011). Aβ oligomers are difficult to isolate as they quickly convert into other forms (Benilova et al., 2012). Different researcher use diverse protocols and designate different nomenclatures to identify them (Aβ*56 as a good example,(Lesne et al., 2006)). Basically,

apart from vague target (Aβ), limited CNS penetration of drugs and AD patient heterogeneity might be responsible for recent setbacks in phase 3 clinical trials.

Many high profile failures in late phases of clinical trials definitely did dampen the progress but did not decrease the spirit of AD drug discovery. Numerous phase 3 clinical trials that are in progress will validate these targets in near future. Recently, at Alzheimer Association International Conference (AICC)Vancouver 2012 (http://www.alz.org/aaic/), phase 2 data from Gammagard trial reported stabilisation of 4 AD patients for more than three years which is encouraging (Relkin, 2012). Also, many novel drugs like EVP-6124, a small-molecule partial agonist selective for the alpha-7 nicotinic acetylcholine receptor, improved cognition and function in Phase 2 clinical study (Strobel, 2012a). Certainly, we are dealing with a complex disease which may need more than one silver bullet to cure and prevent.

2.2 NEUROINFLAMMATION

Inflammation is body's response to noxious stimuli. Celsus described four cardinal signs of inflammation: redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*). Virchow added the fifth sign as loss of function (*functio laesa*). Tissue vasculature plays a pivotal role in this classic model of inflammation. In fact, local vasodilatation ensures excess blood supply, thereby resulting in redness and heat at the site of inflammation. Increased vascular permeability resulting in leakage of plasma proteins and fluid causes swelling. Bradykinins, prostaglandins and other lipid mediators, provide molecular signals necessary for cellular infiltration and heightened pain sensitivity. Therefore, tissue vasculature has an important role in maintenance of inflammatory responses. Further, classic triple response described by Lewis in 1924 featuring flush, flare and wheal are also typical vascular consequences of a cutaneous irritation (by stroking metal). Different cellular, molecular and genetic inducers regulate various phases of inflammation. Regulation of inflammation is important for maintaining homeostasis. Immune cells in circulating blood play a very crucial role in initiation, maintenance and resolution of inflammation (Serhan et al., 2010). Resolution of inflammation is equally important to limit collateral damage caused by inflammation. During resolution phase of inflammation, infiltration of macrophages and monocytes ceases and their apoptosis ensue; mediators like lipoxins, resolvins, protectins, prostaglandin D and transforming growth factor beta-1 (TGF-β-1) play an important role in resolution of inflammation (Serhan and Savill, 2005; Serhan et al., 2007).

Inflammation in general can be characterized by nature of stimuli and duration of stimuli. Acute inflammatory responses arise from short-lived stimuli like tissue injury caused by a knife cut. Chronic stimuli in comparison are due to stimuli for prolonged durations like those seen during allergies or systemic infections. Chronic inflammatory processes are known to be driving forces behind molecular pathogenesis of many diseases. Inflammatory diseases can be classified as allergic, neurodegenerative or autoimmune diseases. In case of allergic diseases, an exaggerated inflammatory response to foreign antigen drives the disease as happens for example in bronchial asthma. In case of neurodegenerative diseases, an abnormal toxic protein assembly or conformation when deposited initiates a cascade of inflammatory events as happens in AD. Lastly, in case of autoimmune diseases, uncontrolled inflammatory responses against self-antigens are at the root of disease pathogenesis in condisions such as multiple sclerosis and rheumatoid arthritis.

Inflammatory responses occurring within central nervous system (CNS) microenvironment are termed as "neuroinflammation". Neuroinflammation is simply brain's response to stimuli like infection, mechanical or chemical injury, ischemic shock or abnormal protein deposits etc. These responses are deemed beneficial if neuroinflammatory processes cease after stimuli is removed. However, in many neuropathological conditions, these processes fail to cease either due to persistent stimuli or failure of normal resolution mechanisms (Serhan et al., 2008). Chronic neuroinflammation is detrimental and causes collateral damage to CNS, further deteriorating homeostasis (Glass et al., 2010). Various CNS diseases caused by bacteria, viruses, prion bodies, and abnormal protein conformations always display a chronic component of neuroinflammation. Mechanical brain injuries due to accidents etc. involve initial acute inflammatory response followed by a much prolonged chronic inflammation. The cellular mediators differ during acute and chronic inflammatory conditions. These

cellular mediators are amenable to immunomodulation which may further impact prospective or ongoing inflammatory responses. Immunomodulation of microglia is a promising tool for researchers who wish to control neuroinflammatory responses occurring inside CNS.

2.2.1 Inflammation vs. neuroinflammation

Inflammatory responses within brain contrast significantly with those occurring outside CNS. Owing to the presence of blood-brain-barrier (BBB) and skeletal structures surrounding the brain, it is not feasible to manifest classic signs of inflammation, described by Celsus. Table 2.2a summarizes the main differences of inflammation outside brain and inside brain.

Table 2.2 a: Inflammation differs inside and outside brain.

INFLAMMATION	
Outside Brain	Inside Brain
Rednesss, heat, swelling, pain and loss of function are present.	Swelling and pain are mostly absent.
Both innate and adaptive immune systems are involved.	Restricted and controlled involvement of innate and adaptive immune cells.
Depending on stimulus mostly neutrophils, macrophages, B cells andT cells and other immune cells are present.	Microglia are the key immune cells.
Antigen presentation by dendritic cells.	Dendritic cells are absent.
Lympahtic system is involved.	Lymphoid organs are absent.
Abundant expression of MHC proteins.	Very low constitutive expression of MHC.
Immune cell recruitment and functions are normal.	Very restricted entry and significantly dampened B cell and T cell functions.

Glial cells, microglia and astrocytes, are distributed ubiquitously in the brain parenchyma, such that noxious stimuli (external or internal) are quickly intercepted by their long actively sensing processes. Armed with basic host-defence mechanisms and extensive molecular coordination, these cells aim to provide efficient immune surveillance in spite of restricted immune cell access into CNS. In the absence of cellular and molecular components of blood, inflammation is supported by these resident microglial cells and astrocytes, in addition to their other housekeeping functions. There are also similarities in inflammatory processes inside and outside brain, of which worth mentioning are the roles of cytokines and chemokines. Further, NFkB transcription factors regulate pro-inflammatory gene expression inside as well as outside brain. One should note that these differences in part stem from much believed "immune privilege" status of brain.

For long, brain has been thought to be an immune privileged organ. Tissue grafts, tumors, viruses, bacteria when introduced into brain parenchyma evade immune detection consolidating this traditional view. Most of the time term "immune privilege" is linked inaccurately with BBB. Immune privilege is a relative term applicable only to brain parenchyma (Galea et al., 2007). Brain meninges, choroid plexus, circumventricular organs and ventricles lack "immune privilege" status. Afferent arm of immune system comprising of professional antigen presenting cells (dendritic cells) is typically lacking in CNS (Galea et al., 2007) and this deficiency contributes towards cellular basis of immune privilege. In the absence of cellular transport for antigens, soluble antigens reach nearest cervical lymph nodes by taking fluid route via perivascular spaces of capillaries and arteries to elicit an immune response which is further skewed towards humoral rather than cell mediated responses (Harling-Berg et al., 1999). This suggests that the afferent arm of immune system is deficient in brain. In the efferent arm, the major obstacle for incoming immune cells in CNS is their apoptotic cell death, executed by Fas ligand engagement. All CNS cells express Fas ligands (Bechmann et al., 1999). Furthermore, high local concentrations of anti-inflammatory cytokines like interleukin-10 (IL-10), TGF-β-1 and presence of gangliosides suppress efficient T-cell activity within the brain parenchyma (Strle et al., 2001; Malipiero et al., 2006; Irani et al., 1996). Even if peripheral immune cells survive this initial roadblock, very low constitutive expression of major histocompatibility complex (MHC) within CNS (Perry, 1998) dampens antigen recognition. Modulation by astrocytes and microglia also greatly influence normal functioning of immune cells within CNS (Trajkovic et al., 2004; Magnus et al., 2005). Furthermore, the immune privilege status of CNS is severely compromised during neuroinflammation, probably due to breach of BBB and local immunostimulation by chemokines and cytokines. Therefore, inadequacies of adaptive and innate immune system, age and anatomical location of brain region all together determine the immune status of CNS (Galea et al., 2007). These shortcomings of immune system seem to be necessary as CNS has restricted capacity to regenerate post-mitotic, non-regenerating neurons if lost during a full-fledged inflammatory reaction (Horner and Gage, 2000).

Innate immune system of brain can be activated by a plethora of physiological and pathological stimuli. Circulating Lipopolysaccride (LPS), bacterial and viral components during systemic infections (meningitis), tumors, infiltration of infectious agents into CNS (encephalitis), energy and metabolic dysregulation, neural dysfunction, traumatic injuries of physical and chemical nature, accumulation of misfolded proteins etc. constitute stimuli

lasting either acutely or chronically in CNS with a global or local consequences (Glass et al., 2010).

2.2.2 Mediators of inflammation

Since the beginning of 20th century, microglia were known by many names such as "Nissl's Stäbchenzellen," "Gitterzellen," or "third element of Cajal". Microglia are the major resident immune cells of CNS and were known long for their migratory and phagocytic functions (Penfield, 1925). They represent approximately 5-10% of all brain cells. Microglia execute many important physiological house-keeping functions. In normal conditions, microglia exhibit quiescent phenotype, show anti-inflammatory and neuroprotective functions, and are constantly surveying their microenvironment for any internal or external cues that might endanger brain homeostasis (Streit, 2002). By using *in vivo* two-photon imaging, microglial cells in cortex of live mouse were found to be very active in their presumably resting or non-activated state (Nimmerjahn et al., 2005). Bacteria, viruses, injuries, cellular fragments of dead cells, apoptotic cells, mis-folded protein deposits etc. activate microglia. Activation of microglia can be characterized by changes in its cellular morphology and induction of many genes related to innate immune system. Activation by LPS is the gold standard for studying microglia activation under experimental conditions (Hanisch and Kettenmann, 2007). Other than tissue surveillance, microglia also carries out synaptic pruning (Ekdahl, 2012). Microglia are also important mediators of tissue regeneration and neurogenesis. In CNS lesions of leeches, accumulation of microglia was necessary for axonal sprouting suggestive of their beneficial role in CNS injuries (Ngu et al., 2007).

Evidently, microglia needs to acquire appropriate activation phenotype in order to display diverse effector functions related to tissue surveillance and repair. CNS macrophages (analogous to activated microglia) displaying M1/M2 phenotype, at the opposing ends of activation scale, is too simplistic model (Gordon, 2003). This classification is akin to Th1 and Th2 responses of T cells. Both LPS and IFN-γ induce M1 phenotype in microglia/macrophage via activation of TLRs and thereby produce proinflammatory cytokines, chemokines, induce oxidative stress, promotes Th1 reponses, and cytotoxicity. In contrast, M2 macrophage is activated by IL-4 or IL-13 promotes down regulation of proinflammatory mediators and further promotes tissue remodelling (Gordon, 2003). Expression of mannose and galactose receptors, production of ornithine and polyamines through the arginase pathway, high expression of chitinase 3-like 3 (YM1) and release of cytokines like IL-4,IL-13, IL-10 etc. charecterize M2 phenotype. In addition, downstream signalling pathway like STAT1 and STAT3/STAT6 promotes M1 and M2 polarizations respectively (Sica and Mantovani, 2012). Further, M2 phenotype is sub divided into M2a (IL-4,Arginase-1, YM-1,IL-13) for stopping proinflammatory pathways, M2b (Il-10 and CCL-1) for remove reactive oxygen and nitrogen species produced during inflammation and M2c for tissue repair and remodelling (Varnum and Ikezu, 2012). Many subsets of microglia/macrophages with varying degrees of classic (M1) and alternate (M2) effector functions in diverse acute and chronic stimuli, arising from a wide array of physiological and pathological conditions, only further complicates this model of microglia/macrophage activation.

Microglia in CNS are important players of innate immune inflammatory responses in various neurological conditions like Parkinson's disease (Rogers et al., 2007), HIV dementia (Garden, 2002), multiple sclerosis (Muzio et al., 2007), amyotropic lateral sclerosis (Dewil et

al., 2007), and AD (Mandrekar-Colucci and Landreth, 2010). Microglia respond to a wide variety of noxious stimuli by producing soluble pro-inflammatory mediators that specifically target removal of bacteria, viruses, debris from injured tissue or locally deposited abnormal proteins(Kettenmann et al., 2011).

Many of the inflammatory mediators like complement proteins (Osaka et al., 1999) and cytokines (Feuerstein et al., 1994) are known to cause neuronal damage apart from affecting neuronal functions. CNS specific expression of TNF in neurons caused seizures, ataxia and early death resulting from widespread infiltration of Tcells into CNS, astrocytosis, microgliosis and focal demyelination (Probert et al., 1995). In a different model, expression of TNF in astrocytes caused chronic inflammatory encephalopathy further consolidating the detrimental role of inflammation on maintaining neuronal structure and function (Stalder et al., 1998). Proinflammatory mediators are also known to inhibit trophic factors like brain-derived neurotrophic factor (BDNF) thus indirectly contributing towards neurodegeneration (Nagatsu and Sawada, 2005). Moreover, acute neuroinflammatory processes can lead to excitotoxicity followed by microglial activation and neuronal degeneration, which can be suppressed by NMDA receptor antagonists (Morimoto et al., 2002). As nitric oxide (NO) production by astrocytes is involved in NMDA receptor mediated neuronal death, neuroinflammation can be further mechanistically linked to excitotoxicity (Hewett et al., 1994). Anti-NMDA receptor encephalitis also links NMDA receptors to neuroinflammation. This acute neuroinflammatory condition involves production of autoantibodies against NMDA receptor thus precipitating seizures, confusion and amnesia in patients (Irani et al., 2010). Further, antibodies caused NMDA receptor internalization and their levels correlated with clinical severity of disease. Even in the absence of any explicit structural damage to neurons, chronic treatment with proinflammatory cytokine TNF inhibited long-term potentiation (LTP) in a dose-dependent way (Tancredi et al., 1992). Similarly, cytokine interleukin-6 (IL-6) decreased LTP in hippocampal CA1 region along with concomitant inhibition of mitogen activated protein kinase(MAPK)/extracellular signal-regulated kinase (ERK) phosphorylation that was further reversed by tyrosine kinase inhibitors (Tancredi et al., 2000). In addition, an inverse relationship between interleukin -1 beta (IL-1β) in dentate gyrus (DG) and LTP in synapses of perforant pathway was reported in aged, stressed as well as in rats pretreated with IL-1β suggesting that inflammatory cytokines induce formation of reactive oxygen species that trigger lipid peroxidation, and that these changes further lead to depletion of membrane arachidonic acid that correlates with LTP (Murray and Lynch, 1998). Inducible nitric oxide (iNOS) produced by microglia activated by Aβ was also shown to be responsible for inhibition of LTP (Wang et al., 2004). All these data indicate that neuroinflammatory processes are detrimental for synaptic plasticity and normal neuronal function.

Chemokines and the cells bearing chemokine receptors play a very important role in supporting neuroinflammatory processes of CNS (Veenstra and Ransohoff, 2012). In fact, their role has been studied in various neuropathological conditions like traumatic brain injury (Semple et al., 2010), multiple sclerosis (Holman et al., 2011) and AD (Lee et al., 2010; Cho et al., 2011). Chemokines facilitate chemotaxis, tissue extravasation and modulation of lymphocyte function. Neuroinflammatory responses ensuing bacterial and viral infections result in massive infiltration of immune cells like neutrophils, monocytes and lymphocytes into the affected tissue. This immune cell infiltration is mediated by chemokines like

interleukin-8 (IL-8),interferon-gamma-inducible protein 10 (IP-10) and macrophage chemoattractant protein 1 (MCP-1) (Luster, 1998). Furthermore, proinflammatory cytokines like interferon gamma (IFN-γ) are also capable of inducing chemokine production that further perpetuates inflammation (Owens et al., 2005).

Cytokines are capable of crossing blood-brain barrier (BBB) thereby forming a direct link between periphery and CNS. Moreover, cytokines in CSF might be absorbed back into circulation. Also, there is increasing evidence of cytokine involvement in various pathomechanisms of neuroinflammation. IL-1β induces expression of IL-6 production and inducible nitric oxide synthase (iNOS) activity in astrocytes. Further, it also activates microglia, increases production of macrophage colony-stimulating factor (M-CSF), which in turn induces production of S100β from astrocytes, thus launching a self-perpetuating cycle (Griffin et al., 1998). IL-6 promotes astrocytosis, microgliosis and stimulates release of acute phase proteins. Moreover, IL-6 knockout mice exhibit faster acquisition and facilitation of radial maze learning suggestive of its physiological role in memory processes (Braida et al., 2004). TNF is neurotoxic (Combs et al., 2001) but can also have protective roles by preventing neuronal apoptosis. Overexpression of TNF in astrocytes of aged transgenic mice resulted in increased deposition of amyloid in cerebral blood vessels and meninges indicating amyloidogenic potential of cytokines (Wyss-Coray et al., 1997).

Microglia, astrocytes, and macrophages in culture release an inducible form of nitric oxide (iNOS) upon stimulation with LPS (Corradin et al., 1993; Galea et al., 1992). Expression of iNOS and NO has been described in many neurological disorders like experimental autoimmune encephalitis (EAE) (Willenborg et al., 1999), cerebral ischemia (del Zoppo et al., 2000), AIDS dementia (Hori et al., 1999) and ALS (Almer et al., 1999).

Cyclo-oxygenases are known to important mediators of inflammation in health, and their role in many inflammatory disorders is well appreciated (Dubois et al., 1998). Primary astrocytes and microglia cultures exposed to LPS or cytokines are capable of synthesizing cyclooxygenase-2 (COX-2) (O'Banion et al., 1996; Bauer et al., 1997). In rat microglial cultures prostaglandin E2 (PGE_2) is a major enzymatic product of COX-2 and capable of further inducing COX-2 in microglial cells in a paracrine or autocrine manner (Minghetti et al., 1997). The microglial receptor for PGE_2, EP2 subtype (EP2), inhibits phagocytosis and enhances neurotoxic activities (Shie et al., 2005a; Shie et al., 2005b). Accordingly, deletion of EP2 receptor in APP/PS1dE9 mice resulted in reduction in lipid peroxidation in ageing mice, reduced oxidative stress, suppressed Aβ levels and reduced Aβ deposition (Liang et al., 2005). So, it appears that neuroinflammation caused oxidative stress leads to enhanced amyloid pathology via EP2 signalling.

Both microglia and astrocytes express Toll-like receptors (TLRs) (Lehnardt, 2010; Gorina et al., 2009). Bacteria, viruses and other microbial stimuli are mediated by pathogen associated molecular patterns (PAMPs). PAMPs activate TLRs, which in turn via induction of NFκB, increase gene transcription of IL-1β, resulting in production of inflammatory pro-peptides. Such pro-IL-1β peptides are cleaved by caspase-1 to release active IL-1β (Martinon and Tschopp, 2004) (Hanamsagar et al., 2012). Function of yet other innate immune receptors, nucleotide-binding domain leucine rich repeat containing receptors (NLRs), is necessary for activation of caspase-1. Upon activation by diverse stimuli such as adenosine triphosphae (ATP), uric acid, bacterial ribonucleic acid (RNA), and bacterial muramyl dipeptide, the

function of NLRs is dependent on assembly of large complexes of inflammasomes. Inflammasomes recruit and activate caspase-1, thus complimenting pro-inflammatory response of initial TLR activation by PAMPs. Such dual innate signalling ensures effective protection from microbial infections in CNS (Chakraborty et al., 2010). In case of stroke or trauma (sterile tissue injury), damage associated molecular patterns activate TLRs while NLRs are activated by endogenous substances like aggregated peptides (Halle et al., 2008) or uric acid crystals. ATP from damages CNS tissue also can also be sensed by NLRs via purinergic receptor activation.

2.2.3 NF-κB as mediator of neuroinflammation

NFκB proteins, which were initially discovered as nuclear factor enhancing expression of κ-immunoglobulin light chains of B-lymphocytes, are highly conserved during evolution and are expressed by all cell types. NFκB transcription factors comprise of 5 subunits, namely RelA (p65), RelB, c-Rel, NFκB1 (p50; p105) and NFκB2 (p52; p100). These subunits can exist in cytoplasm as heterodimers or homodimers. All NFκB proteins show Rel homology domains that are necessary for dimerization, nuclear localization and DNA binding. Normally, NFκB proteins are bound to inhibitory IκB proteins in cytoplasm. Again there are three different inhibitory IκB proteins IκBα, IκBβ and IκB$_\varepsilon$ are identified and they show characteristic ankyrin repeat domains. Interestingly, B-cell lymphoma 3 protein homolog (BCL-3), p105 and p100 also can act as inhibitory proteins as they also possess ankyrin repeat domains (Li and Verma, 2002). Many diverse inflammatory stimuli such as LPS, TNF, and ligands for TLRs activate their cognate receptors thus initiating a signalling cascade that ends by phosphorylating the inhibitor of NFκB kinase complex (IKK complex). This complex is composed of IKK1, IKK2 and NFκB essential modulator (NEMO). Activation of NFκB pathway is controlled by activation of IKK (see figure 2.2a). Activation of IKK complex leads to phosphorylation of IκB and further to its polyubiquitylation and proteasomal degradation. Thereby NFκB dimers are realeased in the cytoplasm and enter the nucleus, where they initiate expression of many genes involved in immune and inflammatory responses (Pasparakis, 2009).

Induction of NFκB mediated gene transcription in response to environmental, mechanical, chemical and microbial stimuli is an adaptive response that aims to bring back homeostasis. However, maladaptive responses are not uncommon during pathophysiological conditions. Aberrant activation of NFκB transcription factors have been implicated in a plethora of neuroinflammatory conditions and vice versa. In addition, mutations in key NFκB genes have been implicated in many diseases(Mattson and Camandola, 2001).

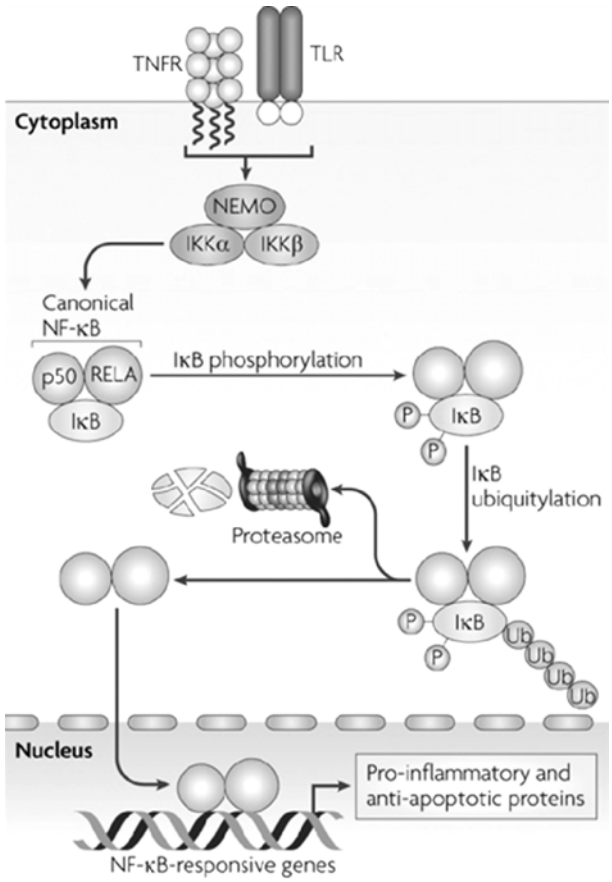


Figure 2.2a. Canonical NF-κB pathway. *Adapted* from Nature reviews Immunonology(2009) 9:778

2.2.4 Constitutive NFκB activity is important for learning and memory

Constitutive NFκB activity has been reported in several rodent brain regions including the amygdala, hypothalamus, olfactory lobes, cortex and hippocampus (Schmidt-Ullrich et al., 1996; Bhakar et al., 2002). One needs to exercise reasonable caution with immunohistochemical reports of staining p65 or p50 in neuronal tissue, as data related to antibody specificity in western blots and immunostaining from tissues from knockout mice appeared contradictory (Herkenham et al., 2011). Antibodies raised against the C-terminus containing transactivation domains were reported to be much more successful in specificity tests than those raised against the N-terminus proto-oncogene c-Rel (REL) homology domain or nuclear localisation sequence (NLS) region. Probably amino acid sequences are conserved in many proteins, which can account for this non-specificity. Genuinely, very low amounts of

p65 and p50 inside cells can sometimes be the cause for negative staining, but in such situations raising the antibody concentration to an extent of seeing cellular labelling amounts to nonspecific staining. One should be especially careful when interpreting neuronal staining patterns in the hippocampus because of the high neuronal density in this brain area. Namely, high density renders cells to appear as specifically stained, especially at lower magnifications (Herkenham et al., 2011). Immunostainings have shown basal NFκB activity in many glutamatergic neuron populations in the cortex and hippocampus (Kaltschmidt et al., 1993; Kaltschmidt et al., 1994; Kaltschmidt et al., 1995). The fact that this basal activity can be blocked by glutamate antagonists and calcium channel blockers indicates that it might be involved in physiological synaptic functions. Similarly, presence of inducible NFκB in biochemically purified synapses indicates that NFκB activity is necessary for maintaining synaptic function at the molecular level (Kaltschmidt et al., 1993; Meffert et al., 2003). In contrast, ablation of NFκB reduced synaptic densities in *stratum lucidum* of the hippocampus (Kaltschmidt and Kaltschmidt, 2009). Moreover, it has been found that knockout of the p65 subunit (also, *Tnfrsf1a* $^{-/-}$) or forebrain specific NFκB inhibition in neurons in mice results in not only in poor performance on spatial navigation tests but also reduced LTP and LTD (Meffert et al., 2003; Fridmacher et al., 2003). Recently, NFκB has been shown to regulate the protein kinase A / cyclic AMP-responsive element-binding protein (CREB) pathway (Kaltschmidt et al., 2006). In addition, evidence from chemically induced brain injury in wild-type and *Nfkb1* null mice demonstrate that NFκB regulated genes are involved in neuroplasticity in the hippocampus (Kassed et al., 2002). All this evidence indicates a prominent role for neuronal NFκB activity in learning and memory functions.

2.2.5 NFκB in neuroprotection and neurodegeneration

Typically, NFκB activation is implied in neuronal death induced by glutamate toxicity (Grilli et al., 1996), haloperidol induced oxidative stress (Post et al., 1998) and focal ischemia (Schneider et al., 1999). However, some *in vitro* studies have shown that pre-treatment of neuronal cells with TNF and lymphotoxin-alpha (LT-α) protects neurons against Aβ induced neurotoxicity via activation of NFκB (Barger et al., 1995). Cultured neurons pretreated with 0.1μM Aβ40 or 2ng/ml of TNF protected cerebellar granule cells from cell death induced by 10μM of $A\beta_{40}$ via NFκB activation (Kaltschmidt et al., 1999). Similarly, activation of NFκB was found protective against ferrous sulphate mediated oxidative stress (Kaltschmidt et al., 2002), glutamate and Aβ neurotoxicity (Goodman and Mattson, 1996). Protective anti-apoptotic effects on cultured neurons by NFκB activation was shown to be primarily mediated by induction of Mn-superoxide dismutase, inhibition of peroxynitrate formation and suppression of lipid peroxidation (Mattson et al., 1997). Furthermore, induction of NFκB activation increased levels of anti-apoptotic proteins and was strongly neuroprotective against recombinant adenovirus induced neuronal death in one study (Bhakar et al., 2002). Similarly, in three different mouse models *in vivo* preconditioning with 3 min sublethal ischemia or 5 mg/kg kainic acid or 500nM of linolenic acid led to rapid activation of NF-κB, which could be blocked by NFκB inhibitor diethyldithiocarbamate or kappaB decoy DNA (Blondeau et al., 2001). All these data support physiological neuroprotective and anti-apoptotic effects of NFκB activation in CNS. Along similar lines, transgenic overexpression of

IkBα super-repressor in forebrain neurons inhibited NFκB activation (Fridmacher et al., 2003) and augmented neurodegeneration induced by kainic acid or oxidative stress (Fe^{+2}).

Opposing roles of NFκB activation in various in vitro models of neuronal apoptosis and survival were ascribed to the balance of the p65 and c-Rel subunits (Pizzi et al., 2002). Metabotropic glutamate receptor type 5 activation drives c-Rel dependent antiapoptotic pathway responsible for neuroprotection against Aβ peptide (Pizzi et al., 2005). Under ischemic stress, unbalanced activation of p65/*Nfkb1* dimers over p65/c-Rel leads to cell death by inducing transcription of proapoptotic Bim and Noxa genes while p65/c-Rel dimers induce antiapoptotic Bcl-xL genes (Sarnico et al., 2009). Neuroprotective role for another NFκB sub unit was demonstrated in *Nfkb1* null mice. Mice deficient in *Nfkb1* gene were treated with trimethyltin, 3-nitropropionic acid (mitochondrial toxin) or kainic acid (excitotoxin), they displayed aggravated neuronal damage compared to wild-type animals (Kassed et al., 2004; Yu et al., 2000; Yu et al., 1999). In transient middle cerebral artery occlusion (tMCAO) model, massive neurodegeneration in the hippocampus and striatum was evident with Flouro-Jade staining (marker of apoptotic cells). When compared to non-transgenic littermates, *Nfkb1*-/- mice showed an 8-fold increase in hippocampal Fluoro-Jade staining, indicating a pivotal role for NFκB activation in neuronal survival (Duckworth et al., 2006). This is in direct contrast to studies which showed reduction of infarct volume in *Nfkb1*-/- mice (Schneider et al., 1999). Similarly, in permanent MCAO model, *Nfkb1*-/- mice developed a smaller infarct than wild-type controls (Nurmi et al., 2004).

In *Nfkb1*$^{-/-}$ mice, all CNS cells including neurons and microglia are deficient in the *Nfkb1* gene. Thus it becomes difficult to untangle NFκB activation or suppression in a cell specific manner, which is pivotal to explain contrasting roles of NFκB in neuroprotection and neurodegenration. When mice with microglia specific IKK-2 ablation were subjected to transient middle cerebral artery occlusion (tMCAO), they displayed a 10% reduction in infarct size compared to their wild-type littermates (Cho et al., 2008), indicating that NFκB activation in neurons is important for survival but NFκB activation in microglia is deleterious for neuronal survival. Similar observations were also made in astroglia as well. Cultured cortical astrocytes when treated with $A\beta_{42}$ showed NFκB activation in dose-dependent manner. However, this NFκB activation in astrocytes resulted in increased iNOS expression and NO production (Akama et al., 1998), indicating a deleterious role of NFκB activation in astrocytes. Spinal cord injury in transgenic mice with NFκB inhibition specifically in astrocytes (expressing dominant negative form of IκBα under GFAP promoter) showed improved functional recovery, decreased lesion volume, reduced pro-inflammatory expression of cytokines and chemokines, and salvaged white matter (Brambilla et al., 2005). Present data indicates that NFκB signalling in astrocytes might regulate chemokine (C-C motif) ligand 2 (CCL2) induced infiltration of immune cells to the lesioned brain. Similarly, in the EAE model, inhibition of NFκB signalling (NEMO and IKK-2 ablation) prevented expression of pro-inflammatory cytokines, chemokines and adhesion molecules, resulting in a milder pathology (van Loo et al., 2006).

Understanding the mechanisms involved in the proliferation of neural stem cells in adult CNS may reveal novel drug targets for the treatment of many neurodegenerative diseases. A recent *in vitro* report showed that neural stem cells isolated from subventricular zone when

treated with 10ng/ml of TNF, induced IKK-2 mediated NFκB activation and up regulation of cylin D1 expression, resulting in increased cellular proliferation (Widera et al., 2006). Furthermore, NFκB subunit p65 and p50 were found to be necessary for normal growth and expansion of neuropshere cultures (Young et al., 2006). Similarly, a recent *in vivo* study found a 50% reduction in number of newly born neurons in the hippocampus of adult *Nfkb1*$^{-/-}$ mice compared to wild-type controls (Denis-Donini et al., 2008), which further strengthens the role of NFκB signalling in adult neurogenesis. All these data consolidate the notion that targeting NFκB signalling in glial cells is a good idea for curtailing pro-inflammatory consequences of CNS diseases, whereas in neurons and neural stem cells, augmenting canonical NFκB signalling might be beneficial for survival and protection from apoptosis as well as for boosting adult neurogenesis.

Several genetically engineered mouse models have been developed to elucidate the roles of NFκB family members. However, one has to be mindful of potential caveats while interpreting the phenotypes of these mice. Several of these mice exhibit embryonic lethality; therefore much of the observations are limited to only early stages of development. One has to also appreciate the fact that there exists considerable redundancy among the transcriptional factors in cell type and developmental stage-specific manner. In addition, the propensity of crosstalk at the level of IKKs and transcription factors further confounds interpretation of results from these transgenic mice. As seen in *Nfkb1*$^{-/-}$ mice, loss of NFκB activity might as well disrupt other important intracellular signalling pathways (tumor progression locus 2 (Tpl2)/ERK signalling). Furthermore, IKKs are also known to phosphorylate proteins other than those involved in the NFκB pathway. In spite of the above limitations, genetically engineered mice have enormously expanded our understanding about both canonical and alternative pathways of NFκB activation.

Canonical NFκB pathway offers many good targets for controlling inflammatory diseases. However, chronic inhibition of this vital pathway might result in precipitation of unwanted side effects as this pathway is pivotal in regulating many physiological functions. NFκB c-Rel was shown to be one such good candidate for neuronal survival in various *in vitro* toxicity models (Pizzi et al., 2005). Furthermore, mGlu5 receptor mediated expression of MnSOD and Bcl-X_L are both transcriptional targets of c-Rel. In contrast, p105 has a much more restricted role in regulating immune responses and hence is a better target than IKK of the canonical pathway.

2.3 NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

Neuroinflammation has been implicated in many neurodegenerative diseases like Parkinsons's disease, stroke and AD (Akiyama, 1994). In addition, plethora of inflammatory processes and abnormal innate immune responses are observed in brains of people with AD (Akiyama, 1994; Wyss-Coray and Mucke, 2002). Several cytokines, chemokines, complement proteins and other proteins typically associated with immune and inflammatory processes were abnormally expressed in AD (Akiyama et al., 2000). Such neuroinflammatory processes in AD may begin quite early in the course of the disease, which is indicative of causation. However, it is unclear whether neuroinflammation may be a driving force for the disease, or might even induce beneficial responses that may slow or modify the disease progression.

2.3.1 Evidence from humans

Epidemiological and genetic evidence supports the idea of an early involvement of inflammatory processes in AD. The Honolulu-Asia aging study reported an increase in C-reactive protein 25 years before the diagnosis of dementia in Japanese-American men (Schmidt et al., 2002), potentially suggesting an early involvement of inflammatory processes during AD pathogenesis. Also, patients taking anti-inflammatory drugs like NSAIDs showed decreased risk for developing dementia indicating that inflammation might play a critical role in the AD pathogenesis (McGeer et al., 1990; in t'Veld et al., 2001; Launer, 2003). Recently, genome-wide association studies as well hint to a role for inflammatory genes like clusterin (CLU) and complement receptor 1 (CR1) in AD (Hollingworth et al., 2011). Gene expression analysis from post-mortem AD tissue also indicated an upregulation of many proteins and transcription factors typically associated with neuroinflammation (Colangelo et al., 2002). Genes related to NF-κB, IL-1α, CCL-2 and TNF induced protein 2 were consistently shown to be increased in AD patients, whereas upregulation of complement 4A and 4B, CD68 and glial fibrillary acidic protein (GFAP) genes in transgenic AD mice was similar to that in humans (Wyss-Coray, 2006). However, several gene microarray studies in humans with AD did not overlap consistently, highlighting issues with reproducibility and reliability caused by differences in experimental setups, inter-individual differences and differences in statistical procedures used. Moreover, immune responses of inbred mice might be different from genetically heterogeneous human population, and in addition, chronic transgenic overexpression of APP in these mice might cause an aggravated activation of innate immune responses, which may not be representative of human AD patients (Wyss-Coray, 2006). Thus validation in various mouse models and ultimately in humans is necessary to make gene expression data to be more reliable. Genetic differences with *APOE* also seemed to modulate immune responses (Lynch et al., 2003a; Lynch et al., 2003b).

Early studies established increased expression of immune system-associated antigens in white and grey matter samples from both controls and AD patients. Furthermore, by using immunohistochemistry microglia, astroglia, macrophages, pericytes were identified as chief cellular mediators (Luber-Narod and Rogers, 1988). Microglia or macrophages expressing histocompatibility glycoprotein HLA-DR were conspicuously found to be present surrounding lesions of AD (McGeer et al., 1987)(McGeer et al., 1988). Moreover, brains of normal elderly showed meagre HLA-DR immunoreactivity, whereas virtually all neuritic plaques of AD brains co-localized with HLA-DR signal (Rogers et al., 1988). Triple

immunohistochemistry on post-mortem entorhinal and hippocampal AD brain tissue revealed spatial relationships between Aβ plaques, microglia and astrocytes (Dandrea et al., 2001). These studies show diversity of cellular mediators surrounding Aβ deposits. More recently, *in vivo* imaging of activated microglia using PET with benzodiazepine ligand (R)-PK11195 showed higher regional binding (entorhinal, temporoparietal and cingulated cortex) in AD patients than in normal controls (Cagnin et al., 2001). Plasma levels of cytokines like IL-12, IL-16, IL-18 and TGF-β-1 were found to be higher in AD patients than controls, and they correlated with disease progression (Motta et al., 2007). Secretion of cytokines like IL-2 and IFN-γ were implicated in moderately severe AD patients, whereas reduction in TNF and IL-3 was implicated in mild stages of AD, suggesting a defect even in peripheral immune responses (Huberman et al., 1994). However, measurements of peripheral cytokines and chemokines in AD and controls were inconsistent but surely reflected defective immune responses (Lee et al., 2009). Peripheral monocytes and macrophages from AD patients displayed defects in immune functions (Beloosesky et al., 2002; Khansari et al., 1985) while frontal cortex of AD brains showed a higher expression of the CD45 marker than that of controls. In addition, CD45 immunopositivity was localized to microglial plasma membrane further consolidating their role in AD progression (Masliah et al., 1991). These observations established the role of immune cells enduring neuroinflammatory processes in AD. Furthermore, early ultrastructural studies indicated that microglia might not be able to phagocytose Aβ but instead be involved in the formation of fibrillar Aβ plaques (Frackowiak et al., 1992; Wisniewski and Wegiel, 1994; Wegiel et al., 2000).

2.3.2 Evidence from cell cultures and mouse models

Neuroinflammation may also be a driving force in the progression of AD neuropathology. Immunostimulation with proinflammatory cytokines increased mRNA levels of BACE (Sastre et al., 2003). Similarly, treatment of human astrocytes with TGF-β-1 elevated APP mRNA levels (Amara et al., 1999). Also, exposure of NT2 neurons to oxidative stress increased BACE expression and its activity (Tamagno et al., 2002). Furthermore, IL-1β exposure increased APP synthesis by 6-fold in primary human astrocytes and by 15-fold in human astrocytoma cells (Rogers et al., 1999). All these data indicate that neuroinflammation regulates Aβ production at multiple levels. *In vitro* studies show that neurotoxic mediators released by activated microglia are responsible for neuronal injury (Meda et al., 1995). Stimulation with Aβ increased CD40 expression on microglia, which when further treated with CD40 ligand induced TNF expression and caused neuronal loss, suggesting that CD40-CD40 ligand interaction is important for Aβ neurotoxicity (Tan et al., 1999). Activated microglia of inflamed CNS differ from resting quiescent microglia of normal CNS with respect to overexpression of CD45 marker (Sedgwick et al., 1991). Microglial CD45 activation inhibits proinflammatory signalling pathways, such as CD40 ligand induced activation of the src-family kinases, lck and lyn (Tan et al., 2000b), and also Aβ induced microglial activation of p44/42 mitogen-activated protein kinases (Tan et al., 2000a).

Systemic inflammation is known to increase brain amyloidosis in mice (Guo et al., 2002). In many human amyloid depositing mouse models, neuroinflammatory LPS stimuli increased Aβ and tau pathologies (Qiao et al., 2001; Sheng et al., 2003; Kitazawa et al., 2005), supporting the idea that inflammation is a driving force for AD progression. It is important to note that in these early studies LPS was not given directly into the brain by intracerebral or

intrahippocampal injections, but was administred either intracerebroventricular or intraperitoneal, suggesting that both innate and adaptive immune systems responses might have played role in aggravating Aβ pathology. Modulation of innate as well as adaptive immune responses in transgenic APP mice was achieved by various genetic and pharmacological tools that could profoundly influence specific molecular components of neuroinflammatory mechanisms. For example, stereotactic brain injections of recombinant adeno-associated viral vector expressing proinflammatory TNF in 3xTg-AD mice resulted in infiltration of inflammatory immune cells and caused neuronal death (Janelsins et al., 2008). This along with previous systemic LPS injection experiments suggests that neuroinflammation not only aggravates early Aβ and tau pathologies but is also detrimental for neuronal survival. In line with this observation, inhibition of soluble TNF signalling by dominant negative TNF prevented accumulation of early Aβ plaques in LPS treated 3x-TgAD mice (McAlpine et al., 2009). Surprisingly however, more complete blocking of TNF signalling by genetic ablation of TNF receptor 1 and 2 in 3xTg-AD mice resulted in aggravated amyloid and tau pathologies suggestive of a critical role of TNF-signalling in microglia mediated uptake and clearance of extracellular Aβ (Montgomery et al., 2011).

On similar lines, reducing CD45 expression either pharmacologically or genetically worsened amyloid pathology. Gene deletion of microglia activation marker CD45 in APP/PS1 mice increased cerebral amyloidosis, elevated Aβ oligomer levels and caused neuronal loss due to neurotoxic TNF and IL-1β releasing phenotype of microglia (Zhu et al., 2011). In concert, mouse Aβ vaccine trials showed increased CD45 expression by microglia associated with greater reductions of fibrillar Aβ (Wilcock et al., 2001). Therefore, modulation of CD45 seems to be a promising target if one wishes to influence amyloid pathology. Many other mediators of inflammation like complement proteins, TGF-β, CCR2, CX3CR1, CD40, and microglia themselves were manipulated to influence inflammation in the context of amyloid pathology. Activation of complement proteins plays a pivotal role in AD neuroinflammation. In Tg2576 mice, deficiency of C1q protein did not change amyloid pathology but significantly subdued inflammatory reactions of both astrocytes and microglia surrounding Aβ plaques and decreased loss of synaptophysin and microtubule-associated protein 2 (MAP-2) markers (Fonseca et al., 2004). However, very old APP mice with complement C3 deficiency showed increased Aβ deposition in the brain and suppressed iNOS and TNF with elevated interleukin-4 (IL-4) and IL-10, indicative of a M2 microglia activation (Maier et al., 2008). Furthermore, inhibition of C3 activation by expressing soluble complement receptor-related protein y, a complement inhibitor, in brains of APP mice also resulted in 2-3 fold higher Aβ burden and prominent neurodegeneration (Wyss-Coray et al., 2002). Similarly, increased expression of C3 in APP mice overexpressing TGF-β was associated with greater reduction in Aβ loads (Wyss-Coray et al., 2001). Therefore, activation of C3 was found to be beneficial for ameliorating Aβ pathologies. Likewise, expression of TGF-β-1 in astrocytes of APP mice resulted in reduction of Aβ in brain parenchyma (Wyss-Coray et al., 2001). In line with this observation, neuronal inhibition of TGF-β in APP mice resulted in Aβ accumulation and neurodegeneration (Tesseur et al., 2006). In contrast, genetic ablation of TGF-β signalling in Tg2576 mice also mitigated parenchymal, cerebrovascular amyloid and Aβ by 90%, which might be due to an increased infiltration of peripheral macrophages into CNS (Town et al., 2008). Thus, it appears that CNS overexpression of TGF-β or peripheral inhibition of TGF-β on macrophages is beneficial for ameliorating Aβ pathologies.

Chemokine receptor CCR2 in microglia mediates accumulation of microglia around sites of neuroinflammation and deficiency of CCR2 in Tg2576 mice impaired microglia accumulation, elevated Aβ levels by suppression of Aβ degrading enzyme neprilysin and accumulated Aβ deposits much earlier (El Khoury et al., 2007). Therefore, CCR2 dependent microglia accumulation seems to be protective in early stages of AD. Deficiency of microglial chemokine CX3CR1 or fractalkine receptor prevented layer 3 neuron cell loss in 3xTg-AD mice suggesting a critical role for Fractalkine-CX3CR1 in neuron microglia communication (Fuhrmann et al., 2010). Microglia directly interact with Aβ via TLR-2, TLR-4 and CD14, triggering microglia mediated inflammatory activation as well as microglia mediated Aβ clearance (Fassbender et al., 2004)(Reed-Geaghan et al., 2009). Innate immune response elicited by TLRs is mediated by myeloid differentiation factor 88 (MyD88) resulting in activation of AP-1 and NFκB transcription factors (Akira et al., 2001). However, MyD88 deficient microglia in APP mice, produced by MyD88 deficient reconstruction of bone marrow, improved cognitive functions, attenuated pro-inflammatory TNF-α, IL-1β and CCL-2 gene expression, and reduced Aβ burden by enhanced phagocytosis (Hao et al., 2011). In spite of overwhelming evidence supporting the role of microglia in clearance of Aβ plaques, a recent study involving complete microglia ablation in APP mice, however, suggested their limited role in formation and maintenance of Aβ plaques (Grathwohl et al., 2009).

Transgenic *APP* mice mimic many features of AD neuroinflammation. In APP/PS1dE9 mice used in our studies, neuroinflammation is typically characterized by accumulation of microglia around Aβ deposits as early as 4 months of age. This is also the same time when these mice deposit their first plaques. In addition to microgliosis, many markers of activated microglia like CD45 expression increases (See figure 5.1.4e-C). Microgliosis is clearly evident around Congo red positive fibrillar Aβ deposits. Both resident microglia and peripheral macrophages can be detected by CD45 immunostaining. Using anti-CD45 monoclonal antibody as marker, it is also possible to see peripheral macrophage infiltration into the brain parenchyma. In APP/PS1dE9 mice this infiltration begins around 12 months of age. Interestingly, cognitive deficits in Morris water maze show up in these mice around this age. Such infiltrating cells are characterized by their round cell shape, relatively smooth surface bearing few to no processes and an intense CD45 immunostain signal (See figure 5.1.4e-D,E,F). Such wandering cells can often be seen in the grey and white matter, hippocampus, thalamus, striatum, meninges, choroid plexus, and around circumventricular tissues of brain. Even though they appear to be infiltrating from periphery, it is difficult to ascertain their exact origin as there is no protein marker available that is faithfully restricted to either peripheral or CNS compartments. Another microglia marker, Iba-1, is also elevated considerably but more conspicuously in microglia that are present in the immediate vicinity of Aβ plaques. With Iba-1 immunohistochemistry, one can easily differentiate between activated and quiescent resting microglia (See figure 5.1.4e-A,B). Other markers like CD11b, and CD68 are also elevated in activated microglia. CD68 is a lysosomal marker of macrophages indicative of putative phagocytosis (See figure 5.1.4d-C). Inflammation in other transgenic AD mouse models appears more or less similar. Neuroinflammation in Tg2576 mice is further characterized by the presence of TNF and IL-1β immunopositive microglia around fibrillar Aβ, while IL-6 immunopositivity was confined to astrocytes surrounding thioflavin positive Aβ deposits (Benzing et al., 1999a).

2.3.3 Mediators of inflammation in AD

Microglia from aged human brains are rich in cytokines, chemokines and complement mediators of inflammation (Lue et al., 2001). Apart from these, cyclooxygenases, iNOS, MMP-9, VEGF, APOE are significant mediators of inflammation in the AD brain. There is *in vitro* evidence that Aβ can directly activate cytotoxic classic complement pathways (Rogers et al., 1992). Similarly, Aβ also stimulated production of chemokines like C-C motif chemokine 3 (MIP-α) and C-C motif chemokine 4 (MIP-β) mRNA in macrophages and MCP-1 in astrocytes (Smits et al., 2002). In addition, infiltration of immune cells require upregulation of chemokines, and several of these chemokines and their receptors have been reported to be upregulated in AD brains with intact blood-brain barrier (Xia and Hyman, 1999) (Heneka et al., 2010).

Cytokines as mediators of inflammatory processes in AD are released by microglia and astrocytes surrounding Aβ deposits. IL-1β, TNF, and TGF-β-1 are among the many cytokines involved in AD. Treatment with IL-1 has been shown to increase APP gene expression in human umbilical vein endothelial cells (Goldgaber et al., 1989). Another cytokine, TGF-β, was also shown to be modulating APP gene expression in microglial BV-2 cell line (Monning et al., 1994). A transformed human monocyte cell line, THP-1, when stimulated by Aβ also induced production of TNF (Klegeris et al., 1997), suggesting that cytokines can induce APP expression and Aβ in turn can as well induce cytokine production. Many of the pro-inflammatory cytokines in AD are produced by microglia. Aβ and IFN-γ synergistically activate microglia by triggering production of reactive nitrogen intermediates and TNF causing neuronal cell injury *in vitro* (Meda et al., 1995). Apart from fibrillar Aβ, even Aβ oligomers can stimulate adult microglia to release TNF (Floden and Combs, 2006). Freshly dissolved $A\beta_{40}$ stimulated microglia to release IL-1β while freshly dissolved $A\beta_{42}$ stimulated microglia to release IL-1α and IFN-γ (Lindberg et al., 2005). Microglia can be stimulated by Aβ in the same manner as IFN-γ and LPS, thereby unleashing a cytotoxic proinflammatory responses, which stand in contrast to those microglial responses stimulated by IL-4 cytokine (Butovsky et al., 2005). This clearly suggests that cytokines can tune and tame microglial responses towards a direction defined by pathology and physiology of brain microenvironment. Moreover, inflammatory cytokines released by activated microglia may cause activation of astrocytes, which in turn may further induce APP expression and thus amplify the pro-inflammatory cytokine production to a never ending cycle (Ho et al., 2005). Besides, presence of many inflammatory cytokines such as IL-1β, TGF-β. IL-10, TNF, IL-6 was detected in the brain of Tg2576 AD mice, where much of the cytokine signal was restricted to Aβ plaque microenvironments (Benzing et al., 1999b; Apelt and Schliebs, 2001) (Sly et al., 2001). Furthermore, progressive age-dependent upregulation of IL-12 and IFN-γ mRNA was detected by *in situ* hybridisation technique in cerebral cortices of Tg2576 mice (Abbas et al., 2002).

Even though cyclooxygenases are involved in neuroinflammation, their role in AD is controversial. Some studies have indicated increased expression of COX-2 in the AD brain (Ho et al., 2001), while some other studies reported decreased COX-2 in late stages of AD (O'Banion et al., 1997). PGE_2 is an end product of COX-2, and EP2 is principle receptor for PGE2. PGE_2 signalling via EP2 receptor was found to be promoting age-dependent oxidative

damage and increased amyloid burden in APP/PS1 mice via BACE1 activity(Liang et al., 2005).

Apolipoprotein E (ApoE) is associated with neurodegenerative diseases, including AD and Parkinson's disease. In addition to its role lipid transport and pathogenesis of atherosclerosis, it is also known for its immunomodulatory properties. Strong induction of circulating TNF, IL-1β and IL-6 levels by LPS injection was inhibited by ApoE co-administration, suggestive of its cytokine modulatory effects (Van Oosten et al., 2001). *Apoe* deficient mice respond to bacterial infections by producing higher circulating TNF level than controls (de Bont et al., 2000). *Apoe* deficient mice also express higher levels of circulating cholesterol that might further aggravate inflammatory responses (Martens et al., 2008). Upon LPS stimulation, *Apoe* deficient mixed glia cultures produced higher amounts of TNF and IL-6 than wild–type controls. Similarly, intravenous injection of LPS to *Apoe* deficient mice upregulated brain levels of *Tnf* and *Il6* (Lynch et al., 2001). Moreover, ApoE can modulate CNS immune and inflammatory responses in an isoform specific manner. Mouse *Apoe* when replaced with human *APOE4* displayed greater serum and brain levels of TNF and IL-6 than mice with human *APOE3* upon intravenous LPS injection (Lynch et al., 2003b). Similarity of CNS and systemic inflammatory effects in both *Apoe*$^{-/-}$ and *APOE4* mice suggest that at least some *APOE4* effects might be reflective loss of function of ApoE protein.

Recently, it was shown that microglia had higher expression of vascular endothelial growth factor (VEGF) and its receptor Flt-1 in AD brains than in nondemented controls (Ryu et al., 2009). Subsequently, VEGF receptor subtype Flt-1 has been shown to play an important role as microglial chemotactic receptor necessary for mobilizing microglia around Aβ plaques. Furthermore, this Flt-1 dependent mobilization of microglia resulted in neurodegeneration (Ryu et al., 2009).

In AD brains, iNOS expression in microglia was clearly detected by immunohistochemistry (Heneka et al., 2001). More specifically, increased iNOS expression was seen associated with Hirano bodies (eosinophilic rod like inclusions), Aβ plaques and NFTs of AD brains than in control brains (Lee et al., 1999)(Wallace et al., 1997). Injection of fibrillar Aβ into rat striatum resulted in increased activation of microglia and astrocytes that also expressed iNOS, suggesting that Aβ can activate glial cells which in turn can release inflammatory mediators (Weldon et al., 1998). Apart from microglia and astrocytes in AD brain, NFTs and neuropil threads also express iNOS (Vodovotz et al., 1996). In addition, AD brains showed higher levels of nitrotyrosine in neurons than controls indicative of peroxynitrate involvement in oxidative damage associated with AD (Smith et al., 1997).

2.3.4 Neuroinflammation in Alzheimer's disease - Good or bad?

Neuroinflammation in AD is characterized by the presence of reactive astroglia and microglia around Aβ plaques. In addition, presence of complement proteins, pro-inflammatory cytokines, chemokines, infiltration of peripheral monocytes and brain edema are also signs of detrimental neuroinflammation in AD (McGeer and McGeer, 2001). Activated microglia and astroglia release many neurotoxic factors, such as reactive oxygen species, excitatory neurotransmitters, proteases, nitric oxide, which ultimately results in loss of neurons, synapses and cognition (Akiyama et al., 2000). Such microglial activation in the presence of pro-inflammatory cytokines has been shown to hinder degradation or

phagocytosis of Aβ plaques (Koenigsknecht-Talboo and Landreth, 2005)(Akiyama et al., 2000; Hickman et al., 2008). Moreover, proinflammatory cytokines TNF and IL-1β may impair synaptic plasticity and cause memory impairment (Wang et al., 2005). The above mechanisms constitute representative examples for detrimental neuroinflammation in AD.

Astute application of the immunological principles to target Aβ peptide in experimental AD mice uncovered unexpected beneficial potential of inflammatory processes and ushered a new avenues for disease modifying strategies. Brain processes involving activation of microglia, such as traumatic brain injury were reported to reduce Aβ burden in APP mice (Morgan et al., 2005). This particular activation of innate immune response was deemed to be beneficial. Subsequent investigations revealed mechanisms like Fcγ mediated phagocytosis of Aβ by activated microglia (Bard et al., 2000), suppression of reactive astrocytosis by active vaccination against Aβ (Schenk et al., 1999), reduction of Aβ load by stimulation of microglia by LPS (DiCarlo et al., 2001), and infiltration of peripheral macrophages into CNS (Malm et al., 2005b), these constitute good examples of beneficial neuroinflammation in AD.

Currently, there is no evidence that suggests an early role of inflammation in AD. All the evidence from genetic mouse models suggests that neuroinflammation is a driving force in AD rather than a cause. Inflammatory mediators in AD interact at multiple cellular, molecular and transcriptional levels to facilitate mechanisms underlying neurodegeneration and neuroprotection in AD. Therefore, for modifying AD disease progression one strategy is to balance detrimental and beneficial effects of neuroinflammation, which requires dynamic engagement of innate immune system, and to a minor extent, even elements of adaptive immune system. This incessant relationship between immune cells and neuroinflammation opens up a large untapped opportunity to specifically modify progression of AD. Mechanisms like suppression of activated microglia and astroglia, switching cytokine profiles between Th1 and Th2, protection of neurons and synapses, and fostering neurogenesis are tangible objectives to regulate immune responses. Experimental and therapeutic approaches in this direction offer many immunomodulatory avenues for the treatment of AD.

2. 4 IMMUNOMODULATORS FOR ALZHEIMER'S DISEASE

Undisputable involvement of cellular and molecular mediators of innate immune system in pathogenesis of various neurodegenerative diseases is well appreciated (Bordon, 2010; Goni et al., 2010). Microglia, astroglia and neurons are the major cellular mediators while, Toll-like receptors, cytokines, chemokines etc. are some important molecular mediators. Under aseptic conditions, these cellular and molecular mediators regulate diverse neuroinflammatory and immune responses to a variety of endogenous and exogenous stimuli. Any experimental intervention through pharmacological or genetic approaches bears consequences on both innate and adaptive arms of immune system thereby influencing neuroinflammatory milieu. Such modulators of immune responses may be immunostimulants, immunosuppressant drugs, vaccines, adjuvants or monoclonal and polyclonal antibodies. Neurotransmitter glutamate recently joined the ranks of immunomodulators. Metabotropic glutamate receptor 4 (mGluR4) in dendritic cells modulates regulatory T cells and supresses neuroinflammation in a mouse model of multiple sclerosis (Hansen and Caspi, 2010). In another example, transgenic mice expressing human APOE4, upon LPS stimulation had elevated brain expression of TNF-α and IL-6 than APOE3 counterparts suggesting isoform–specific immunomodulatory properties of Apo-E (Lynch et al., 2003a). Furthermore, treatment with small ApoE-mimetic peptide in these mice suppressed systemic and brain neuroinflammation. Thus, immunomodulation by ApoE can be beneficial in a variety of neuroinflammatory conditions associated with stroke, multiple sclerosis, septic shock, traumatic brain injury and Alzheimer's disease (Lynch et al., 2003a). In the context of AD, immunomodulators can simply provide temporary relief from the toxicities associated with Aβ peptide or at least decrease, if not cease, the impact of ongoing neuroinflammation by suppressing the neurotoxic activation of glial cells and by influencing network of cytokines or chemokines etc. In addition, immunomodulators may also switch beneficial cellular phenotypes such as phagocytic and neurogenic microglia. In AD therapeutic pipeline, NSAIDs, Peroxisome proliferator-activated receptor gamma (PPAR-γ) agonists, statins, thalidomide and other TNF inhibitors, omega-3 fatty acids, curcumin, sodium valproate, alpha-7 nicotinic receptor agonist, Aβ vaccines, monoclonal Aβ antibodies, human intravenous immunoglobulins, umbilical cord stem cells, NFκB inhibitors like pyrrolidine dithiocarbamate (PDTC) are some examples of potential immunomodulators.

2.4.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Some epidemiological studies indicate that rheumatoid arthritis patients on NSAID treatment are less likely to develop AD (McGeer et al., 1990; Launer, 2003). The Canadian Study of Health and Aging also showed reduced risk of AD with history of arthritis, usage of NSAIDs, or use of corticosteroids (Anonymous 1994). Furthermore, the Rotterdam study revealed that consistent long-term use of NSAIDs reduced the risk of AD by 80% (in t'Veld et al., 2001). Moreover, protective effect of NSAIDs was strongly dependent on the treatment duration and *APOE* genotype. In some studies, NSAID mediated protection to AD was limited to only those with *APOE epsilon 4* allele (Szekely et al., 2008);(Pasqualetti et al., 2009). Several mechanisms were hypothesized for the protective effect of NSAIDs, such as anti-inflammatory, neuroprotective, anti-amyloidogenic, and anti-Aβ aggregational effects.

NSAIDs are clinically widely used for pain, fever and inflammation. They act by inhibiting cyclooxygenase enzymes leading to suppression of prostaglandin synthesis (Smith et al.,

2000). Certain NSAIDs like ibuprofen and naproxen are nonselective for COX isoforms (COX-1 vs. COX-2). NSAIDs like celecoxib, rofecoxib, diclofenac, nimesulide etc are COX-2 selective. As inflammation is a silent component of AD pathology, these classic anti-inflammatory drugs were deemed to confer relief to AD patients. Epidemiological and experimental data from in vitro cultured cells and transgenic APP mice further revealed many underlying mechanisms. As Aβ peptide is the prime drug target in AD, early experiments investigated anti-amyloidogenic effects of NSAIDs. Aspirin not only prevented Aβ aggregation *in vitro* but also reversed β-sheet conformation of preformed Aβ fibrils at clinically relevant doses (Thomas et al., 2001). This property was also seen in a number of NSAIDs like indomethacin, naproxen, ketoprofen, ibuprofen, celecoxib and rofecoxcib (Gasparini et al., 2004). Similar anti-aggregation effects were also demonstrated by naproxen and ibuprofen using PET molecular imaging probes for *in vitro* and *in vivo* Aβ binding (Agdeppa et al., 2003). In addition, NSAIDs may also induce expression of amyloid binding proteins like transthyretin, whose function is to sequester and prevent Aβ aggregation (Gasparini et al., 2004). Besides, NSAIDs like indomethacin, ibuprofen, and nimesulide increased expression of APP protein and were further shown to stimulate non-amyloidogenic α-secretase processing of APP through activation of MAP kinases (Avramovich et al., 2002). Effects of NSAIDs on secretion of Aβ largely depended on the type of cell culture system employed (Gasparini et al., 2004). R-flurbiprofen directly targeted γ-secretase and hence lowered $A\beta_{42}$ levels. COX inhibition is the principal mode of action for NSAIDs (Smith et al., 2000). However, Aβ lowering effect of NSAIDs was also reported to be independent of COX inhibition (Weggen et al., 2001). In this study by Weggen et al. sulindac sulphide, ibuprofen and indomethacin lowered $A\beta_{42}$ levels in cultured cells by more than 80% irrespective of the cell-type used. In the same study, however, aspirin, naproxen (non-secetive COX inhibitor), meloxicam (preferential COX-2 inhibitor), SC-560 (selective COX-1 inhibitor) and celecoxib (selective COX-2 inhibitor) failed to show any Aβ lowering effects.

Transgenic Tg2576 mice fed on chow mixed with ibuprofen for 6 months caused reductions in IL-1β, GFAP, microglia, dystrophic neurites, and suppressed amyloid pathology by ~50% (Lim et al., 2000). Similarly, in a comparative study 8 of 20 commonly used NSAIDs lowered $A\beta_{42}$ in APP transgenic mice after acute dosing suggestive of their potential in AD therapeutics (Eriksen et al., 2003). In Tg2576 mice, short treatments with ibuprofen but not with naproxen caused reductions of Aβ levels (Weggen et al., 2001). Microglial reactivity, as measured by CD45 and CD11b expression, was decreased alongside lowering of $A\beta_{42}$ in response to buprofen treatment (Yan et al., 2003). Nitric oxide releasing derivatives of flurbiprofen, HCT 1026 and NCX 2216 also showed Aβ lowering effects (Gasparini et al., 2004; Jantzen et al., 2002). Early and chronic ibuprofen treatment regimens resulted in reductions of Aβ consistently in several APP mouse models. In another study, indomethacin treatment for 7 months in Tg2576 mice decreased brain levels of Aβ but also interestingly suppressed NFκB activity significantly (Sung et al., 2004). Cognitive impairment and memory functions of Tg2576 mice were restored by naproxen, ibuprofen and the selective COX-2 inhibitor MF-tricyclic (Kotilinek et al., 2008). This beneficial effect was not due to lowered $A\beta_{42}$ or suppression of inflammatory cytokines like TNF and IL-1β, but depended inversely on PGE_2levels suggestive of COX-2 inhibition by NSAID treatment (Kotilinek et al., 2008).

In spite of epidemiological and experimental evidence, clinical trials with NSAIDs have been disappointing possibly due to many reasons like inadequate dose or treatment duration or wrong therapeutic window (Heneka et al., 2011). A short, 12-month randomized controlled clinical trial for ibuprofen in mild to moderate AD patients, however, showed no significant deterioration of cognitive status in a subset of *APOE epsilon 4* carriers, in spite of no overall treatment differences (Pasqualetti et al., 2009). Alzheimer's disease anti-inflammatory prevention trial (ADAPT) comprised of naproxen, celecoxib or placebo treatment arms was initiated in cognitively intact elderly with familial history of dementia. However, this trial was stopped because of cardiovascular safety concerns (Heneka et al., 2011). Clinical trials with nonselective NSAIDs or newer COX selective drugs were not found to be beneficial in acute treatment setup for AD symptoms. A COX-inactive NSAID, tarenflurbil (Flurizan), which is a R-enantiomer of flurbiprofen was shown to possess Aβ lowering effects. However, a multicenter, randomized, placebo-controlled phase 3 trial in mild AD patients failed to slow down cognitive decline or loss of activities of daily living in mild AD patients (Green et al., 2009). A detailed account of epidemiological, experimental, clinical details of various NSAIDs can be found in the comprehensive review by (Imbimbo et al., 2010).

2.4.2 Peroxisome proliferator activated receptor gamma

Immunomodulatory effects of NSAIDs can also be mediated by activation of PPARγ activity. In fact, typical NSAIDs like ibuprofen, indomethacin, fenoprofen and flufenamic acid are PPARγ ligands (Lehmann et al., 1997). PPARγ is widely present in adult and developing brain as well as in spinal cord (Heneka and Landreth, 2007). Of its two isoforms, PPARγ1 is specifically present in adipose tissue, whereas PPARγ2 is present in other tissues like liver and skeletal muscle Upon activation PPARγ forms heterodimers with retinoid X receptor; heterodimers then recruit co-activators and bind to PPAR responsive elements present in promoter regions of many genes. PPARγ can also inhibit gene expression without binding to gene promoters by transrepression of several genes regulated by NF-κB, activator protein 1 (AP-1), signal transducers and activators of transcription (STAT-1) transcription factors (Heneka et al., 2010). PPARγ plays a critical role in controlling inflammatory processes and mediators. PPARγ agonists and ligands are known to inhibit expression of several proinflammatory genes, such as cytokines IL-1β, TNF and IL-6, chemokines like MCP-1, COX-2, and metalloproteinase 9 (Landreth and Heneka, 2001). Furthermore, iNOS mediated cell death in response to LPS treatment of cerebellar granule cells was prevented by activation of PPARγ (Heneka et al., 1999). Agonists of PPARγ also inhibited neurotoxicity associated with Aβ activated microglia by suppressing expression of IL-1β, TNF andCOX-2 (Combs et al., 2000). In addition, stimulation of neuroblastoma cells transfected with APP by cytokines was inhibited by ibuprofen and indomethacin treatment, which was further suppressed by PPARγ anatagonists (Sastre et al., 2003). Further *in vivo* studies demonstrated that acute treatment of APP transgenic mice with PPARγ agonist like pioglitazone suppressed activation of microglia and astrocytes, and reduced expression of COX-2 and iNOS along with suppression of BACE (Heneka et al., 2005);(Sastre et al., 2006), suggesting that acute effects of PPARγ involve rapid inhibition of inflammatory responses and negatively modulating amyloidogenesis. However, during chronic treatment paradigms, amyloid lowering properties of PPARγ activators was reported to be limited, although immunomodulatory negative effects on inflammation was replicated (Yan et al., 2003).

2.4.3 Vaccination strategies for Alzheimer's disease

Solomon and colleagues were the first to demonstrate that antibodies raised against Aβ protein prevented aggregation of Aβ monomers and disintegrated preformed Aβ fibrils (Solomon et al., 1996; Solomon et al., 1997). This *in vitro* observation laid foundations for future immunotherapeutic approaches for AD. This strategy received a major impetus with the first *in vivo* proof of concept reported by Schenk et al. (Bard et al., 2000; Schenk et al., 1999). In PDAPP mice, vaccination with pre-aggregated synthetic $A\beta_{1-42}$ peptide induced development of Aβ antibodies, which then prevented deposition of Aβ and reduced preformed Aβ plaque load. In addition, treatment also decreased dystrophic neurites and inflammatory gliosis. These results were soon replicated by many groups independently (Weiner et al., 2000; Das et al., 2001) and were complemented by reports showing reversal of cognitive impairment in amyloid producing transgenic mice (Morgan et al., 2000)(Janus et al., 2000). However, one study reported limited ability of Aβ vaccination to alleviate learning deficits in immunized mice in spite of 60% reduction in amyloid plaque pathology (Chen et al., 2007). In this study only a subset of mice with low cortical Aβ levels displayed spatial memory benefits due to Aβ immunisation. Nevertheless, these initial studies demonstrated the potential of Aβ vaccination strategies to alleviate amyloid pathology and cognitive impairment in mice. In Tg2576 mice, however, active vaccination seemed to be efficient in preventing Aβ deposition in young mice but not in older mice with preformed amyloid plaques (Das et al., 2001). Also, in non-human primates like Caribbean vervets (Lemere et al., 2004) and aged canines (Head et al., 2008), Aβ clearing effects with Aβ vaccination was demonstrated to be efficacious. Active Aβ vaccination with even non-fibrillar and non-toxic $A\beta_{40}$ as well resulted in development of high titres of Aβ antibodies, which alleviated amyloid pathology in transgenic AD mice (Sigurdsson et al., 2001).

Immune system of elderly patients might be less competent to induce immune response against Aβ peptide. In addition, sometimes immune responses to Aβ vaccine are exacerbated and difficult to control. Probably due to same reasons, a phase 2 clinical trial in AD patients with Aβ vaccine AN 1792 (comprising of full length synthetic $A\beta_{42}$ and QS-21- a strong Th1 inducing adjuvant), could induce Aβ antibodies only in 19% of patients, and more importantly, was prematurely stopped due to development of aseptic meningoencephalitis in 6% of patients (Orgogozo et al., 2003).

In an effort to improve safety and efficacy, many novel second generation vaccines have been developed. An optimal combination of antigen, adjuvant and route of delivery are critical for the success of active vaccination strategy (Lemere, 2009). The presence of adjuvants along with antigen stimulates the host immune system to effectively mount immune responses. Conventional adjuvants like complete and incomplete Freund's adjuvant (Sigurdsson et al., 2001), Alum (Asuni et al., 2006) or heat shock protein 70 homolog of E.coli (Koller et al., 2004) were initially tested but lacked desired optimal efficiency. T-cells recognize Aβ epitopes on the C-terminal end of Aβ peptide from 16-42 amino acids (Lemere, 2009). Therefore, in order to avoid T-cell responses, N-terminal fragments of Aβ peptide from 1-15 amino acids (constituting B-cell epitopes) were preferred as antigens with minor modifications to enhance immunogenicity (Cribbs et al., 2003); (Monsonego et al., 2003). Immunogens like dendrimeric $A\beta_{1-15}$ (Seabrook et al., 2007) consisting of 16 copies of $A\beta_{1-15}$ on a branched lysine tree, two tandem linear repeats of $A\beta_{1-15}$ linked by two lysines with or

without N-terminal three amino acid sequence RGD or attachment of T1 T-helper cell epitope by two lysines to $A\beta_{1-15}$ were also developed(Maier et al., 2006). It was already known that T cell responses based on their cytokine release profile can be characterized as Th1 (cell mediated and proinflammatory) or Th2 type (humoral and anti-inflammatory). In mice, induction of antibodies predominantly of IgG1 and IgG2b sub-classes are indicative of anti-inflammatory Th2 cytokine signalling whereas IgG2a sub-class results from pro-inflammatory cytokine signalling (Abbas et al., 1996). Th2 cytokine profiles were preferred over Th1 in order to avoid T-cell mediated meningoencephalitis. Vaccinations with papillomavirus-like particles expressing 1-9 amino acid region of Aβ on viral capsid surface (Aβ-VLP) resulted in efficient humoral response at the same time avoiding induction of T cell responses (Zamora et al., 2006), suggesting that viral based vaccines are potentially safe and effective alternative candidates for AD vaccine (Chackerian et al., 2006). Add ravetch ref

Furthermore, appropriate route of administration in addition to suitable adjuvant are important to achieve high titres of Aβ antibodies after vaccination in mice. Intranasal immunization with fibrillar $A\beta_{40/42}$ plus adjuvant LT(R192G) (a mutant form of E.coli heat labile enterotoxin) resulted in higher titres of Aβ antibodies than in combination with adjuvants like monophosphoryl lipid A (MPL)/trehalose dicorynomycolate (TDM) emulsion or cholera toxin B subunit. However, subcutaneous vaccinations with the same antigen and adjuvants were not as efficient as intranasal routes (Maier et al., 2005). Transcutaneous route of vaccine delivery of aggregated $A\beta_{1-42}$ with cholera toxin adjuvant as well resulted in higher titres of Aβ antibodies of IgG1 subclass and reduced brain Aβ loads by 50% without any pro-inflammatory consequences of vaccination (Nikolic et al., 2007). Furthermore, this approach was not associated with any T-cell infiltration or cerebral microhemorrhage. Remarkably, one study reported decreased vascular amyloid and microhemorrhages with clinically approved Alum as adjuvant (Asuni et al., 2006).

Limitations and side effects of active Aβ immunotherapy fostered development of passive immunisation methods. In passive immunisation strategy, either monoclonal or polyclonal antibodies reactive to Aβ peptide are administered by intravenous or intraperitoneal routes. In PDAPP mice, injection of N-terminal specific Aβ antibodies not only crossed blood-brain-barrier but also decorated plaques and induced clearance of Aβ deposits by Fcγ receptor mediated microglial phagocytosis (Bard et al., 2000). However, in PDAPP mice, passive administration of m266 antibody directed towards the middle region of Aβ, prevented Aβ deposition, decreased biochemical levels of Aβ in brain, and increased Aβ immune complexes in plasma and CSF, indicating an efflux of Aβ from CNS in support of the peripheral sink mechanism (DeMattos et al., 2001). More direct evidence came from studies with multi-photon microscopy, where plaque clearance was demonstrated within 3 days of application of Aβ antibody directly to the cortex (Bacskai et al., 2001). Subsequent mechanistic studies revealed that Aβ N-terminally directed antibodies were efficient in clearing plaques, providing neuronal protection. Moreover, affinity of antibodies to FCγ receptors on microglia was found to be more important than affinity to Aβ itself (Bard et al., 2003). In triple transgenic AD mice, passive immunisation with Aβ antibodies not only cleared extracellular plaques but also decreased intracellular Aβ and tau pathologies (Oddo et al., 2004). Behavioural and memory benefits were also reported with passive immunisation protocols (Wilcock et al., 2004b). However, removal of Aβ plaques was not essential for obtaining cognitive benefits. (Kotilinek et al., 2002; Dodart et al., 2002). In addition, Aβ passive

immunotherapy prevented synaptic loss and reversed gliosis (Chauhan and Siegel, 2002), preserved dendritic morphology and structural plasticity in brains of PDAPP mice (Spires-Jones et al., 2009), and finally also protected from Aβ oligomer mediated loss of neuronal function (Klyubin et al., 2005; Klyubin et al., 2008). Passive immunisation with Aβ antibodies while efficacious in removal of Aβ plaques also resulted in Aβ antibody interactions with vascular amyloid causing cerebral amyloid angiopathy and microhemorrhages in mice (Wilcock et al., 2004b; Pfeifer et al., 2002; Racke et al., 2005).Microhemorrhages are consequences of antibodies reacting to Aβ present in the walls of cerebral blood vessels, thus triggering an inflammatory reaction. This is particularly critical in old people with frail blood vessels. All these AD mouse data indicate overall efficacy of passive immunisation strategy provided that the treatment begins before onset of robust plaque pathology in brain and blood vessels.

Mechanistically, both active and passive immunisation strategies rely on efficient clearance of Aβ from brain. Three main prominent mechanisms for immunotherapy have been proposed (Weiner and Frenkel, 2006; Morgan, 2011; Morgan, 2009; Morgan, 2005). First, Aβ antibodies prevent and interfere with aggregation of Aβ monomers into amyloid fibrils or even disintegrate pre-established Aβ plaques. Second, Aβ antibodies decorate plaques in the brain, which are subsequently recognized by Fcγ receptors on microglia. Thus, activated microglia removes Aβ deposits by phagocytosis. The third mechanism relies on the fact that there is a dynamic equilibrium between Aβ within the brain and outside the brain, thus binding of Aβ in the periphery by antibodies instigates an efflux of Aβ from CNS into plasma. The first two mechanisms will require antibodies to cross blood-brain-barrier while the third mechanism does not require antibodies to enter CNS parenchyma. All these mechanisms might be mutually exclusive and independent (Citron, 2004). Currently, nine passive immunisation trials and six active immunisation trials are in various stages of clinical development (Lemere and Masliah, 2010b) and trial results due in 2012-2013 will be critical in shaping future drug discovery programs in AD. Recently, antibodies have also been used to interfere with APP interaction with BACE1 site (Arbel-Ornath et al., 2010). Immunotherapeutic strategies for AD are destined to be disease modifying treatments as removal of Aβ from AD brains may delay or even reverse progression of this disease. Modulation of host immune system or influencing immune functions of microglia by Aβ antibodies irrespective of being passively transferred or actively produced constitutes their immunomodulation potential.

2.4.4 Miscellaneous immunomodulatory drugs

Among the other potential immunomodulators for AD are interferon-β and glatiramer acetate. Interferon-β is a type I interferon immunomodulatory cytokine which can be induced by ligands for TLRs. There are very few studies which explored immunomodulatory effects of interferon-β in the context of AD pathology. Nevertheless, they are known to inhibit production of IL-1β by regulating imflammasome either by mechanisms downstream of STAT activation or by induction of IL-10 (Gonzalez-Navajas et al., 2012). They are also known to shift proinflammatory Th1 cytokines milieu to Th2 anti-inflammatory profiles. Thus, their ability to influence innate immune responses and cytokines networks indicates their potential for use in AD. Glatiramer acetate has been tested in AD mice and was shown effective in clearing Aβ plaques and switch microglia phenotype by expressing CD11C

dendrite cell marker producing insulin growth factor 1 (IGF-1) (Butovsky et al., 2006). This treatment also induced neurogenesis and confered cognitive benefits to mice. Immunomodulatory effects of drugs on microglia can be monitored indirectly by measuring CSF levels of chitotriosidase, which is an indicator of activated microglia. Such immunomodulatory effects on microglia/ macrophages were previously reported by natalizumab treatment in multiple sclerosis patients (Olsson et al., 2012) and may be as well used for AD trials as biomarker of microglial activity. Anti-cancer drug mitoxantrone can block Aβ oligomerization (Colombo et al., 2009), and has been known to possess immunomodulatory properties by suppressing proliferation of CNS inflammatory cells and inhibiting the regulation of pro-inflammatory cytokines (Derwenskus, 2011). Immunomodulatory properties of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSC) were reported to improve both memory impairments and neuropathology (Lee et al., 2012). These cells not only reduced Aβ deposition, BACE-1 levels and tau hyperphosphorylation but also suppressed proinflammatory cytokines, elevated anti-inflammatory cytokines, and increased alternatively activated microglia (Lee et al., 2012). Similar anti-inflammatory and immunomodulatory effects were also reported by bone marrow-derived mesenchymal cells (Lee et al., 2010). Immunomodulators like thalidomide and its derivative supidimide were reported to favour nonamyloidogenic processing of APP via the α-secretase pathway (Avramovich et al., 2002). Further, neuronal and cognitive deficits associated with LPS induced chronic neuroinflammation was rescued by 3,6 dithalidomide, an analog of thalidomide possessing anti-TNF activity, indicating behavioural benefits of immunomodulation (Belarbi et al., 2012). Temporal and dose-dependent production of many cytokines and chemokines by intracerebroventricular injection of Aβ peptide or LPS was also suppressed by treatment with immuonodulators like glucocorticoids or IL-10 (Szczepanik and Ringheim, 2003). Many novel synthetic and natural agonists of Toll-like receptors are also being developed to specifically harness neuroprotective functions of innate immune system. Such immunomodulators may prevent detrimental effects of neuroinflammation and alter the course of disease progression, exemplifying a disease modification strategy in AD.

2.5 HUMAN INTRAVENOUS IMMUNOGLOBULIN FOR ALZHEIMER'S DISEASE

In 1991 Mönning et al. first reported Aβ immunoreactivity in human serum and CSF (Mönning et al., 1991). Subsequently, B-cells isolated from AD patients were reported to secrete antibodies that were reactive to Aβ plaques and blood vessels in AD brains (Gaskin et al., 1993). Several studies thereafter tried to characterize immune responses against Aβ by measuring autoantibodies in the brain, CSF and serum. So far, the evidence for the presence of Aβ autoantibodies in AD remains ambiguous. One study (Baril et al., 2004) demonstrated that subjects ranging from one-year-old to elderly showed low and variable titres of Aβ autoantibodies in the serum. Furthermore, no significant difference Aβ antibody titres were found between AD and control subjects. Likewise, another study (Hyman et al., 2001) found it quite common for normal healthy people above 65 years age to show small titres of autoantibodies against Aβ. However, since titres of Aβ antibodies in this study were far below those acquired during experimental immunotherapy, it was concluded that low affinity interactions of these antibodies may not be clinically useful either for diagnostic purposes or to therapeutically impact Aβ in brain or CSF or plasma (Hyman et al., 2001). In contrast, a study using tissue microarrays on AD and control brains demonstrated that antibodies reactive to Aβ are common in the elderly and that these antibodies can decorate Aβ plaques inside the brain (Kellner et al., 2009), indicative of therapeutic potential for autoantibodies against Aβ protein. Similarly, another microarray study also reported the presence of natural antibodies against Aβ and other toxic amyloidogenic assemblies in the serum and CSF of AD patients and control subjects (Britschgi et al., 2009). Interestingly, in one study, Aβ antibodies in the CSF of AD patients were found to be 38% lower than those in healthy subjects (Du et al., 2001).

Several studies thereafter have also reported significantly lower serum Aβ antibodies in AD patients than in elderly controls (Weksler et al., 2002; (Yang et al., 2005) Song et al., 2007). This phenomenon was not attributed to age-related decline in normal immune responses (Weksler et al., 2002). Not only serum titres, but also avidity of serum Aβ antibodies was significantly reduced in AD patients. However, the avidity of Aβ antibodies was not influenced by the age of the subjects in another study (Jianping et al., 2006). Interestingly, a study (Moir et al., 2005) showed significantly reduced titres of autoantibodies to oligomeric cross-linked Aβ protein species in AD patients but no differences in autoantibodies to unmodified Aβ monomers. Similartly, large immune complexes of autoantibodies against Aβ protein were selectively decreased in AD patients in another study (Henkel et al., 2007). In stark contrast to aforementioned studies, one study (Nath et al., 2003) reported significantly increased serum auto Aβ antibody titres in AD patients. However, only 3 of 11 AD patients showed presence of Aβ antibodies in ventricular CSF (from autopsy). Furthermore, in this study none of the 31 normal control subjects showed any detectable antibodies to Aβ. Similarly, another study (Mruthinti et al., 2004) using affinity purified IgGs from plasma showed four-fold higher titres of Aβ antibody in AD patients than in unaffected individuals. Likewise, after applying appropriate ELISA dissociation protocols, levels of serum Aβ autoantibodies were significantly elevated in AD patients than controls in a third study (Gustaw et al., 2008).

Thus, currently available literature shows decreased, unchanged or increased Aβ autoantibody levels in AD patients compared to normal subjects. The discrepancy in these studies can in part ascribed to technical caveats associated with ELISA measurements of protein complexes under investigation. Significant quantities of both Aβ protein and Aβ autoantibodies are bound to complexes, and therefore measuring total amounts of antigen or antibody following unmasking is critical for their precise estimation (Weksler et al., 2005). Furthermore, recent studies indicated very high non-specific binding properties of human intravenous immunoglobulins (hIVIG), such that even the reverse sequence of $A\beta_{1-42,}$ BSA or plain buffer solution could show positive optical density values (Klaver et al., 2010b);(Klaver et al., 2010a)(Klaver et al., 2010a)(Klaver et al., 2010a)(Klaver et al., 2010a)(Klaver et al., 2010a)(Klaver et al., 2010a)Klaver et al., 2010a);Klaver et al., 2010b). Notably, all the previous studies that measured Aβ autoantibodies (Dodel et al., 2002; Szabo et al., 2008) described no controls for the specificity of antibody binding to Aβ protein. Despite all this ambiguity about Aβ autoantibodies in serum or CSF, there is little doubt about the central role for immune system in regulating Aβ homeostasis.

Finally, Aβ antibodies in commercial preparations of hIVIG were investigated and shown to be reactive to Aβ monomers or oligomers (Klaver et al., 2010a). Detailed examination of epitope specificity of natural Aβ antibodies contained in human intravenous immunoglobulins (hIVIG) indicated the presence of conformational Aβ antibodies that were presumably formed from the neo-epitopes formed during aggregation of Aβ peptides to oligomers and fibrils (Szabo et al., 2008) (Britschgi et al., 2009). Roughly < 0.2% of antibodies in commercial hIVIG preparation has been reported (Gammagard S/D) to be reactive to Aβ protein (O'Nuallain et al., 2006). However, owing to the presence of antibodies reactive to toxic Aβ assemblies (O'Nuallain et al., 2008), therapeutic viability of hIVIG for treatment of AD was taken seriously by many investigators.

hIVIG, is not a pharmaceutical product but a collection of pooled polyclonal natural and acquired IgG antibodies. The subset of Aβ antibodies in hIVIG preparations are natural antibodies in the sense that they have not been produced in response to sensitization to any foreign antigen or due deliberate immunization. Much of the natural antibodies in healthy people are autoantibodies that are polyreactive to a wide array of self-antigens (Kazatchkine and Kaveri, 2001). Human body makes IgG which is purified by commercial fractionators from pooled plasma of more than 10000 healthy volunteers. Among the purification methods are Cohn-Oncley process (cold ethanol precipitation), Kistler-Nitschmann process, ion-exchange chromatography methods, and caprylate treatment followed by ion-exchange chromatography (Martin, 2006)(Radosevich and Burnouf, 2010). Typical plasma pools range from 4000 to 50000 litres. The World Health Organisation (WHO) guidelines require more than 1000 donors per lot. Hence, hIVIG represents a broad spectrum of antigenic repertoire that would be present in normal serum. Sometimes this property might as well cause dilution of any clinically useful components. Companies invest efforts and resources to minimise batch to batch variation, in order to maintain and ensure reproducible biological functions of purified immunoglobulins. Ensuring normal pharmacokinetics, pharmacodynamics and functional efficacy of hIVIG formulations largely depend on preservation of native molecular structure of Fab and Fc domains of IgG (Radosevich and Burnouf, 2010). According to a review comparing physic-biochemical properties of commercially available hIVIG

preparations (Boros et al., 2005),the half-life of hIVIG is approximately 21 days. Commercial preparations of hIVIG contain intact IgG molecules with trace amounts of IgM and IgA. For some IgA deficient patients there is risk of developing immune response (anaphylaxis) to IgA upon hIVIG infusion. These preparations are formulated as liquids with high IgG concentrations (100mg/ml) and are intended for intravenous use. Formulations also included stabilizers like polyols (sorbitol), sugars (maltose, glucose), or amino acids (glycine, L-proline, isoleucine) to prevent aggregate formation. Sodium content (0.9%) and osmolality of hIVIG preparations are controlled to reduce infusion-related adverse events. Optimal pH 4.0 to 4.5 also prevented aggregation and which upon administration would be rapidly neutralized by buffering capacity of blood. Therefore, buffering agents are added to maintain stability and prevent aggregation. Typically, hIVIG preparations have approximately neutral pH. Recently, pure (>98%) high IgG concentration (200mg/ml) stabilized by proline was developed for subcutaneous self-administration by patients with necessary training (Jolles and Sleasman, 2011). Presence of L-proline in the formulation prevents IgG aggregation during storage and decreases viscousity of the liquid formulation. Such formulations with shorter infusion times and better patient compliance are commendable advances in the field of hIVIG therapies.

hIVIG has a long history of being safe and effective therapy (van Doorn et al., 2010. Caution is exercised and all the necessary steps are taken in accordance with good manufacturing practice (GMP) protocols to avoid risk of viral transmission. Currently hIVIG is used as replacement therapy for primary and secondary immune deficiencies. It is also indicated in the treatment of autoimmune and systemic inflammatory diseases like idiopathic thrombocytopenic pupura (ITP), Guillian-Barré syndrome, multifocal motorneuropathy, chronic inflammatory demyelinating polyneuropathy(CIDP), and myasthenia gravis (Ballow, 2005)(Kaveri et al., 2011). Adverse reactions to hIVIG are seen in less than 5% of the patients. They include headaches, chills, nausea, fatigue, myalgia, arthralgia and back pain (Ryan et al., 1996)(Spurlock and Prittie, 2011). Patients with hypertension, diabetes, renal disease, age, hyperviscosity and dehydration are at higher risk for adverse events. More than 90% of hIVIG associated adverse acute renal failures was found to be due to sucrose containing hIVIG preparations (Orbach et al., 2005)(Cheng and Christmas, 2011). Decent safety profile and presence of Aβ reactive antibodies in hIVIG prompted many investigators to carry out clinical trials that were designed to test if hIVIG treatment will be beneficial for AD patients.

Many small published clinical studies have suggested beneficial effects of hIVIG treatment in AD. In a small clinical study comprising of 7 patients with varying neurological disorders, treatment with 0.4 g/kg hIVIG (Octagam) for three consecutive days significantly decreased CSF Aβ levels and elevated serum levels of Aβ after hIVIG treatment (Dodel et al., 2002). In another study by the same group (Dodel et al., 2004), 5 AD patients were treated with 0.4 g/kg hIVIG (Octagam) for three consecutive days every 4 weeks for 6 months. Following hIVIG treatment, AD patients showed 30% decrease in CSF total Aβ levels, 233% increase in serum total Aβ levels and a modest improvement in ADAS-cog (Alzheimer's Disease Assessment scale – Cognitive subscale) scores but no change in MMSE (Mini-mental State Examination) scores. This was the first demonstration that hIVIG treatment can bring about clinical benefits to AD patients. A subsequent open-label phase 1 trial in mild AD patients (Relkin et al., 2009) revealed many interesting clinical aspects of hIVIG treatment. This study comprised of hIVIG treatment for 6 months followed by a 3-month washout period and an additional 9 months of

hIVIG treatment. All 8 patients were initially given a test dose of hIVIG (0.4 g/kg) and then were randomly assigned to one of the doses (0.4 g/kg/2 weeks, 0.4 g/kg/week, 1 g/kg/2 weeks and 2 g/kg/4 weeks). After the first test dose all patients showed significantly higher Aβ antibodies in their serum. The half-life of serum Aβ antibodies was calculated to be 9.3 days which was much shorter than typical IgG (~21 days), suggesting that Aβ antibodies were engaging their endogenous ligands (probably Aβ). After 6 and 9 months of renewed hIVIG treatments, there was a significant decrease in CSF $A\beta_{40}$ and $A\beta_{42}$ from their baselines levels. Increased MMSE scores were also seen during this phase. However, during the 3-month washout period CSF $A\beta_{40}$ and $A\beta_{42}$ levels returned to baseline values and MMSE scores fell. In this study, lower doses of hIVIG were associated with higher MMSE scores than high ones suggesting an inverse dose-response relationship (Relkin et al., 2009). Stability and sometimes even improvement on neurocognitive test scores were also reported in another study (Devi et al., 2008), albeit in a small number of AD patients. Furthermore, data from a subsequent phase 2 trial (Fagan, 2004.) showed treatment effects on brain atrophy, measured by calculating ventricular enlargements rates. Patients receiving hIVIG showed significantly reduced ventricular enlargement rates which also correlated with reduced cognitive decline as measured by CGIC (Clinical Global Impression of Change) and ADAS-cog scores (Relkin et al., 2010). Currently, a phase 3 multicenter clinical trial involving more than 300 patients is underway.

Nearly, 30 years back Imbach and his collegues for the first time demonstrated clinical benefits of high dose IgG in acute ITP patients (Imbach et al., 1981). Since then, clinical usage of hIVIG expanded rapidly but its mechanism of action still remains elusive and mysterious. As only high dose hIVIG showed anti-inflammatory properties, it implied that only a small portion of polyclonal hIVIG were responsible for therapeutic effects. Much of the work done in this regard comes from various mouse models of human antibody-mediated autoimmune diseases such as ITP, RA, CIDP etc. Subsequently, intravenous infusion of mere Fc fragments from hIVIG were also found to be sufficient to treat children with acute ITP (Debre et al., 1993),thereby, implying a role for Fc fragments. Samuelsson and his collegues conclusively demonstrated that Fc fragments of IgG protected mice from experimental immune thrombocytopenia and this protective effect of hIVIG required surface expression of inhibitory FcγRIIB on spleenic macrophages (Samuelsson et al., 2001). Figure 2.5a describes antibody mediated regulation of macrophage activation in health and disease. Furthermore, IgG glycosylation was found to be of necessary for both FcγRIIB binding and anti-inflammatory effects of hIVIG. A single N-linked glycan attached at aspragine 297 in Fc domain of IgG, consisted of a heptameric core with variable branching and terminal sugar residues such as galactose, sialic acid, N-acetylglucosamine and fucose. Nearly, 5% of serum IgG possessed this carbohydrate moiety. Ravetch and his collegues discovered that indeed terminal sialic acid residues of Fc fragments were responsible for anti-inflammatory properties of hIVIG (Kaneko et al., 2006b). Morever, deglycosylation of antibodies in hIVIG by neuraminidase treatment abolished anti-inflammatory effects and as expected upon 20% enrichment with sialylated IgG antibodies, potency of hIVIG to suppress inflammtion enhanced by 10 fold (Kaneko et al., 2006b). Discovery of Fc sialylated IgG antibodies initiated search for its cognate receptor. However, sialylated mouse and human IgG antibodies have less affinity for Fcγ receptors and were found to show high affinity for specific ICAM3-grabbing nonintegrin, related 1 (SIGN-R1) and dendritic cell-specific ICAM-3-grabbing non-

integrin 1(DC-SIGN) receptors. These receptors are present on subset of spleenic macrophages. Consequently, op/op mice, Rag1 $^{-/-}$ mice and spleenectomized mice were not protected by hIVIG from induced experimental arthritis (Bruhns et al., 2003). Further blockade of a receptor, (SIGN-R1) expressed by spleenic marginal zone macrophages completely abrogated anti-inflammatory effects of hIVIG. Similarly, SIGN-R1$^{-/-}$ mice were not protected from induced arthritis either by hIVIG or sialylated Fc fragments (Anthony et al., 2008). Human orthologue of SIGN-R1 is DC SIGN (hDC-SIGN). SIGN-R1 $^{-/-}$ mice when engineered to express human DC-SIGN were both responsive to hIVIG and sialylated Fc fragments. Lastly, hDC-SIGN receptor interaction with sialylated IgG antibodies induced IL-33 in the spleen and blocking IL-33 receptor abolished protection of hIVIG from induced arthritis. IL-33 induces innate Th2 response by inducing IL-4 which in turn induced expression of inhibitory FcγRIIB on macrophages thereby increasing activation threshold for inflammation (Anthony et al., 2011). Thus, it appears that hIVIG exerts its anti-inflammatory effects via a novel innate Th2 mechanism initiated by sialylated antibodies (See figure 2.5b). This pathway explains hIVIG effects only in spleenic macrophages. Interestingly, B cells do not express Sign-R1 and DC-SIGN. hIVIG binds to B cells irrespective of CD22 ligand (terminal sialic acid ligand) and CD22 (expressed by B cells). Also, there are no Fcγ receptors on T cells and only a subset of spleenic and lymph node macrophages express Sign-R1. Yet, hIVIG binds to B cells, Tcells and myeloid cells, presumably interaction with yet to be discovered cell surface receptor/s for hIVIG.

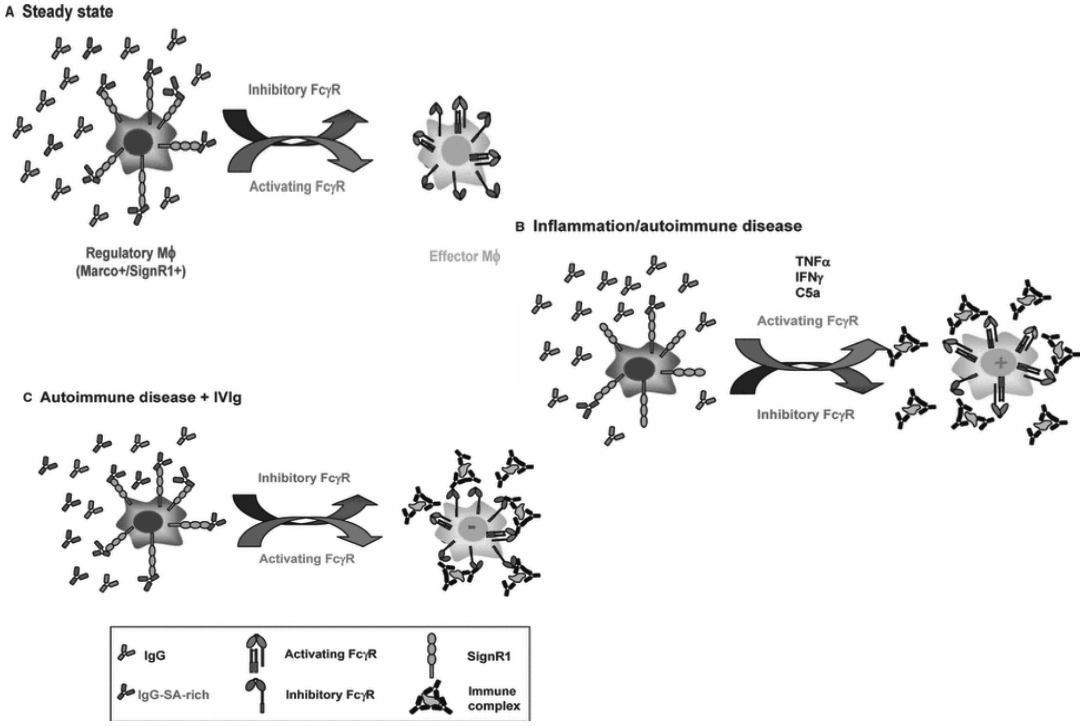


Figure 2.5a. Regulation of macrophage activation by antibodies in health and disease. *Adapted from* Immunological Reviews (2010): 236 :265-275, 15.

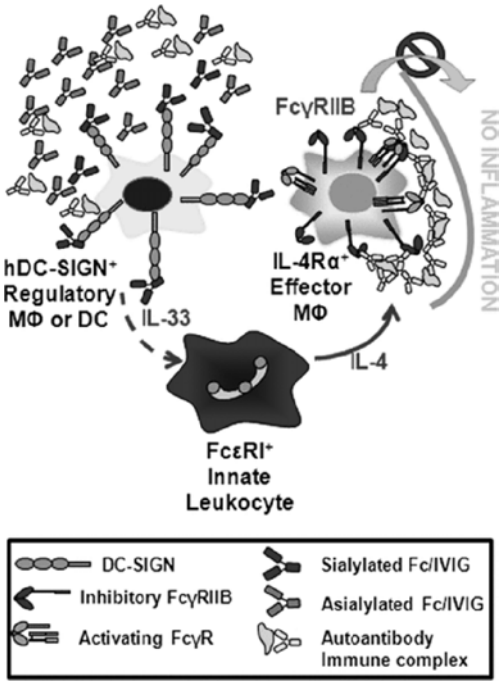


Figure 2.5b. Putative mechanism of action of hIVIG. *Adapted from* Ann N Y Acad Sci. (2012)1253:170-180.

Anti-inflammatory and immunomodulatory functions of hIVIG emerge from many independent and synergistic effects on immune system. The Fab portion mediates antigen binding whereas the Fc portion mediates various effector functions of IgG, which include expression, function and modulation of Fc receptors (Samuelsson et al., 2001); and also inhibition of complement activation (Lutz et al., 1996). Anti-inflammatory effects are also mediated by interference with the cytokine networks (Bendtzen et al., 1993);(Andersson et al., 1993), constituent anti-idiotypic antibodies (Kazatchkine et al., 1994), and by modulation of T- and B-cell activation (Boros et al., 2005). For anti-inflammatory effects, high-dose immunoglobulin by intravenous route was found to be necessary. The mechanism is not yet fully understood but engagement of the Fc portion of IgG with Fcγ receptors on effector cells might be responsible (Vaitla and McDermott, 2010). Also, with higher doses of hIVIG (2 g/kg), inhibition of complement fixation to target cells was proposed as one of the mechanisms working complimentary to microglia mediated changes in cytokine production (Hack and Scheltens, 2004). Neuroprotective potential of hIVIG from complement mediated neuronal death has also been reported (Arumugam et al., 2007). In a different study, same authors also demonstrated capacity of hIVIG to reduce activation of kinases like p38MAPK, c-Jun NH(2) -terminal kinase and NFκB p65 *in vitro* (Widiapradja et al., 2012). In the same study, hIVIG also attenuated Aβ induced accumulation of lipid peroxidation products and upregulated anti-apoptotic protein Bcl-2 in cortical neurons. *In vitro* hIVIG prevented Aβ fibril formation and protected neurons from Aβ induced neurotoxicity (Du et al., 2003). Istrin and colleagues (Istrin et al., 2006) also reported dose- dependent disintegration of fibrillar

$A\beta_{40}$ by hIVIG. Using BV-2 microglial cell line they demonstrated a change in microglial phenotype with ramified morphology along with concomitant increase in CD45 expression. They also reported enhanced microglial phagocytosis of fibrillar $A\beta_{40}$ in response to hIVIG treatment.

In vivo studies of hIVIG in APP mice are scanty. In fact, the current thesis is the only comprehensive report of hIVIG *in vivo* effects in transgenic AD mice. Preliminary studies conducted with Aβ antibody enriched fractions of hIVIG were encouraging. One such study (Wei et al., 2006) showed an increase in serum Aβ levels and also lower CSF Aβ levels in APP mice after treatment with affinity purified Aβ antibodies (extracted from hIVIG). Recently, Dodel et al. reported also improvement in behavioural changes in transgenic AD mice treated with natural Aβ antibodies isolated from hIVIG (Dodel et al., 2011). In the present thesis, study 1 describes the *in vivo* treatment effects of hIVIG in APP/PS1dE9 mice. Various factors like dose of hIVIG, age of mice at treatment onset and treatment duration were considered in the experimental design. In our experiments we set out to find *in vivo* mechanisms of hIVIG in AD mice. Clearly, in the context of neuroinflammatory processes of AD, anti-inflammatory properties of hIVIG may drive inflammation around amyloid plaques towards neuroprotective direction and thereby mediate beneficial effect in AD patients. By preventing the toxicity of amyloid fibrils and modulating microglia function, hIVIG may prove to be helpful. Hence, there is an urgent need to test the mechanisms of hIVIG action in an animal model of AD.

Clinical benefits described in phase 1 and 2 trials prompt for mechanistic explanations for hIVIG effects. In this study, we focused on amyloidocentric as well as amyloid-independent; anti-inflammatory, immunomodulatory effects of hIVIG. In spite of promising initial clinical outcomes, there are many unseen hurdles for hIVIG before it reaches patients. For example, since hIVIG is a pool of polyclonal antibodies obtained from large population of healthy donors, every batch of hIVIG may not be similar with respect to its antibody pool and their biological activities. This variation inherent to vast repertoire of human IgG's might actually limit reproducibility and reliability of any experimental data. However, companies manufacturing hIVIG undertake serious measures to reduce this biological variation between various batches of hIVIG.

In our experimental setup, serious considerations were also needed to the possibility of mice developing neutralising antibodies or anti-human IgG antibodies in response to hIVIG treatment. Such antibodies are not commonly seen in humans and hence this limitation needs to be addressed if clinical implications of the present experimental study to remain valid. Antibodies are not conventionally known to cross the blood–brain-barrier. However, there are specific receptor mediated transport mechanisms like those involving LRP-1, human insulin receptor, transferrin receptor, diptheria toxin receptor and neonatal Fc receptor that can influence antibody movement across blood-brain-barrier (Proulx et al., 2012)(Boado et al., 2007; Deane et al., 2005). Therefore, in order to show any effects inside brain, hIVIG needs to cross this barrier. This is a valid limitation in both humans (clinical studies) and mice (current study). A very small proportion of antibodies in hIVIG is reactive to Aβ; therefore, it will be interesting to see if this is sufficient to detect and opsonise Aβ deposits inside the brain. Furthermore, in our experiment, even if hIVIG crosses the blood-brain barrier and decorates Aβ deposits, the ability of Fcγ receptors present on mouse microglia to detect Fc portions of

human IgG will be under scrutiny, because it will bear implications on microglia mediated phagocytosis. If hIVIG passes all the above hurdles in our experimental setup, reduction in Aβ load should translate into cognitive benefits in mice. For testing cognition in mice, one potential issue is inflammation associated with multiple intraperitoneal injections of human protein during chronic treatment regimens. The abdominal discomfort related to inflammation associated with treatments (hIVIG or saline) can confound any subtle cognitive changes in mice during routine Morris water maze task. Last but not the least, antibody treatments in AD patients and mice were previously associated with vascular bleedings, and thus propensity of microhemorrhages with hIVIG treatment needs to be cautiously evaluated so as to ensure safety. The limitations presented above are mostly related to practical issues concerning our experimental setup.

2.6 NF-KB IN ALZHEIMER'S DISEASE

Several studies point to an important role of NFκB in AD pathology. One study utilising a polyclonal antibody for p65 revealed prominent NFκB immunoreactivity in neurons, their processes, neurofibrillary tangles, and dystrophic neurites of AD patients. In control sections, only weak neuronal staining was noticed. However, this study did not reveal glial immunoreactivity suggesting enhanced expression of neuronal NFκB in areas affected by AD pathology(Terai et al., 1996). In another study, monoclonal p65 antibody was used to stain tissue from histopathologically confirmed AD patients. Among 300 Aβ immunopositive plaques roughly 65% were found to be strongly positive for p65 immunoreactivity. Neurons and astrocytes near the plaques were also positive. Furthermore, p65 staining intensity was correlated with plaque stage (primitive, diffuse, classic) (Kaltschmidt et al., 1997). Involvement of other NFκB family members, p105 and IκBγ, were also found to be elevated in AD brains as compared to age-matched controls (Huang et al., 2005). In addition, several reports identified NFκB in the AD brain tissue as well as in nuclear extracts prepared from AD patients (Boissiere et al., 1997; Lukiw and Bazan, 1998). Furthermore, enhanced expression of IκBα and other NFκB proteins was also found to be associated with neurofibrillary tangles (Yoshiyama et al., 2001). The reactive astrocytes located near the vicinity of Aβ plaques produce many pro-inflammatory mediators like cytokines, chemokines, and iNOS that have been shown to be neurotoxic (Akama et al., 1998; Lee and Brosnan, 1996).Examination of nuclear extracts from AD tissue samples revealed an enhanced NFκB DNA binding, which was shown to be associated with increased COX-2 expression, thus directly linking inflammation to NFκB signalling (Lukiw and Bazan, 1998).

Molecular mechanisms implicating a role of NFκB in AD can be understood from its functional relationship with APP, Aβ, BACE, γ- secretase, RAGE, and Pin 1. In brains of AD patients, both NFκB p65 expression and BACE1 levels were found to be increased. Further, NFκB signalling regulates BACE expression and APP processing, thereby implicating a central role for NFκB in AD(Chen et al., 2011) .In rat primary neurons and post-mitotic neuronal cells, stimulation with Aβ40 resulted in dose-dependent neuronal damage, reduction of anti-apoptotic protein Bcl-XL and selectively induced translocation of NFκB p65 and p50 subunits. Furthermore, these Aβ40 mediated effects were inhibited by a kappaB decoy, acetylsalicylic acid (aspirin) and a selective IKK-2 inhibitor (Valerio et al., 2006). Also, certain NSAIDs like indomethacin have been shown to suppress NFκB resulting in reduction of Aβ in mice (Sung et al., 2004). Therefore, NFκB signalling may modulate production, deposition and clearance of Aβ.

NFκB has been shown to play a role in transcriptional regulation of the *APP* gene. p50 homodimers as well as p50/p65 and p50/c-Rel heterodimers interact with specific sequences located in 5′ regulatory region of the *APP* gene and act as transcription activators (Grilli et al., 1995). Similar NFκB sites have also been found for presenilin and BACE, with NFκB acting as repressor for BACE in non-activated neurons and astrocytes. However, NFκB acts as transcription activator for *Bace1* in activated astrocytes and Aβ-exposed neurons (Bourne et al., 2007). These findings suggest a dual role of NFκB in regulating *Bace1* transcription in cell specific manner. Furthermore, in APP23 mice, deletion of *Tnfrsf1a* gene resulted in > 70% reduction of Aβ pathology, which was further shown to be due to decreased BACE1 levels and activity (He et al., 2007). Subsequent investigations with potent NFκB inhibitors

demonstrated significant reduction in TNF induced *Bace1* promoter activity, indicating that NFκB plays a significant role in regulating transcription of *Bace1* (He et al., 2007).

Gamma-secretase is a critical enzyme for processing APP and to generation of Aβ protein. Presenilin 1, presenilin 2, nicastrin, Aph-1 and Pen-2 are the major constituents of this intramembrane protease (Iwatsubo, 2004). Local and systemic modulation of γ- secretase was reported to induce NFκB induction along with pro-inflammatory molecular mediators like TNF and COX-2 (Nasoohi et al., 2012). Inhibitors of γ-secretase or Notch RNA interference reversed Notch hyperactivity that was typically associated with *Nfkb1*$^{-/-}$ mice. Furthermore, Notch 1 was shown to be an important upstream regulator of NFκB (Schwarzer et al., 2012), thus implying a significant role of NFκB in Notch mediated effects of γ-secretase inhibition. In macrophages following LPS stimulation, treatment with γ-secretase inhibitors prevented activation of Notch signalling, thereby diminishing NFκB mediated inflammatory activity (Monsalve et al., 2009). Therefore, NFκB activity is susceptible to functioning of γ-secretase, which is also the key enzyme for generation of Aβ peptide in AD.

Aβ and tau proteins are known to undergo non-enzymatic glycation and form AGEs (Advanced Glycation End products). Binding of AGEs to Receptors for Advanced Glycation End Products (RAGEs) can initiate NFκB dependent gene transcription (Granic et al., 2009). AGEs also mediate release of cytokines like IL-1β and TNF, which in turn can further activate NFκB signalling (Granic et al., 2009). Levels of pro-inflammatory cytokines are increased in AD, and among these, TNF has been shown to activate NF-κB, which can be critical for neuronal survival (Kaltschmidt and Kaltschmidt, 2009).

Another protein, Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1), has been shown to bind both Aβ and phosphorylated tau proteins, stabilising them in their non-pathogenic functional conformations (Lu and Zhou, 2007). Pin1 has been also shown to bind phosphorylated p65 upon TNF-α treatment, which subsequently results in increased NFκB activation. Pin1 also inhibits ubiquitin-mediated proteolysis of p65 by SOCS1 (suppressor of cytokine signalling-1). Further, Pin1 binding to p65 inhibits IκBα from binding p65, which means more transcriptional activity of NFκB and promotion of neuronal survival (Ryo et al., 2003).

All mechanisms described above show complex consequences of NFκB activation in different physiological and pathological contexts like those related to memory, detrimental inflammatory responses and neuroprotective and cell survival capabilities. All these diverse functions are also tightly regulated by NFκB family transcription factors.

Pharmacological inhibition of NFκB in transgenic AD mice has yielded some conflicting data. Pyrrolidine dithiocarbamate (PDTC) is a well-known NFκB inhibitor with anti-inflammatory (Cuzzocrea et al., 2002) and antioxidant properties in several models of CNS injury (Nurmi et al., 2004; Ahtoniemi et al., 2007; La Rosa et al., 2004). In rats, neuroinflammation induced by intracerebroventricular injection of Aβ were attenuated by PDTC and further conferred cognitive benefits (Cheng et al., 2006). Intraperitoneal daily treatment of APP/PS1dE9 mice with PDTC 50 mg/kg for 5 months suppressed TNF and COX-2 induction and attenuated astrogliosis. However, treatment also resulted in elevation of

brain Aβ42 burden (Zhang et al., 2009). In the same AD mouse line, however, treatment with PDTC (20 mg/kg in drinking water) for 7 months conferred spatial memory benefits via Cu+2 – activated Akt pathway with no accompanying changes to amyloid burden (Malm et al., 2007). Treatment with nonsteroidal anti-inflammatory drugs like indomethacin and flurbiprofen, which are known to reduce NFκB activity, showed marked reductions in Aβ pathologies in mice (Eriksen et al., 2003; Sung et al., 2004). Effects on amyloid and inflammatory pathologies can also be studied in genetic mouse models deficient in key components of the NFκB pathway, such as *Nfkb1* knockout mice.

2.6.1 Gene deletion of *Nfkb1 and* p50 subunit deficiency

NFκB p65/p50 dimer is more common than any other homo- or heterodimers. It is also the major DNA binding dimer in the normal adult rodent brain (Kaltschmidt and Kaltschmidt, 2009). Therefore, it looks temepting to either genetically remove p65 or *Nfkb1* to block canonical NFκB pathway. Actually, p65 gene deletion is lethal for mice. However, gene deletion of *Nfkb1* (precursor protein for p50) produces viable mice (Sha et al., 1995)that are deficient in both p105 and p50, and hence provide an opportunity to study functional and pathological consequences of inhibiting NFκB signalling in health and disease. Precursor protein p105 is processed by proteolytic mechanisms mediated by proteasome. Constitutively most cells maintain equal levels of p105 and p50. However, upon stimulation complete degradation of p105 ensues, thereby releasing all bound Rel subunits to freely translocate into nucleus. Typically, p105 can retain p65, p50 and c-Rel but not RelB in the cytoplasm(Beinke and Ley, 2004). In fact, in mice that lack p105 precursor and IκBγ but express p50 (targeted deletion of COOH-terminal ankyrin domain of p105) indicate that p105 has a regulatory role on p50 homodimer activity and deregulated p50 homodimers in such p105-/- mice might act as transcriptional activators or repressors depending on the cell type. Thus, p105 might mainly function to retain p50 dimers in the cytoplasm (Ishikawa et al., 1998).

The precursor protein p105 also binds to Tpl-2 with high affinity. Tpl-2 functions as MAP-3 kinase that phosphorylates and activates the ERK-1/2 kinase MEK-1/2 kinase. Tpl-2 deficient macrophages display dramatically reduced expression of TNF and COX-2 when stimulated by LPS by virtue of defective ERK-1/2 activation (Waterfield et al., 2003). Furthermore, *Tpl-2* knockout mice are resistant to LPS induced endotoxin shock, suggesting an important role for Tpl-2 in both innate and adaptive immune responses. Furthermore, p105 maintains Tpl-2 protein stability (p105 mice show very low levels of Tpl-2), and binding of p105 also inhibits Tpl-2 MEK kinase activity (Beinke et al., 2003). It was shown that upon LPS stimulation IKK complex also regulates ERK activation via p105 as illustrated in Figure 2.6a (Beinke and Ley, 2004). Therefore, one has to be cautious when interpreting molecular phenotype of these *Nfkb1* null mice, as deletion of *Nfkb1* also results in secondary deficiency of proteins like Tpl-2, ABIN-2 causing combined defects in both NFκB and Tpl-2-MEK-ERK pathway.

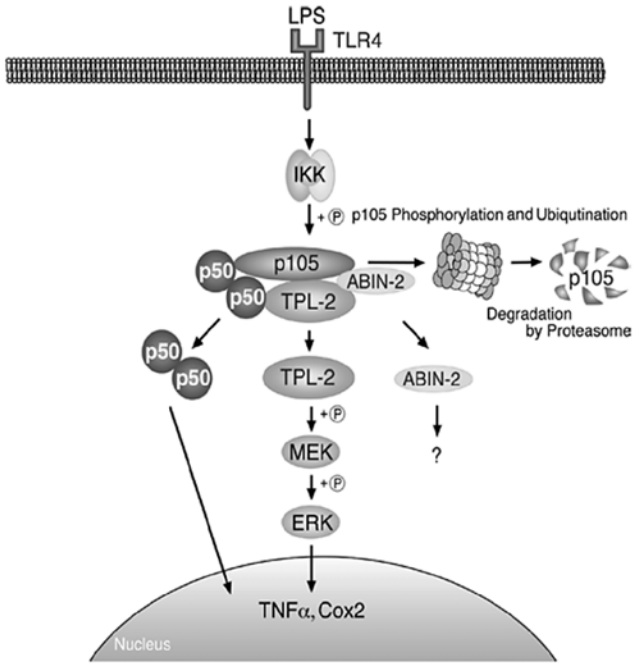


Figure 2.6a. NFκB precursor protein p105 is involved in multiple downstream pathways leading to inflammatory gene exprssion.

Mice deficient in p105 do not produce p50 and display multifocal defects in immune system (Sha et al., 1995). These mice fail to mount a normal humoral response to T-cell dependent antigens. In *Nfkb1* null mice, the same antigen can elicit different patterns of cytokine induction in different cell types. These mice are susceptible to intracellular protozoan parasite *Leishmania major*. Their parasitic lesions fail to resolve, owing to defective Th1 responses. *Nfkb1* null mice are also resistant to both collagen-induced (chronic) arthritis and methylated BSA/IL-1-induced (acute) models of arthritis. In contrast, intragastric infection with *Helicobacter hepaticus* causes more severe colitis in these mice owing to overproduction of pro-inflammatory cytokines in the gut(Beinke and Ley, 2004; Beinke et al., 2003). Homodimers of *Nfkb1* lack transactivation domains and may act as repressors of gene transcription. Therefore, in *Nfkb1* null mice some effects may arise, in addition to loss of *Nfkb1* function, also as a result of depression of NFκB target genes (Kaltschmidt and Kaltschmidt, 2009). Cortical neurons of *Nfkb1* null mice distinctly exhibited reduced neuritic branching, loss of varicosities and Notch 1 signalling hyperactivation. Treatment with γ-secretase inhibitor or Notch RNA interference reversed abnormal cortical morphology, suggesting cross-talk between NFκB and Notch signalling (Bonini et al., 2011). Lack of *Nfkb1* gene also severely affected the survival of newborn cells in dentate gyrus implicating *Nfkb1* gene functioning in survival of new neurons of dentate gyrus (Denis-Donini et al., 2008).

Mice with *Nfkb1* gene deletion can provide us with a unique opportunity to study the effects of blocking canonical NFκB pathway, albeit with certain caveats. Studies investigating Aβ and neuroinflammatory mechanisms of AD in these genetically manipulated mice may further our understanding of the role of NFκB in AD pathogenesis.

3 Aims of the study

The main objective of this study was to explore the therapeutic potential of experimental immunomodulation in the pathogenesis of Alzheimer's disease using experimental mouse models. More specific aims of the thesis are listed below:

1. To investigate in transgenic mouse model, the hypothesized mechanisms of action behind the beneficial effects of human polyclonal intravenous immunoglobulin (hIVIG) treatment observed in early phase AD clinical trial patients? (hIVIG study)

2. To assess the effect of hIVIG treatment on amyloid and inflammatory pathologies and cognition in a mouse model of Alzheimer's disease (hIVIG study)

3. To determine the role of *Nfkb1* gene on amyloid and inflammatory pathologies of genetically engineered APP/PS1 mice (NFκB study)

4. To study the interaction between chronic neuroinflammation caused by amyloid pathology in APP/PS1 mice and acute neuroinflammation induced by a local LPS injection, and the role of NFκB *Nfkb1* subnit in the inflammatory responses (NFκB study)

4 Materials and methods

4.1 ANIMALS

We employed two different genetically engineered mouse lines in our studies. To model AD we used APPswe/PS1dE9 (APP/PS1) mice that harbor human APPswe mutation (K595N and M596L) and human *PSEN1* with deletion of exon 9 co-integrated in the same transgene (Jankowsky et al., 2004). The mice were backcrossed for nine generations to C57BL/6J mice. APP/PS1 mice develop progressive amyloid pathology beginning as early as 4 months of age (Garcia-Alloza et al., 2006) and cognitive deficits around 12 months of age (Minkeviciene et al., 2008). Nontransgenic wild-type littermates (referred to as wild-type mice) were used as controls.

To study the role of NFκB in neuroinflammation we purchased mice deficient in *Nfkb1* gene (p105) from Jackson laboratories (Sha et al., 1995). These mice were backcrossed to the C57BL/6J strain for 6 generations and maintained as homozygotes. We crossed *Nfkb1* knockout mice with APP/PS1 mice and established mouse lines carrying mutated *APP* and *PSEN1* transgenes on *Nfkb1* null (*Nfkb1*$^{-/-}$) or wild-type (*Nfkb1* wild-type) background. These novel mice were viable and did not show any overt physical or behavioural defects at birth. Homozygous *Nfkb1* wild-type carriers (either on APP/PS1 or wild-type for this transgene) served as controls. The housing conditions (National Animal Center, Kuopio, Finland) were controlled (temperature +22°C, light from 07:00 to 19:00; humidity 50-60%), and fresh food and water were freely available. The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

4.2 EXPERIMENTAL DESIGN

Our hIVIG study comprised of four independent experiments, each of which differed with respect to age of mice at onset of treatment and treatment duration. The source of hIVIG used in this study was commercially available Gammagard Liquid (Baxter A/G, Austria). Table 4.2a gives summary of all the experiemnts in this study and table 4.2b details all the parameters assessed in this study. Below are the details and design of individual experiments.

Experiment 1 (IVIG-1): Four-month-old APP/PS1 mice received a single injection of either 0.1 g/kg (low dose) or 1 g/kg (high dose) of hIVIG intraperitoneally. Mice were sacrificed either one day or one week after hIVIG injection (N = 6 in each group). A separate group of mice received three weekly injections of hIVIG at the high dose (N=8). The control group received 0.01 ml/g of saline. Mice received ~ 0.3 ml of either hIVIG solution or normal saline. Concentration of Aβ in the brain and serum was analysed by ELISA. Microglial reactivity was assessed by CD45 immunoreactivity. Table 4.2a gives more details about this experiment. Development of anti-human IgG antibodies in serum was assessed from the hIVIG group that had 3 weekly injections (group 6).

Experiment 2 (IVIG-3): Aged mice (14-months) were treated with bi-weekly intraperitoneal injections of either highdose hIVIG (N = 18), control IgG (N = 8) or saline (N= 15) for 3 weeks. Control IgG is hIVIG depleted with anti-Aβ antibodies. Acute treatment effects on spatial memory were assessed by the Morris water maze task. Mice were sacrificed at the age of 15 months a week after they received the last injection. Development of anti-human IgG antibodies was assessed from serum samples. Amyloid and microglial pathologies were evaluated in histological sections. Vascular microbleedings were assessed by Perl's hemosiderin staining.

Experiment 3 (IVIG-6a): Young (4-months) APP/PS1 mice received once weekly intraperitoneal injection of either high dose hIVIG (N = 16) or saline (N = 14) for 3 months. After testing cognitive functions, mice were sacrificed a week after last injection. Development of anti-human IgG antibodies was assessed in serum samples. Brain sections were evaluated for amyloid and microglial pathologies. See table 4.2b for more details.

Experiment 4 (IVIG-6b): Young (4-months) APP/PS1 mice received once weekly intraperitoneal injection of either high dose hIVIG (N = 14-16) or saline (N = 15) for 8 months. After carrying out behavioural tests for cognition, mice were sacrificed a week after last injection. Development of anti-human IgG antibodies was assessed in serum samples. Brain sections were evaluated for amyloid and inflammatory pathologies.See table 4.2b for more details.

Table 4.2a. Summary of experiments in hIVIG study.

Experiment	Group	Treatment	Dose	Injection schedule	sacrifice	N
IVIG-1 4-month old APP/PS1 mice	1	Saline	10ml/kg	1	1 day after last injection	6
	2	hIVIG	0.1g/kg	1	1 day after last injection	6
	3	hIVIG	0.1g/kg	1	1 week after last injection	6
	4	hIVIG	1g/kg	1	1 day after last injection	6
	5	hIVIG	1g/kg	1	1 week after last injection	6
	6	hIVIG	1g/kg	Once weekly; 3 weeks	1 day after last injection	8
IVIG-3 14-month old APP/PS1 mice	1	Saline	10ml/kg	Twice weekly; 3 weeks	1 week after last injection	15
	2	hIVIG	1g/kg			18
	3	Control IgG	1g/kg			8
IVIG-6a 4-month old APP/PS1 mice	1	Saline	10ml/kg	Once weekly; 14 weeks	1 week after last injection	14
	2	hIVIG	1g/kg			16
IVIG-6b 4-month old APP/PS1 mice	1	Saline	10ml/kg	Once weekly; 40 weeks	1 week after last injection	15
	2	hIVIG	1g/kg			16

Table 4.2b Summary of tests/analyses carried out in hIVIG Study

Tests/analyses	IVIG-1	IVIG-3	IVIG-6a	IVIG-6b
Behavioural Tests				
Marble test				X
Exploratory activity				X
Novel objection recognition				X
Morris water maze		X	X	X
ELISA measurements				
Anti-human IgG antibodies		X	X	X
Soluble $A\beta_{40}$	X	X		X
Insoluble $A\beta_{40}$	X	X		X
Soluble $A\beta_{42}$	X	X	X	X
Insoluble $A\beta_{42}$	X	X	X	X
Serum $A\beta_{40}$	X	X	X	X
Histology				
Congo Red staining			X	X
microhaemorrhage staining		X		
Immunohistochemistry				
Anti-human IgG	X	X	X	X
Amyloid beta		X	X	X
CD45	X	X	X	X
Iba-1a				X
CD68				X
GFAP				X
Confocal analysis of microglia				X
Doublecortin				X
RT-PCR analysis for *Tnf* and *Il1b*				X

Our NFκB study comprised two individual experiments. In both experiments, we used our novel mouse line deficient in *Nfkb1* gene on APP/PS1 background (*Nfkb1*$^{-/-}$). Mice with intact *Nfkb1* gene on APP/PS1 background (*Nfkb1* wild-type) served as littermates controls. The two experiments differed from each other with respect to chronic stimuli arising from age-dependent Aβ deposition and acute stimuli from LPS injection. Table 4.2c gives summary of all the experiments in NFκB study and table 4.2d details all the parameters assessed in this study.

In the first experiment (age series), we followed *Nfkb1*$^{-/-}$ and *Nfkb1* wild-type mice (both on APP/PS1 background) for progression of amyloid and microglia pathologies across three ages: 3.5, 8 and 12 months. Mice from the last cohort also participated in behavioural tests to reveal any possible abnormal behavioural phenotypes.Availability of mice of either genotypes and sex was considered carefully. Female APP/PS1mice were used at 3.5 months age because they devlop higher magnitude of amyloid pathology much earlier than male

mice. Also, at 12 months age, subtle effects of *Nfkb1*$^{-/-}$ on amyloid pathology might be easier to detect in male APP/PS1 mice rather than in female mice with robust amyloid pathologies. Table 4.2c provides further details.

In our second experiment (LPS injection), we gave intrahippocampal LPS and saline injections to *Nfkb1*$^{-/-}$ and *Nfkb1* wild-type mice (both on APP/PS1 background). Mice were sacrificed 7 days after injections and their brains were evaluated for Aβ and microglial pathologies using immunohistochemistry.

Table 4.2c. Summary of experiments in NFκB Study

Experiment	Age (months)	Sex	N for each genotype			
			APP/PS1 Wild-type & *Nfkb1* wild-type	**APP/PS1 Wild-type & *Nfkb1* $^{-/-}$**	**APP/PS1 & *Nfkb1* wild-type**	**APP/PS1 & *Nfkb1* $^{-/-}$**
Age series	3.5	Females			11	11
	8	Males			7	8
		Females			11	6
	12	Males	14	9	15	8
LPS injection	12	Female			10	5

Table 4.2d. Summary of tests/analyses carried out in NFκB Study

Tests/analyses	Age series			LPS Injection
	3.5 months	8 months	12months	
Behavioural Tests				
Marble test			X	
Exploratory activity			X	
Rotarod test			X	
Elevated plus maze			X	
Morris water maze			X	

ELISA measurements				
Soluble $A\beta_{40}$				
Insoluble $A\beta_{40}$		X		
Soluble $A\beta_{42}$			X	
Insoluble $A\beta_{42}$	X	X	X	
Congo red staining			X	
Immunohistochemistry				
Amyloid beta	X	X	X	X
CD45		X	X	
Iba-1a			X	X
CD68			X	X
RT-PCR analysis for *Tnf* and *Il1b*			X	

4.3 BEHAVIOURAL TESTS

Mice were caged individually and were allowed to adopt to laboratory conditions for about a week before initiating any behavioural tests. During this time the person running the behavioural tests got acquainted with the mice, and *vice versa*, and the mice got used to human handling. We used clean tissue paper for mouse handling. Also the person handling the mice were advised not to apply any strong deodorants or perfumes, and care was taken that their clothes did not emit strong odours (e.g. garlic, ginger, other food materials). A placid mouse comfortable feeling mouse will not bite or elicit a quick escape response (jump) when placed smoothly on handler's palm but rather will leisurely explore restrain boundaries in order to find exit route towards its home cage. During the first day of handling almost every mouse jumped off the palm or tried to bite, or defecated and urinated. However, over 2-3 days they got used to human handling. Loud noise, startling sounds, or pungent smells from adjoining rooms were taken into account before beginning the behavioural tests. Any abnormal events encountered during the tests, either related to mice or test system, were documented for future reference.

4.3.1 Marble burying test

This is a very simple test for neophobia, where one can easily spot anxious mice in a cohort. Mice were caged individually and the cage was filled with double bedding. Nine glass marbles (diameter 1 cm) were arranged in a 3 x 3 array (figure. 4.1-A) and left overnight in the cage. During the night the mouse will typically hide the marbles under the bedding. Next morning, the number of visible marbles was counted. A marble with less than 10% of its visible surface were considered hidden. An anxious mouse should cover more marbles than a mouse that is at ease with the novel objects. However, a highly anxious mouse will not touch any of the marbles. Another task confounder is that some mice carry marbles to their nests.

4.3.2 Exploratory Activity

Mice were tested in a dimly lit and quiet room. Observation cages (26 cm x 26 cm x 39 cm, TruScan® Coulbourn Instruments, CO, USA) equipped with two rings of photodetectors

enabled separate monitoring of horizontal (XY-move time) and vertical activity (rearing). Attached computer software detected spontaneous locomotor activity of the mice and computed various parameters such as ambulatory distance, time engaged in stereotypic movement and number and time of vertical entries (rearing). After gently placing the mouse in the observation cage, the investigator left the room. Activity was measured for 10 min (figure 4.1-B). At the end, mouse was gently returned to its home cage. Faeces /urine were removed and the cage floor was cleaned with 70% alcohol before placing the next mouse in the test cage. Ambulatory distance, rearing time, and stereotypy time were measured.

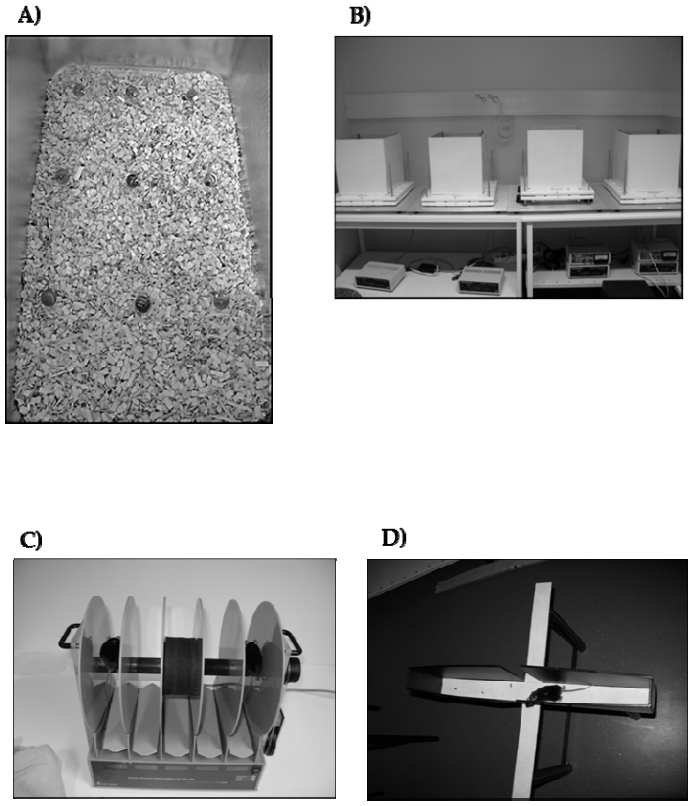


Figure 4.1. **(A)** Mouse home cage displaying arrangement of marbles. **(B)** Test chambers for monitoring exploratory behaviour of mouse. **(C)** Rotarod apparatus with mouse on trial. **(D)** Elevated plus maze set up for testing anxiety in mice

4.3.3 Rotarod test

Motor coordination of mice was tested by using the Rotarod apparatus, which consists of a horizontal rotating rod with vertical dividers (figure 4.1-C). Test was carried out in a quiet and well-lit room. The mouse was gently placed on the rod facing away from the investigator and allowed to adopt to the stationary rod for 30 s. For the next 30 s, the rod revolved with a

constant speed to let the mouse get accustomed to a rotating rod. Then, for the next 8 min, the speed of rotation was increased stepwise to challenge the mouse's balance. The test ended when the mouse fell off the rod or when a cut-off time of 8 min had passed. The apparatus was cleaned of faeces /urine stains with 70% alcohol before placing the next mouse on the rod.

4.3.4 Elevated plus maze

Fear and anxiety of mice was assessed by elevated plus maze. The maze comprised a plus-shaped elevated platform (40 cm from floor), with two opposite arms open (length 30 cm, width 5 cm, ledge 0.25 cm) and two opposite arms enclosed with high walls (30 cm x 5 cm x 15 cm). All four arms merged into a square platform (5 cm x 5 cm) in the center (figure 4.1-D,). A camera mounted on top allowed the investigator sitting in adjacent room to monitor the movements of mice on the maze. This test was carried out in a quiet, dimly lit room. The mouse was gently placed on the maze centre, and the investigator left the room. Each mouse was allowed to explore the maze for 5 min. Mice typically spent most of the time in the closed arms and made only short visits to the open arms. Highly anxious or stressed mice would remain in the closed arms, while mice treated with anxiolytics would spend more time exploring the open arms. The number of visits and percentage of total time spent in open and closed arms were calculated and reported. Data from only those mice making more than 5 moves between arms during the 5-min test period were included in the statistical analysis.

4.3.5 Novel object recognition test

To test recognition memory, we used novel object recognition task which is based on the spontaneous tendency of mice to preferentially explore novel objects. A pair of exactly similar looking objects were kept overnight in the home cage, leaving the mouse with enough time to explore and acquaint themselves with the objects. Next morning the objects were removed for 4 h before the commencement of actual test. The Test was carried out in a well-lit room. In the actual test, a new pair of objects was introduced into the mouse cage. Among these two objects, one was identical to the object introduced during the previous night while the other one was novel. The number of visits to explore the object was recorded. The test was repeated in total with three different pairs of objects during 4 successive days.

4.3.6 Morris water maze

Morris swim task ('water maze') is a classic test for assessing spatial learning and memory in rodents. This test was carried out in a quiet and well lit-room. The apparatus consists of a black cylindrical plastic water tank (Figure 4.2-A) of 120 cm diameter. A black square escape platform (14 cm x14 cm) was located 1 cm below the water surface. The water temperature was maintained at 21°C. The pool was filled on the evening before the scheduled test day.

Two days before the actual test, mice received alley training. The mice were gently dropped into water at one end of a black plastic alley (10 cm x 14 cm x 25 cm). A hidden platform (below surface of water) was placed half-way inside alley. This way the mice learned to climb onto the platform and find that it provided an escape from the water. If the mouse did not find the platform in 20 seconds, it was gently guided towards the hidden platform. The mouse was allowed to stay on the platform for 10 s before it was returned to

their home cage. Each mouse received 5 daily trials for two days. The mouse was gently dried with a paper towel and kept under a heater before replacing into the home cage to prevent hypothermia.

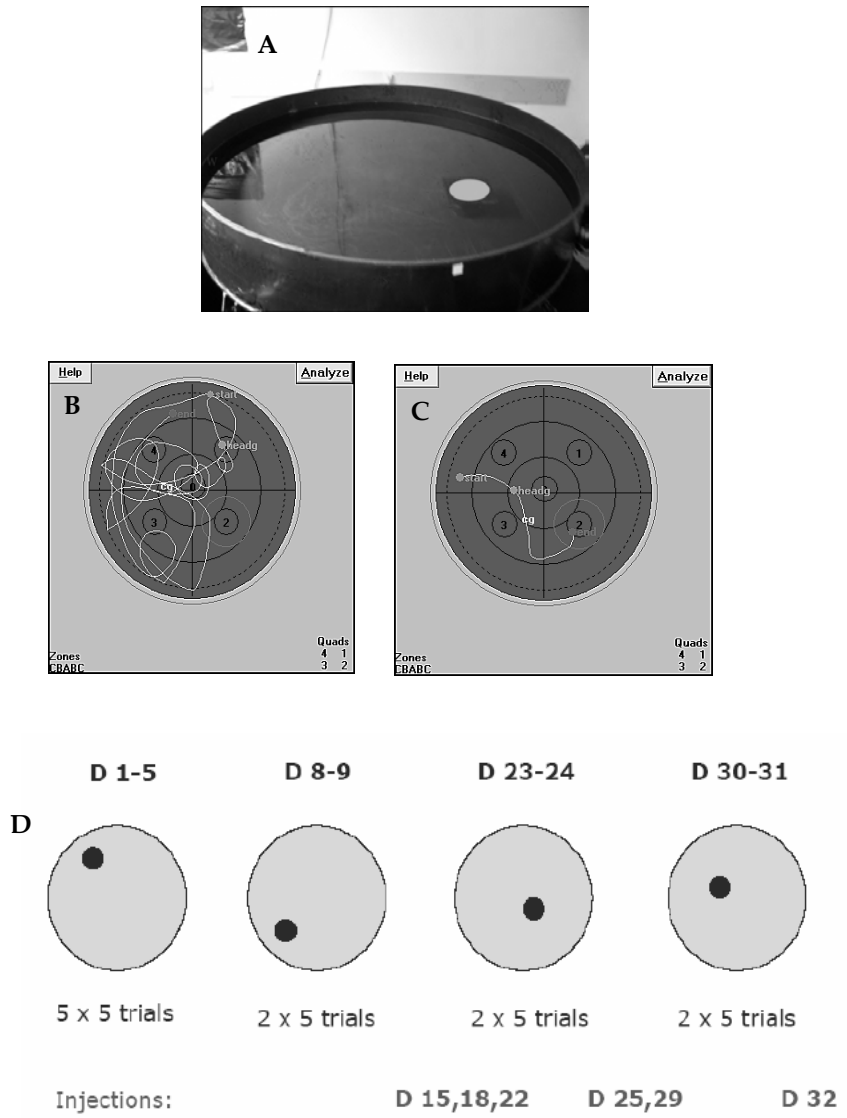


Figure 4.2. Morris water maze set up **(A)** for testing spatial memory in mice. **(F)** Swim tracks of mouse that did not learn to find platform and that of mouse **(C)** that remembered to locate the platform. **(D)** IVIG-3 spatial memory test protocol**.** First 5 days, each mouse received 5 trials/day during which they learned to remember the platform location. On day8-9, platform was moved to new location, mice had to learn the new platform location. Tretament begins and acute effects on memory were evaluated on day 23-24 and day 30-31.

In the learning phase of the task, each mouse received 5 trials per day for 5 days. Each trial started from one of the four randomly selected locations on the pool periphery (North, East, West, and South), so that the mouse was facing the wall. The position of the hidden platform was fixed during all trials on the first 5 days. If the mouse failed to find the platform within 60 s, it was gently guided towards it. After reaching the platform, the mouse was let to stay there for 10 s before transferred to warm waiting area to wait for the next trial. A ceiling camera connected to a video tracker recorded the swim pattern which was recorded and analyzed by a computer software (HVS Image, Hampton, UK) Typically the mice were tested in groups of five, so that each mouse got at least 5-minutes of rest between the trials. On first day, it took for the mouse long to reach the platform (figure 4.2-B), but by day 5 all mice learned to reach the platform by taking the shortest route (figure 4.2-C). In IVIG-6 experiments, day 5 was a probe trial without the platform to determine the search bias of the mouse. A target zone was defined as a 30 cm diameter circle centered on the previous platform location. Since target zone comprised 6.25% of the total surface area of tank, in a probe trial of 60 s, a randomly swimming mouse would spend 3.75 s in the target area. If significantly exceeding this time, it was considered to have a learned search bias. Escape latency, swim path length, swimming speed, and the time spent in target zone (vicinity of platform area) during probe trial were determined by the HVS software.

In the IVIG-3 experiment, a more complicated test protocol was applied (figure 4.2.D). We first let the mice to learn the location of the hidden platform during 5 daily trials over 5 days as described above. Instead of the probe trial without the platform, the platform was moved to a new location on days 8 and 9 to assess learning of a new platform location while the task principle should already be familiar to the mice. All this was first done without any treatment. After three weeks of hIVIG treatment we tested learning to two more new platform positions over two days (days 23-24 and 30-31). This protocol was successfully applied to testing the impact of amyloid plaque deposition in very old APP transgenic mice (Chen et al., 2000).

4.4 INTRAHIPPOCAMPAL LPS AND SALINE MICROINJECTIONS

Under general anesthesia (halothane, 4% for induction, 1.3 % for maintenance). the mouse was placed in a stereotactic apparatus (David Kopf, model 940, Tujunga, CA, USA). The level of inhaled gases was monitored on capnometer (Capnomac Ultima, GE healthcare, UK; O_2: 25-26% ; N_2O: 68%). A rectal thermometer probe was inserted and body temperature was maintained with the aid heating pad. The skull was exposed and a small burr hole was drilled on the skull. The injection needle connected to a Hamilton (Reno, NV, USA) syringe was aimed at the following coordinates: A/P -2.7 mm and M/L ± 3.0 mm from bregma, and , D/V -2.7 mm from dura. After inserting the needle to the desired D/V coordinates, we waited for 30 s before beginning the injection at a rate of 0.2 µl/min. A total volume of 1 µl is injected during 5 min. After finishing the injection we waited for 2 min before slowly pulling the needle up. The injections were made bilaterally. The left hippocampus received saline and the right hippocampus received 4 µg/µl LPS (Sigma-Aldrich St. Louis, MO, USA; L6511) injection. The wound was then cleaned and sutured. The animal was allowed to recover in an incubator overnight. Mice did not receive any post-operative analgesic drugs.

Female mice (~12 months age) from *Nfkb1* wild-type (N=10) and *Nfkb1*-ko (N = 5) genotypes under APP/PS1 background were used. A week after injections mice were sacrificed and their brains were retrieved for routine immunohistochemistry.

4.5 SAMPLING PROCEDURES

On the day of sacrifice, mice were weighed. They were euthanized with excess dose of pentobarbiturate-chloralhydrate (Equithesin i.p injection, ~0.3 ml). In the hIVIG study, terminal blood samples by cardiac puncture (~0.4 ml or more) were collected into Eppendorf tubes (Sarstedt,Numbrecht, germany) and stored on ice (no more than 3 h), centrifuged at 9000 rpm for 20 min(Eppendorf centrifuge 5415D,NY,USA), after which the serum was separated and stored in a -70 °C freezer. Roughly, 50µl was collected for estimation of antihuman IgG antibodies and rest was used for ELISA measurements of serum $A\beta_{40}$. In IVIG-6b experiment, additional blood sample was taken from mice at 7.5 month age by saphenous vein puncture method for estimation of anti-human IgG titres.

After terminal blood sampling, the mouse was transcardially perfused with ice-cold saline (0.2 ml heparin added) for 7 min. The brain was removed, divided into left and right hemibrains, and the left hemibrain including the intact olfactory bulb and cerebellum was cut on a ice cold iron block. Hippocampus was retrieved and three cortical blocks dissected (frontal, parietal and temporal) and stored at -70 °C. The right hemibrain was immersion fixed in a freshly prepared 4% paraformaldehyde for 4 h, transferred to 30% sucrose solution overnight and further transferred to an antifreeze solution where it was stored at -20 °C. For the hIVIG study, coronal sections of 35 µm were cut with a freezing slide microtome, while for the NFκB study, brains were cut with a cryostat into 20µm thick coronal (LPS injection experiment) or sagittal (age series) sections.

4.6 IMMUNOHISTOCHEMISTRY

Following systematic random sampling scheme, four free-floating sections 400 µm apart from each other, starting from septal hippocampus (-1.3 mm from bregma) were collected for various immunostainings. Similarly, six to seven sections each 210 µm apart starting from septal hippocampus (-1.3 mm from bregma) were collected for doublecortin immunostaining. For NFκB study, four to six sections at 400 µm intervals per animal were sampled. All immunostainings were made with free-floating sections except for LPS experiment in NFκB study were immunostaining was performed on mounted sections. Briefly, free floating sections were washed in phosphate buffered solution (PBS) overnight. Next day, after washing in PBS, sections were treated with 0.3 % H_2O_2 in methanol for 30 min to block endogenous peroxidase. For Aβ immunostaining, sections were additionally boiled with 0.05 M sodium citrate (85 °C) for 30 min after which they were cooled in PBS. Free-floating sections were later blocked with 10 % normal goat serum for 30 min. After washing with TBS-T (Tris-buffered saline with triton) for 5 min, sections were incubated overnight with primary antibodies at room temperature as shown in Table 4.2. After ~18 h of incubation, sections were washed in TBS-T and incubated for 2 h with corresponding biotinylated anti-rat, anti-

mouse, anti-rabbit, and anti-goat secondary antibodies (1:500), followed by incubation with horseradish peroxidise (HRP) labelled streptavidin. Color was developed by reacting nickel diaminobenzidine (Sigma) with hydrogen peroxide for 3 min. Reaction was stopped with phosphate buffer and sections were then mounted, air-dried, cleared in xylene and cover slipped in Depex mounting medium. All staining batches had control sections that followed the same procedures except for omitting primary antibodies.

For LPS experiment, four to six 20 μm thick coronal sections at 400 μm intervals from each animal were mounted on superfrost slides. After blocking endogenous peroxidase with H_2O_2 and blocking with normal goat serum, slides were incubated overnight in primary antibodies: Iba1 (1:250, Wako, Osaka , Japan), CD68 (1:200, AbD serotec, Kidlington, Oxford,UK), CD45 (1:100, Serotec AbD serotec, Kidlington, Oxford,UK), 6E10 (1:1000; Covance, Princeton, New Jersey,USA). Following day, slides were washed with TBS-T and incubated for 2 h with corresponding corresponding anti-rat, anti-mouse, anti-rabbit Alexa-Fluor 568 - conjugated secondary antibodies (1:200, Molecular Probes). Slides were then washed and mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) and cover slipped.

For double fluorescent immunostaining, sections were sequentially incubated with CD45 followed by Iba-1 primary antibodies. Tyramide signal amplification kit (Molecular Probes, paisley,UK) was used for visualizing CD45 with cy-3 label, whereas Alexa Fluor 488 conjugated secondary antibody (1:400; Molecular Probes, paisley,UK) was used for Iba-1 staining. Sections were mounted with Vectashield mounting medium containing DAPI.

For detecting human IgG in mouse brain, free floating sections sections were treated with 0.1 % hydrogen peroxide in methanol, and incubated overnight with rabbit anti-human HRP-labelled antibody. Immunoreactivity was visualized with 0.25 % nickel enhanced diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA).

For triple immunofluorescent staining to study co-localization of Aβ, microglia and human IgG, free-floating sections were washed with 0.1 M Tris-buffered saline (TBS), pH 7.4 and nonspecific binding sites in the tissue were blocked with 5 % normal donkey serum in TBS, to which 0.3 % Triton X-100 had been added (NDS-TBS-T), for 1 h. The sections were then incubated overnight with a mixture of biotinylated monoclonal mouse anti-Aβ17-24 (clone 4G8, Covance Princeton, New Jersey, 1:100 in NDS-TBS-T) and rabbit anti-Iba-1 (Wako Chemicals GmbH, 1:400). Next, the sections were rinsed with TBS and applied to a cocktail of highly purified, carbocyanine (Cy)-conjugated secondary antibodies (all from Jackson ImmunoResearch,Philadelphia,USA, 20 μg/ml in TBS containing 2% BSA): Cy3-coupled streptavidin, Cy5-coupled donkey anti-rabbit IgG and Cy2-coupled donkey anti-human IgG. Sections were washed and mounted in Mowiol.

Table 4.2. Primary antibodies used in immunohistochemistry

Antibody & host	Target	Dilution	Catalogue number & Company	Notes
Anti-human IgG/HRP Rabbit polyclonal	Reacts with gamma-chains of human IgG	1:7500	P0214 DAKO, Denmark	May cross react with mouse IgG.
6E10 Mouse monoclonal	Reactive to 1-16 amino acid residues for human APP protein. Epitope lies within amino acids 3-8 of Aβ (EFRHDS).	1:2000	SIG-39320 COVANCE, USA	Needs citrate pre-treatment and stains Aβ deposits as well as neurons. Works nicely even with alexa-flourophores.
W02 Mouse monoclonal	Recognizes 4-10 amino acid residues of human Aβ protein.	1:30000	MABN10 MILLIPORE, USA	Highly potent antibody for visualizing Aβ deposits.
CD45 Rat	Mouse CD45	1:1000	MCA 1388 AbD SEROTEC, UK	Robust marker for activated spider-like microglia.
Iba-1a Rabbit polyclonal	Reactive to carboxy terminal sequence of human, mouse and rat Iba1 molecule (Ionized calcium binding adaptor molecule 1).	1:5000	01-1974 WAKO, JAPAN	Good marker for both activated and quiescent microglia. Cell bodies and microglia processes are distinctly stained. Works well with alexa-flourophores.

CD68 Rat	Macrosialin is murine homologue of human CD68, both members of lysosomal-associated membrane protein.	1:5000	MCA 1957 AbD SEROTEC, UK	Marker for macrophages. Also, used as marker for phagocytosis.
GFAP Mouse monoclonal	Recognizes human, rat pig GFAP (Principle intermediate filament protein in astrocytes)	1:1000	Clone G-A-5 Sigma-Aldrich, USA	Specific marker for astrocytes.
Doublecortin Goat polyclonal	Reactive to human, mouse, rat doublecortin.	1:1000	Sc-8066 SANATACRUZ USA	Specific marker for newborn adult neurons.

4.7 HISTOLOGICAL STAININGS

For Congo red staining, sections mounted on slides were incubated in saturated alcoholic alkaline NaCl solution for 20 min, followed by incubation in alkaline solution of 0.2% alcoholic Congo red solution. Slides were then washed, dehydrated, cleared in xylene, and coverslipped.

For the microhaemorrhage staining, eight 35 μm coronal brain sections from each mouse were used. Sections were first stained with 2% potassium ferrocyanide in 2% hydrochloric acid for 15 min, followed by a counter stain in a 1% neutral red solution for 10 min.

4.8 IMAGE ACQUISITION, PROCESSING AND DIGITAL IMAGE ANALYSIS

Images (2074×2074) from hippocampi were acquired at 2× (Plan N2×/0.06) objective using an upright optical microscope (OLYMPUS BX40, Tokyo, Japan) with Olympus optical DP50 camera. A flat field image was also acquired in order to correct uneven illumination. Immunopositive areas were quantified using Photoshop CS3 Extended version 10 software (Adobe Systems Incorporated, San Jose, CA, USA). Briefly, hippocampus (the region of interest) was outlined and the immunopositive areas were thresholded for measurements and reported as mean percent immunopositive area for each brain. For double-stained fluorescent sections, images were acquired with confocal Zeiss LSM 700 microscope (Jena, Germany). Eight bit images stacks (1024×1024) were taken with Plan-Apochromat 40×/1.3 oil DIC M27 objective. Individual pixel size in x-y direction was 0.31 μm and 0.41 to 0.49 μm along the z-axis. Subsequent image analysis was done by Image J software. After background subtraction,

individual microglial cell stacks were outlined and separated in x-, y-, and z-planes. Each stack comprising of only one microglial cell was then split into its constituent green, red, and blue channels. A simple Image J macro then applied a threshold value for red and green channels, and created selections for measurements from all the slices of a stack. Integrated intensities, area and volume were then calculated. Maximum intensity projections of microglial cell stacks were then used to measure circularity value for each microglial cell.

4.9 ASSAY FOR ESTIMATING ANTI-HUMAN IgG ANTIBODIES

Microtiter plates (PolySorp, Nunc, MLS, Menen, Belgium) were coated with Gammagard Liquid (Baxter AG, Austria) diluted to 1 μg/mL with coating buffer containing $NaHCO_3$ (0.1 M; Merck,NJ, USA) and Na_2CO_3 (0.1 M; Merck, NJ, USA), adjusted to pH9.6. After overnight incubation and washing with a buffer of Dulbecco's PBS (Gibco, Life Technologies) supplemented with Tween20 (0.05%; Merck,NJ, USA), the plates were blocked with the same buffer plus 1% BSA for 2 h at room temperature. After further washing, dilutions of serum samples and controls were added to the plates and incubated for 2 h at room temperature. Serum samples and the negative control (serum pooled from untreated mice) were pre-diluted at 1:50 in the same buffer but with 0.5% BSA and further serially diluted by a factor of 2. Purified mouse anti-human IgG Fc (Jackson ImmunoResearch,Philadelphia,USA) was pre-diluted 1:250,000 and further serially diluted by a factor of 2 for the positive control of the assay. After washings, the plates were incubated with HRP-conjugated goat anti-murine IgG (1:2,000; Southern Biotech, Alabama, USA) for 1 h at room temperature. Further washing and incubation with enzyme substrate (substrate buffer: $Na_2HPO_4.2H_2O$ (0.1 M; Merck,NJ,USA), $C_6H_8O_7.H_2O$ (0.05 M; Merck), pH5.0, *o*-phenylenediamine-dihydrochloride (1 mg/mL; Sigma-Aldrich, USA), and H_2O_2 (0.03 %; Merck)) for 30 min at room temperature produced a color reaction. The reaction was stopped with H_2SO_4 (2 N; Merck,NJ,USA) and the color intensity was measured with an ELISA plate reader (Synergy BioTek,Winooski,USA) set at a 492-nm wavelength (wavelength correction set to 630 nm). All samples where the difference between the samples' optical density (OD) and the blank OD was ≥0.3 were considered positive. The highest dilution to show a positive result was specified as the titre.

4.10 ESTIMATION OF SERUM AND BRAIN AB LEVELS

For estimating serum Aβ40 levels, samples were diluted at 1:3 with diluting buffer in accordance to the manufacturer's instructions (The Genetics Company,Basel, Switzerland). Standards or diluted samples and antibody conjugate comprising of a detection antibody were applied to the 96-well microtiter plates precoated with capture antibody. Following overnight incubations at 4°C and washings, biotin-streptavidin enzyme conjugate was incubated at room temperature for 30 min. A further incubation with enzyme substrate at room temperature for 30 min gave a colored reaction product. After stopping the reaction, color intensity was measured with a microtiter ELISA plate reader (Multiskan MS, Labsystem, Helsinki, Finland) set at 450 nm wavelength. Standard curve was prepared and sample concentrations of Aβ40 were extracted from standard curve and expressed as pg per ml of serum.

Levels of soluble and insoluble Aβ40 and Aβ42 were determined from the hippocampus block. The tissue was weighed and homogenized in 10× volume of Dulbecco's PBS buffer

(Sigma-Aldrich, St.Louis,USA), containing complete inhibitory mixture (Roche Diagnostics, Mannheim, Germany). Samples were centrifuged at 45,000 rpm (Beckman Ultrafuge, Fullerton, CA,USA) for 2 h at 4°C. Supernatant was diluted at 1:2 and used to analyze soluble levels of Aβ40 and Aβ42. The remaining pellet was resuspended in 5 M guanidine-HCl/50 mM Tris·HCl, pH 8.0 and mixed on a shaker for 3 h at RT. Samples were then diluted at 1:50 with reaction buffer (Dulbecco's PBS with 5% BSA, 0.03% tween-20, supplemented with protease inhibitor cocktail) and centrifuged at 16,000 × g for 20 min at 4°C. Decanted supernantant is further diluted at 1:400 with dilution buffer. Diluted samples were then used to analyze insoluble Aβ40 and42 species. Aβ40 and Aβ42 levels were estimated using ELISA kits (Biosource International,Life technologies, Paisley,UK) in accordance to manufacturer's instructions. Aβ40 andAβ42 levels were standardized to tissue weight and expressed as picograms of Aβ per gram ± SEM.

4.11 QUANTITATIVE REALTIME-PCR

Total RNA was extracted from frozen frontal cortices by TRIzol reagent (Invitrogen, Life technologies, Paisley,UK) according to the manufacturer's instructions. RNA concentration and purity was measured with Nanodrop 1,000 spectrophotometer (Thermo Fisher Scientific,Delaware, USA). cDNA was synthesized from 500 ng of total RNA using random hexamer primers (Promega, Madison, USA) as a template and Maxima reverse transcriptase (Fermentas, Thermo Fisher Scientific,Delaware, USA). The relative expression levels of mRNA encoding mouse *Tnf* and *Il1b* were measured according to manufacturer's protocol by quantitative RT-PCR (StepOnePlus; Applied Biosystems, Life technologies, Paisley,UK) by using specific assays-on-demand (Applied Biosystems, Life technologies, Paisley,UK) target mixes. The expression levels were normalized to ribosomal RNA and presented as fold change in the expression ± SEM.

4.12 STATISTICAL ANALYSIS

All data are given as mean ± SEM. For comparing means of two treatment groups or genotypes, independent-sample Student's t-test was employed. For testing genotype and treatment interaction, two-way ANOVA was employed. For non-parametric data, Mann-Whitney U-test was employed. Two-step cluster analysis was used to segregate microglia populations. All statistical analysis were performed by GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and IBM SPSS statistics version 19 for Windows (New York, USA). For LPS experiment, Linear mixed model analysis was performed on IBM SPSS statistics version 19 for Windows to test within subject (saline vs. LPS) and between subject differences (*Nfkb1* wild-type Vs. *Nfkb1*$^{-/-}$). Statistical significance was set at $P < 0.05$.

5 Results

5.1 HUMAN INTRAVENOUS IMMUNOGLOBULIN (hIVIG) STUDY

We treated APP/PS1 mice with intraperitoneal injections of hIVIG in a series of experiments with different treatment regimens. In general, mice well tolerated both single and multiple hIVIG injections. In chronic treatment regimens, repeated hIVIG injections did result in local tenderness around the injection site, but no peritonitis or swelling was noticed and no abnormal behaviour indicating significant pain. Overall, hIVIG treated mice showed no significant side effects. Table 5.1 summarizes all the key findings of hIVIG study.

Table 5.1. Bird's eye view of key hIVIG study findings.

Assessments	IVIG-1	IVIG-3	IVIG-6a	IVIG-6b
General mouse behaviour	Normal	Normal	Normal	Normal and heightened sensitivity to touch near site of injection. No signs of distress or pain.
Anti-human IgG titres	Present	Present	Absent in majority of mice.	Present but highly variable.
Brain Penetration of human IgG	No	No	Yes. Hippocampus showed human IgG gradient along rostro-caudal axis.	Yes. 1. Hippocampus and cortex show human IgG signal. 2. Rostro-caudal human IgG gradient in hippocampus. 3. Aβ Plaque decoration.
Cognitive Tests	--	No treatment effect	hIVIG treated APP/PS1 mice performed better on probe trial.	1. No cognitive benefits. 2. APP/PS1mice more active.
Amyloid pathology	No change	No change	No change	1. Soluble Aβ increased by 30-50%. 2. No change in Aβ plaques.
Microglia pathology	No change	No change.	No change.	1. Decrease in CD45 microglia by 36% 2. Statistically significant increase in Iba-1 by >38% 3. Changes in type B and type C microglial cells.
Proinflammatory gene	--	--	--	1. Suppression of *Tnf*

expression				gene expression. 2. CD45 microglia correlate inversely with *Tnf and Il1b* gene *expression.*
Neurogenesis	--	--	--	1. Increase in doublecortin positive cells by ~60% in both wild and APP/PS1 mice. 2. Inverse correlation between CD 45 microglia and doublecortin positive cells.
Microhemorrhages	--	No microhemorhages Detected	--	--

5.1.1 Effect of hIVIG in young mice (IVIG-1 study)

In this dose-finding experiment, mice well-tolerated hIVIG injections and there were no signs of peritoneal inflammation or pain around the site of injection. Behaviour of mice post-hIVIG treatment was as well unremarkable.

5.1.1a Effects on brain and serum levels of Aβ protein

At 4 months of age APP/PS1 mice show a few scattered plaques as detected by 6E10 immunostaining in the cortex and hippocampus. ELISA assays were used to determine levels of $A\beta_{40}$ and $A\beta_{42}$ in the parietal cortex. One or three, once weekly injections of either a high or a low dose of hIVIG did not significantly change cortical levels of soluble or insoluble $A\beta_{40}$ and $A\beta_{42}$ (figure 5.1.1a-A-D). Similarly, serum levels of $A\beta_{40}$ were assessed to see if hIVIG treatment caused efflux of Aβ from the brain into the peripheral circulation. As evident from Figure 5.1.1a-E, mice treated with high-dose hIVIG and sacrificed a week after injection showed significantly higher Aβ40 levels than saline group ($F_{(4,12)} = 3.54$, $p = 0.04$). Unfortunately, we ran short of serum samples from group 6 that had 3 weekly injections of high-dose hIVIG. Serum Aβ42 levels were below the level of detection. This increase in serum Aβ40 in the high-dose group prompted us to further evaluate its effects with longer treatment durations.

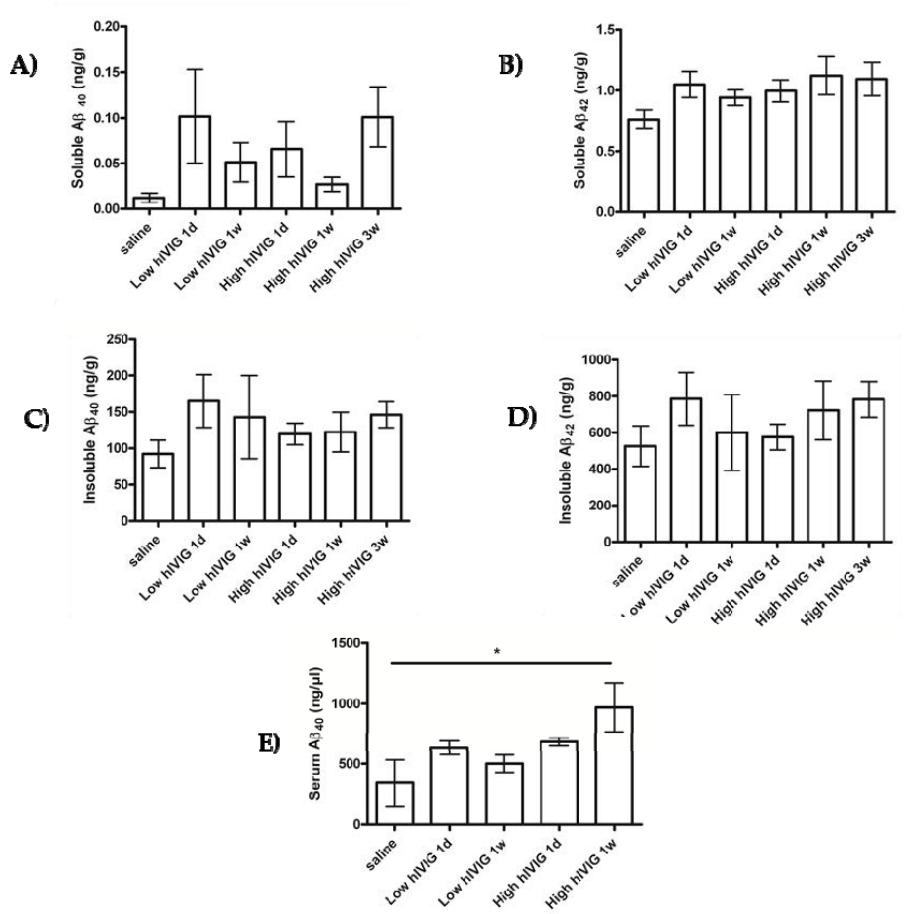


Figure5.1.1a. ELISA measurements of cortical levels of **(A)**soluble $A\beta_{40}$ & **(B)** $A\beta_{42}$ and **(C)** Insoluble $A\beta_{40}$ & **(D)** $A\beta_{42}$. **(E)** Serum $A\beta_{40}$ levels from high hIVIG 3w group is missing.Treatment groups varied with respect to hIVIG dose either low (0.1 g/kg) or high (1g/kg) and time of sacrifice after treatment:either 1day(1d), 1week (1w) or 3 weeks (3w) after treatment.

5.1.1b Effect on microgliosis

In order to assess the acute effects of hIVIG on microglia, CD45 immunostaining was performed and the number of microglial clusters quantified. CD45 signal dovetailed Aβ pathology in young APP/PS1 mice. Usually the immunopositive areas of 6E10 and CD45 staining were overlapping, which indicates an efficient microglial response to Aβ deposition. However, as evident from figure 5.1.1b-A&B, hIVIG treatment with varying dose and duration did not bring about any significant change to microglial responses in either the cortex or the hippocampus (cortex: $F_{(5, 32)} = 0.44$, $p = 0.81$; hippocampus: $F_{(5, 32)} = 0.21$, $p = 0.95$). Again this may be explained by insufficient penetration of hIVIG into the brain. Therefore we proceeded to further test hIVIG with longer treatment regimens.

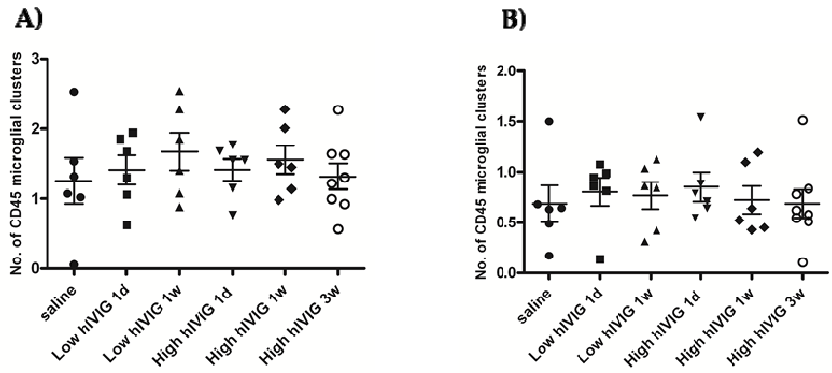


Figure5.1.1b. Number of CD45 positive microglial clusters in **(A)** cortex and **(B)** hippocampus. Treatment groups varied with respect to hIVIG dose either low (0.1 g/kg) or high (1g/kg) and time of sacrifice after treatment: either 1day(1d), 1week (1w) or 3 weeks (3w)after treatment.

5.1.2 Effects of hIVIG in 15-month-old mice (IVIG-3 study)

In this experiment, aged (14-month old APP/PS1) mice well-tolerated high dose hIVIG injections and there were no signs of peritoneal inflammation or pain around the site of injection. Also, there were no noticeable abnormal mouse behaviour post-hIVIG treatments.

5.1.2a Development of anti-human IgG antibodies in APP/PS1 mice

Anti-human IgG neutralising antibodies were analysed by BaxterA/G, Austria. Only titres above 200 were considered significant. None of the mice in saline group developed significant neutralising antibodies (figure 5.1.2a). However, the remaining mice, irrespective of age or number of injections, developed anti-human IgG antibodies. Peak titres in young mice (4 months) were of an order of magnitude lower compared to aged mice (15 months).

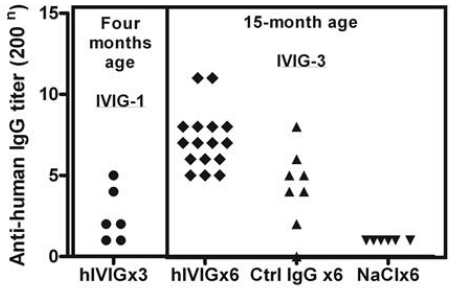


Figure5.1.2a. Titres of anti-human IgG antibodies or neutralising antibodies from IVIG-1 and IVIG-3 experiments.Treaments and corresponding number of injections are displayed on X-axis.

5.1.2b Anti-human IgG staining in hIVIG treated mice

Based on this tentative observation we made plans to initiate our treatments at the age of 4 months in our subsequent experiments. An advantage of treating mice with a human protein is that it should be feasible to locate the human IgG inside the brain by immunostaining. Mere presence of human IgG signal within the brain parenchyma would thus confirm brain penetration of hIVIG. A very nonspecific anti-human IgG staining can be clearly appreciated in brain sections from hIVIG and control IgG groups whereas saline treated sections are completely clean of any signal in figure 5.1.3c-B. Ventricular linings, fimbriae, choroid plexus and some adjoining blood vessels showed strong 3, 3'-diaminobenzidine (DAB) signal indicating that human IgG was inside the brain vasculature but failed to enter brain parenchyma. There was no evidence of Aβ plaque decoration in these mice.

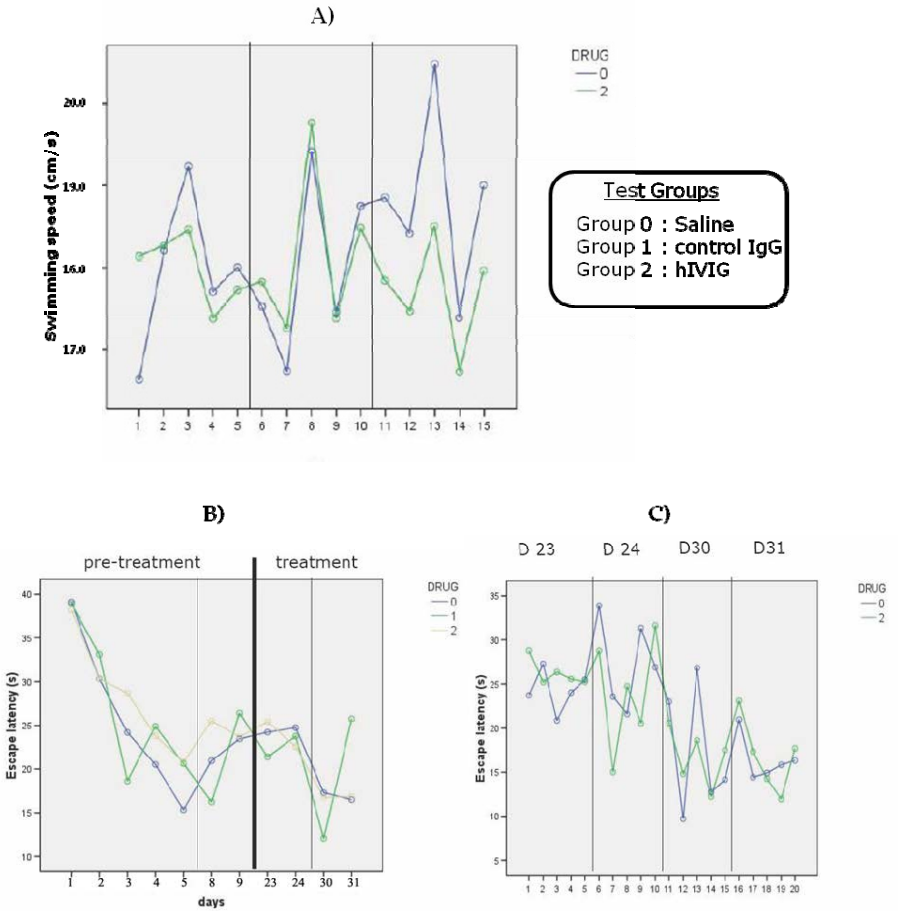


Figure 5.1.2c. Spatial navigation test by Morris water maze. **(A)** Mice in all treatment groups displayed no differences in swimming speeds. **(B)** Escape latencies did not differ among the treatment groups during pre-treatment phase and post treatment phase. **(C)** Trial by trial visualization of escape latencies across treatment groups are also similar.

5.1.2c Cognitive effects of hIVIG on 15-month-old mice

The treatment groups did not differ in their swimming speeds (figure 5.1.2c-A). As evident from the escape latencies (figure 5.1.2c-B), all mice learned the task to the same extent during the first 5 days. Overall, the escape latencies were longer after the platform had been moved to a new position. Figure 5.1.2c-C illustrates the escape latencies on each separate trial for saline and hIVIG treated groups. On some days (D24 and D31), the escape latency was significantly longer than on the previous day, after the platform had been moved to a new location. However, in half of the days (D23 and D30) this was not the case. In addition, one can see an overall trend for escape latencies to shorten towards the end of the experiment. This trend suggests that instead of learning the particular new platform location each day, the mice just learned a more and more efficient general search strategy. Furthermore, the mean latencies of different treatment groups were almost 100 % overlapping, speaking against treatment effect.

5.1.2d Effects of hIVIG on amyloid and microglial pathologies in 15-month-old mice

One week after the last injection, brains and sera were collected for immunohistochemical and ELISA analysis, respectively. As illustrated in figure 5.1.2d-A to D, statistical analysis showed no significant group differences in soluble and insoluble levels of $A\beta_{40}$ and $A\beta_{42}$ (soluble $A\beta_{40}$: $t_{(18)} = 0.20$, $p = 0.85$; insoluble $A\beta_{40}$: $t_{(18)} = 0.20$, $p = 0.85$; soluble $A\beta_{42}$: $t_{(18)} = 0.85$, $p = 0.40$; insoluble $A\beta_{42}$: $t_{(18)} = 0.02$, $p = 0.99$). Similarly, one-way ANOVA analysis of serum levels of $A\beta_{40}$ (figure 5.1.2d-E) revealed no treatment effects ($F_{(2, 16)} = 0.30$, $p = 0.74$). We next assessed brain Aβ plaque load with 6E10 immunohistochemistry. Monoclonal antibody 6E10 recognizes N-terminal 4-9 amino acid residues of Aβ peptide. As shown in figure 5.1.2d-F roughly 8 - 10 % of the cortical area had Aβ positive plaques. However, one-way ANOVA revealed no statistically significant treatment effects ($F_{(2, 37)} = 0.69$, $p = 0.51$). Microglial reactivity (figure 5.1.2d-G) as assessed by CD45 immunostaining, did not differ between the treatments ($F_{(2, 37)} = 1.46$, $p = 0.25$). Inadequate brain penetration of hIVIG, coupled with high anti-human IgG titres in this study might account for for the lack of effects on amyloid and microglial measurements.

5.1.2e Microhemorrhages with hIVIG treatment

See figure 5.1.2e-A to G for some examples of positive profiles of microhemorrhages. Overall, there were 13 positive profiles which were highly suspicious for microhemorrhages. Of these only eight were inside the brain parenchyma and the rest were noticed in choroid plexus. Only one positive profile was noticed in the cortex of saline treated mouse whereas two such profiles were noticed from hIVIG treated mice. Overall, there was no significant difference between groups indicating hIVIG treatment did not significantly increase the risk of microhemorrhages (figure 5.1.2e-H).

5.1.3 Effects of weekly hIVIG treatment for 3 months (IVIG-6a study)

The hIVIG treatments so far had failed to induce any change in the brain amyloid load or alleviate the cognitive impairment of APP/PS1 mice. However, two important factors may explain the poor treatment outcome. Firstly, the magnitude of anti-human IgG titres developed by 15-month-old mice was far higher than those in 4-month-old mice. Secondly, it

was clear that in previous studies there was little brain penetration of hIVIG. Three weeks of treatment was obviously too short for aged mice with massive cerebral amyloid pathology. In this experiment, aged (4-month old APP/PS1) mice well-tolerated chronic weekly high dose hIVIG injections and there were no signs of peritoneal inflammation. Heightened sensitivity to touch was evident around the sites of injection. Also, there were no indications of abdominal pain or distress or abnormal behaviour.

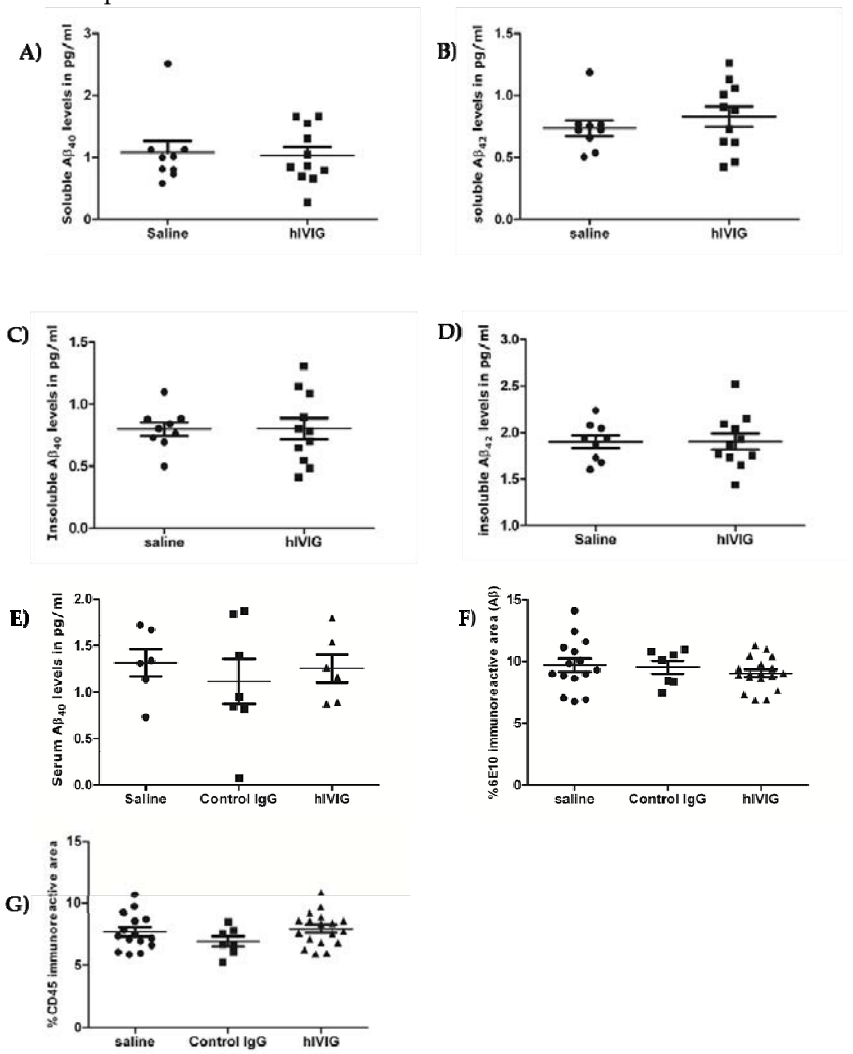


Figure5.1.2d.Quantitative data related to amyloid and microglial pathologies in 15-month old mice from IVIG-3 study. No treatment effects in any of the measured parameters. Biochemical levels of **(A)** soluble $A\beta_{40}$ **(B)**soluble $A\beta_{42}$ **(C)** Insoluble $A\beta_{40}$ **(D)** Insoluble $A\beta_{42}$ **(E)** serum $A\beta_{40}$ from cortex of mice treated with hIVIG or saline.Quantitative immunohistochemical analysis of **(F)** Aβ burden and **(G)** microglial reactivity.

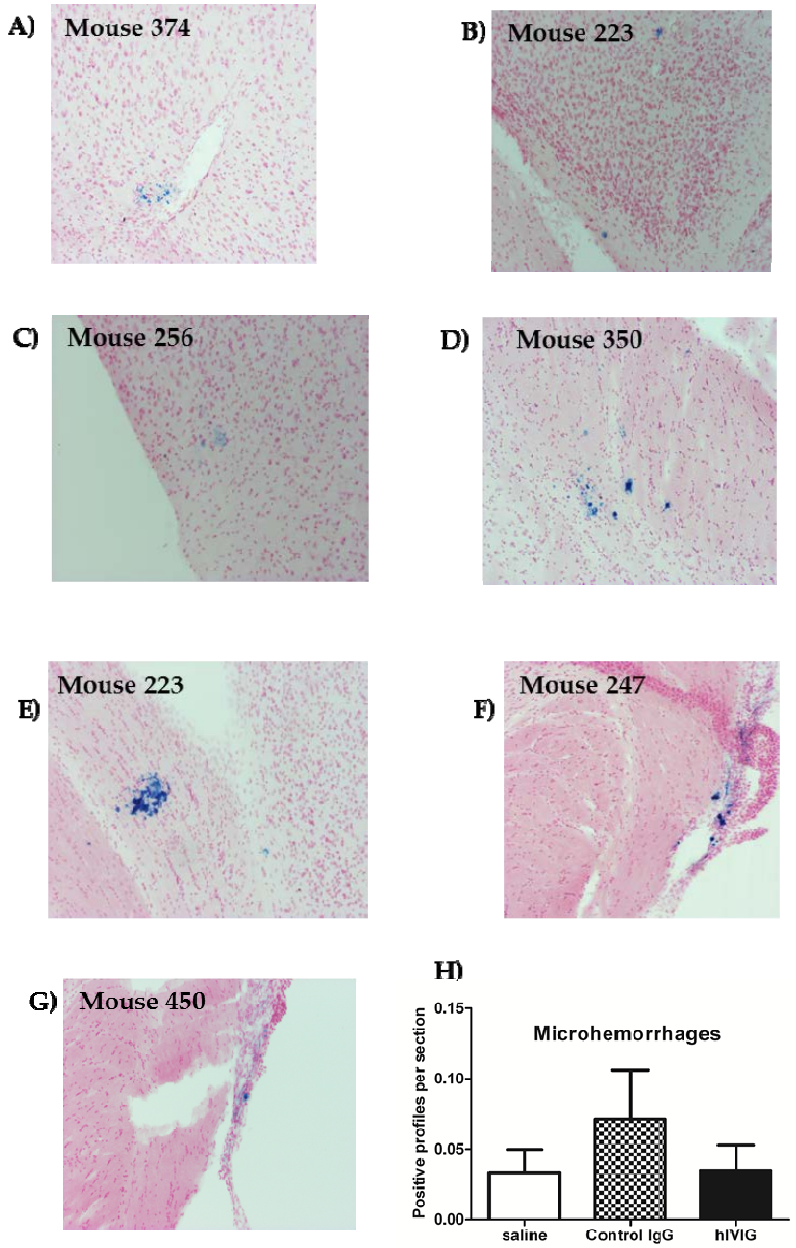


Figure5.1.2e. **(A-G)** Representative images of positve iron staining patterns in mice treated with hIVIG. Mostly, there were common in fimbriae and thalamus regions. **(H)** Quantification of positive microhemorrhage profiles per section.

5.1.3a Effect of 3-month hIVIG treatment on cognition of APP/PS1 mice

As shown in figure5.1.3a-A there were no significant differences between the treatment groups with regard to escape latency on all the four days ($F_{(2, 45)} = 0.88$; $p = 0.42$). On day 4, only a trend for wild-type mice to perform better than APP/PS1 mice emerged. However, a probe trial on day 5 showed a significant difference ($F_{(2, 47)} = 4.53$; $p = 0.16$), such that saline treated wild-type mice ($p = 0.02$), and hIVIG treated APP/PS1 mice ($p = 0.04$) performed statistically better than saline treated APP/PS1 mice (figure5.1.3a-B).

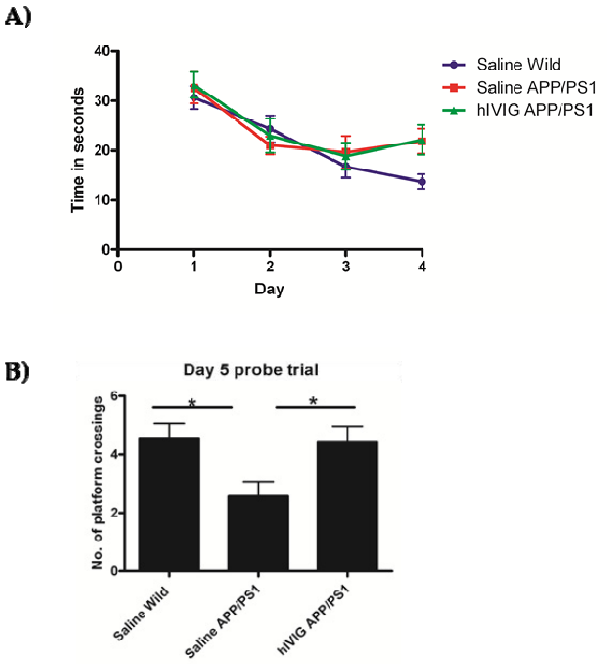


Figure5.1.3a. Morris water maze data from mice treated with hIVIG for 3 months. **(A)** On Day 4 APP/PS1 mice took longer to reach platform than wild mice. **(B)** Probe trial on day 5, showed that saline treated wild and hIVIG treated APP/PS1 mice crossed the platform location significantly more number of times than saline treated APP/PS1 mice.
* indicates $p < 0.05$.

5.1.3b Development of anti-human antibodies during 3-month hIVIG treatment

As shown in figure 5.1.3b, out of 17 mice hIVIG treated mice, only three developed neutralising antibody titres higher than 200. Rest had titres similar to that of saline treated mice suggestive of abated cross-species immune response.

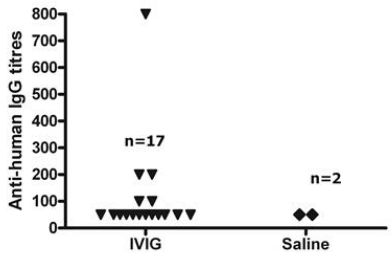


Figure5.1.3b. Anti-human antibodies titres from sera of hIVIG treated mice as estimated by ELISA. Note only 2 mice developed significant titres. Titres > 200 were considered positive.

5.1.3c Successful penetration of human IgG into the hippocampus of APP/PS1 mice

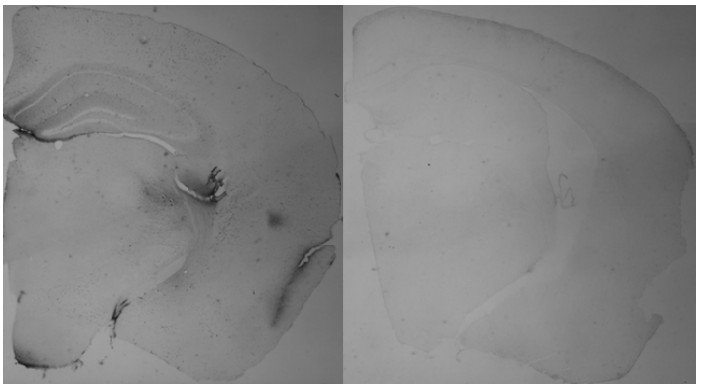
As shown in figure 5.1.3c-A and B, anti-human IgG signal in IVIG-1 and IVIG-3 was quite weak, yet distinctly different from saline treated mice. Notably, even sections from mice treated with control human IgG in the IVIG-3 experiment exhibited a weak signal akin to hIVIG treated mice. Upon careful observation, one could actually identify a clear human IgG signal inside the blood vessels but very scanty staining outside the vessels, indicating that human IgG was indeed present inside the blood vessels but never made its way out in significant quantities into the surrounding brain parenchyma. In the IVIG-6a experiment (figure 5.1.3c-C), where mice received weekly hIVIG injections for 3 months, a clear human IgG signal could be observed within the hippocampus. Importantly, saline treated mice were devoid of any immunoreactivity. Interestingly, not only human IgG signal was concentrated in the hippocampus but there was also a clear gradient in staining intensity across the rostro-caudal axis (figure 5.1.4a-F). Furthermore, Figure 5.1.3c-D also shows scattered intensely stained human IgG aggregates as discrete deposits on the lower blade of dentate gyrus indicative of putative plaque decoration. Overall, anti-human IgG immunostaining in brain sections of these mice indicate successful penetration of human IgG into mouse hippocampus.

5.1.3d Amyloid pathology and reactive microglia assessment after 3-month hIVIG treatment

At the end of experiment, brains were processed and stained with 6E10 antibody to evaluate the total volume of diffuse amyloid plaques and Congo red to stain for dense core plaques. In addition, we used CD45 antibody in order to evaluate microglial activation. As shown in figure 5.1.3d-D and E, treatment did not bring about any significant changes to either 6E10 ($t_{(28)} = 0.44$; $p = 0.66$) or Congo red positive amyloid deposits ($t_{(28)} = 1.60$; $p = 0.12$). CD45 immunopositive area (figure 5.1.3d-F) did not differ between the treatments, either ($t_{(28)} = 0.12$; $p = 0.90$) or similarly, microglial reaction relative to Aβ plaque area did not differ between the treatments ($t_{(28)} = 0.29$; $p = 0.77$). Furthermore, as illustrated in figure 5.1.3d-A to C, no statistically significant differences were found in measurements of soluble ($t_{(27)} = 0.33$; $p = 0.74$) or insoluble levels of $A\beta_{42}$ ($t_{(27)} = 0.09$; $p = 0.93$), or serum $A\beta_{40}$ levels ($t_{(22)} = 0.44$; $p = 0.67$).

A) IVIG-1

hIVIG Saline



B) IVIG-3

hIVIG Control IgG Saline

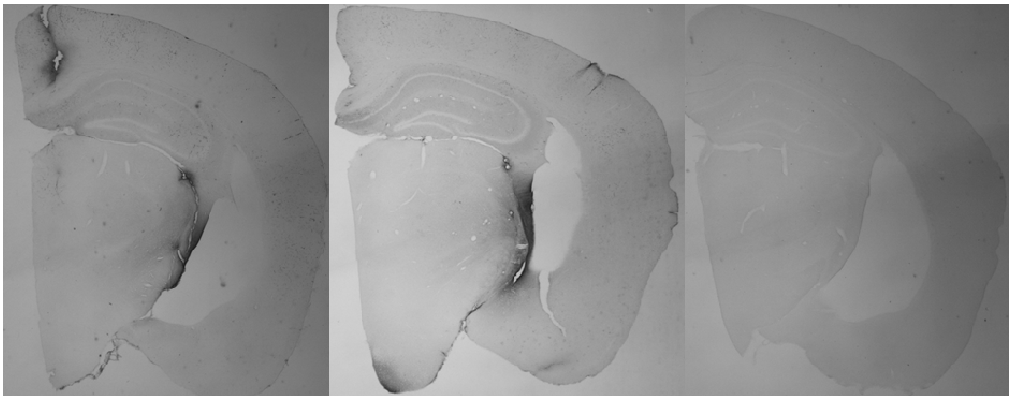


Figure 5.1.3c continued..

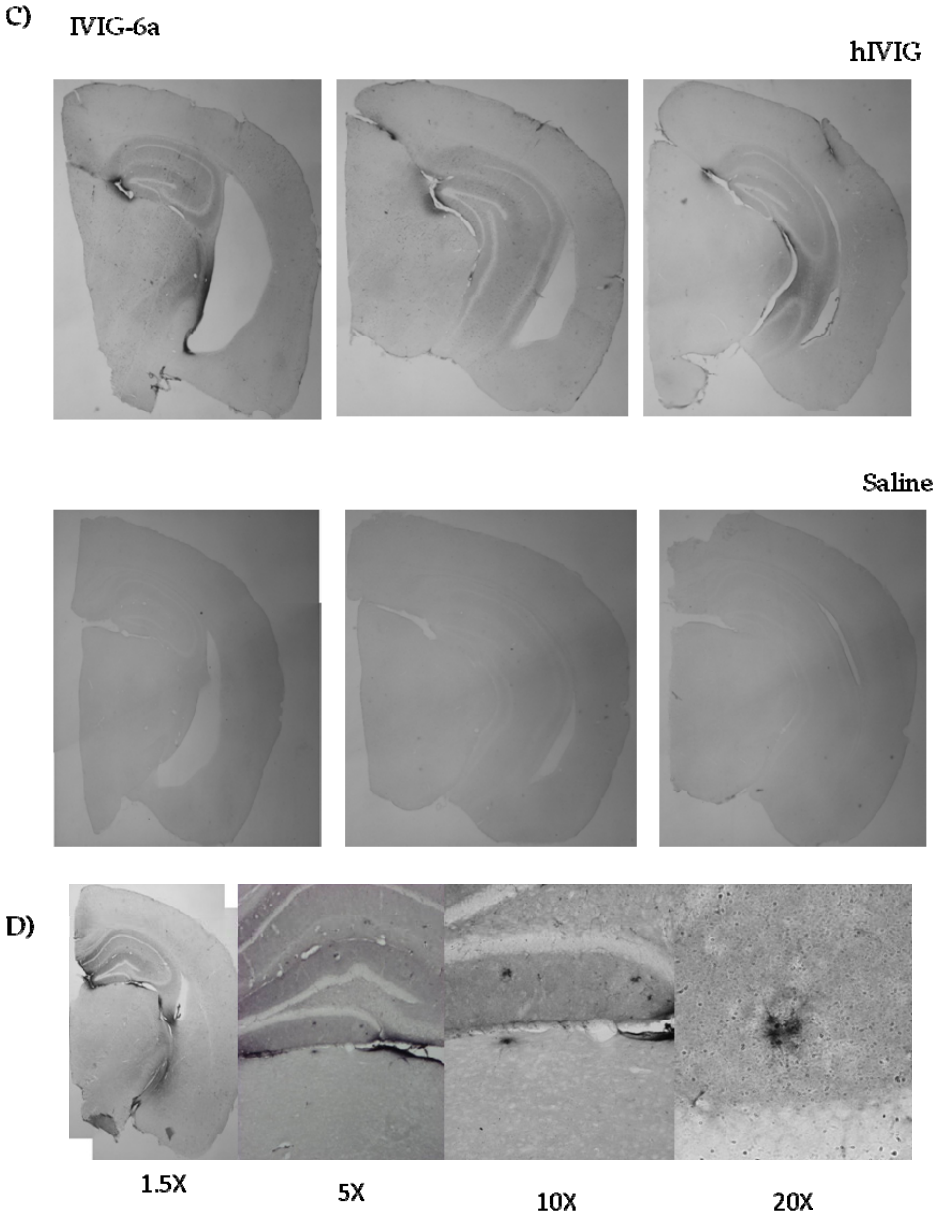


Figure5.1.3c. Anti-human IgG immunostainings. Mouse sections from **(A)** IVIG-1 and **(B)** IVIG-3 experiments showed very weak human IgG signal whereas sections from **(C)** IVIG-6a clearly showed human IgG signal emerging predominantely from hippocampus. Control animals treated with saline were devoid of any anti-human IgG signal. Under high magnification one can clearly see **(D)** dark intensly stained aggregates ofhuman IgG in dentate gyrus.

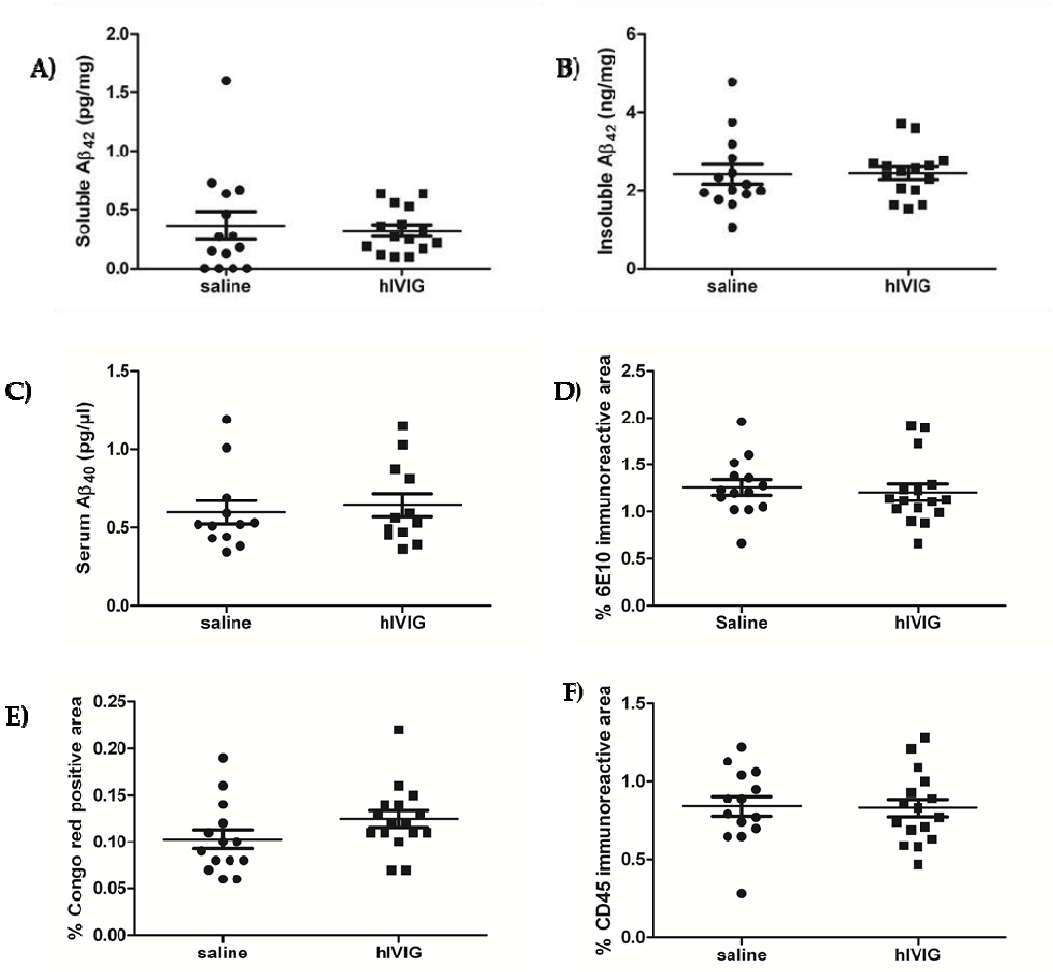


Figure5.1.3d. Hippocampal levels of (**A)** soluble $A\beta_{42}$ **(B)** insoluble $A\beta_{42}$ and **(C)** serum levels of $A\beta_{40}$ measured from IVIg-6a experiment. Mice were 7.5 month old at the end of treatment schedule. **(D, E** and **F)** show quantitative immunohistochemistry data related to Aβ burden, amyloid burden and microglial reactivity, respectively.

5.1.4 Effects of weekly hIVIG treatment for 8 months (IVIG-6b study)

In this experiment, aged (4-month old APP/PS1) mice well-tolerated chronic 8-month weekly high dose hIVIG injections and there were no signs of peritoneal inflammation. Heightened sensitivity to touch was evident around the sites of injection. Also, there was no noticeable abnormal mouse behaviour indicative of abdominal pain or distress. All the mice irrespective of treatment were well groomed and were active in their home-cages.

5.1.4a Development of anti-human IgG titres and brain penetration of human IgG

We took lifetime blood samples at 7.5 month of age and terminal samples at 12-month age in order to assess the development of neutralising antibodies. Interestingly, this time the titres were much more variable than our previous experiment. In contrast to our previous belief, even mice with no detectable anti-human IgG titres at 7.5 months developed high titres by 12-month age and *vice versa*. This indicates that age of mice or the duration of treatment had limited influence over development of neutralising antibodies (figure 5.1.4a-A). We also carried out anti-human IgG immunostainings in mouse brain sections and compared them with anti-human IgG titres measured from sera. As evident from figure 5.1.4a-B, human IgG staining intensity was more or less the same in mice with either low or high anti-human IgG titres. One can see dark deposits of human IgG even in mice with the highest antibody titres. Figure 5.1.4a-C shows the full extent of human IgG deposition and decoration around putative plaques in both the cortex and hippocampus of mouse 389, which developed the highest titres of anti-human IgG antibodies in this experiment. A double staining with congo red and anti-human IgG (figure 5.1.4a-D) shows that this human IgG staining is specific to amyloid plaques.

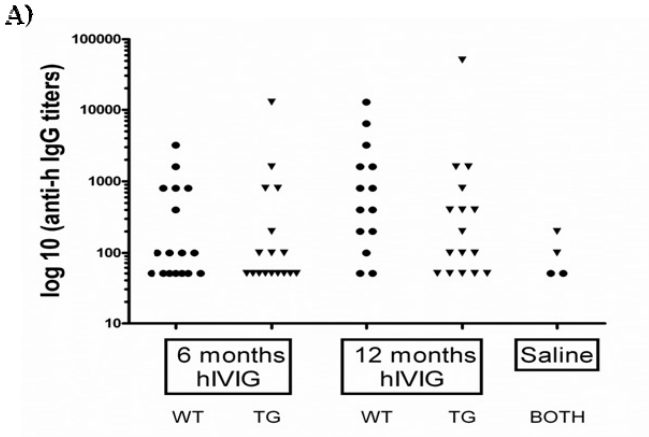
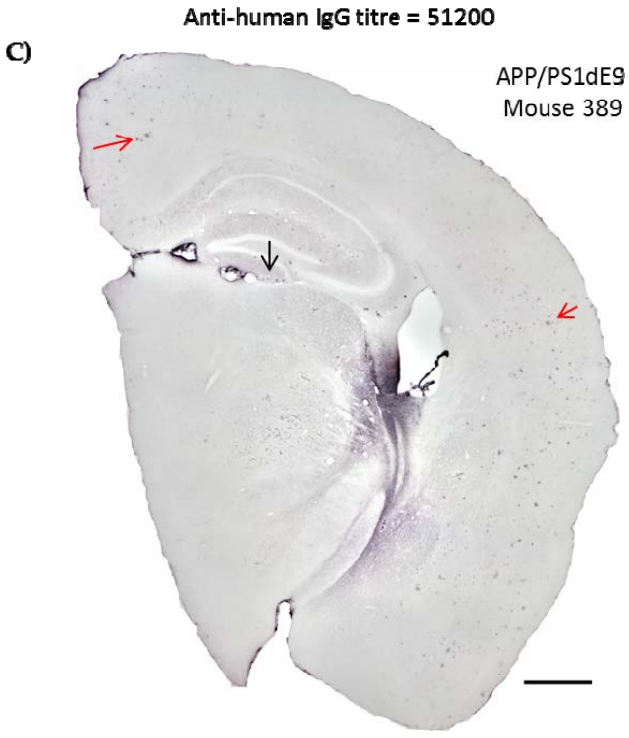
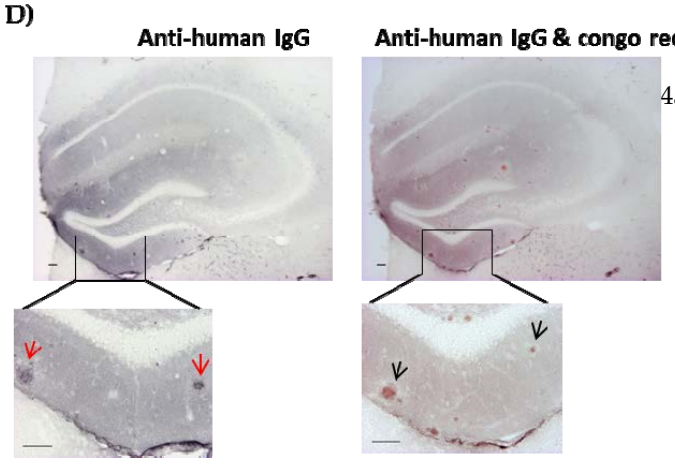


Figure 5.1.4a continued..





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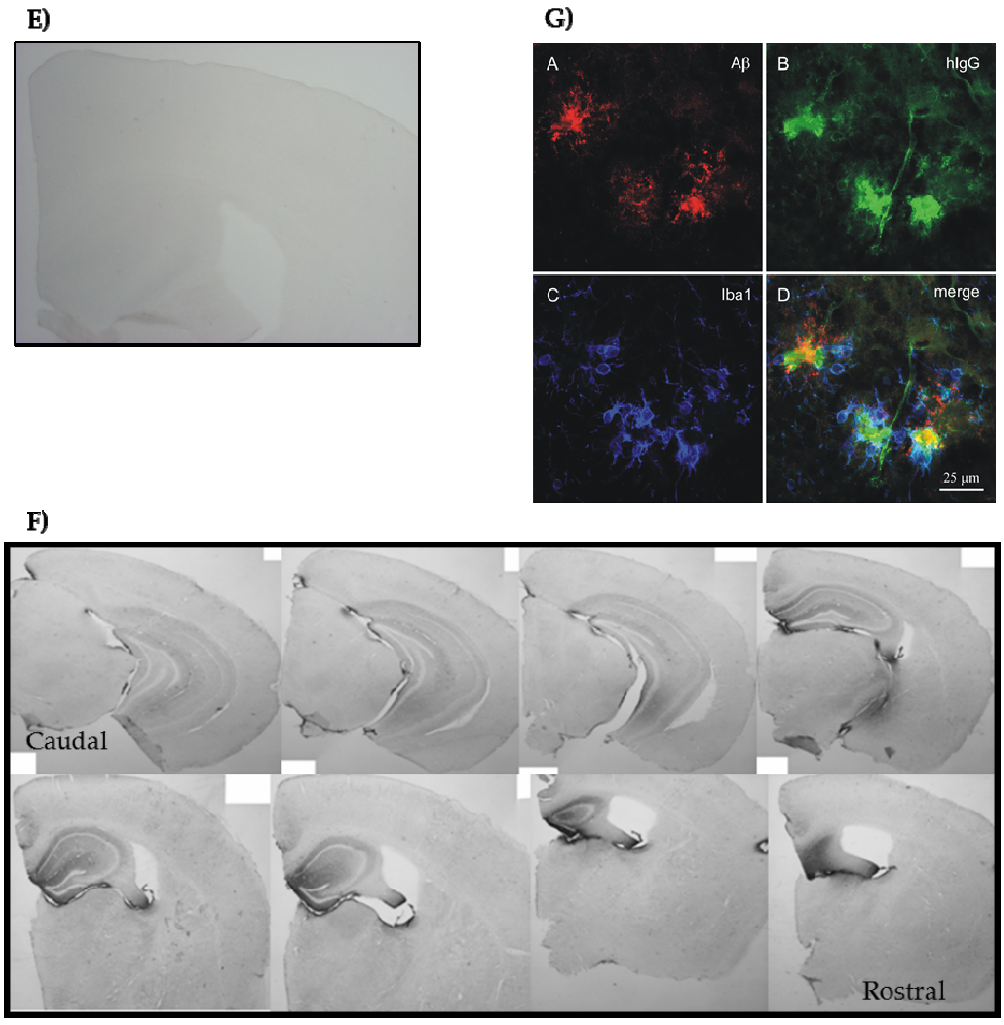


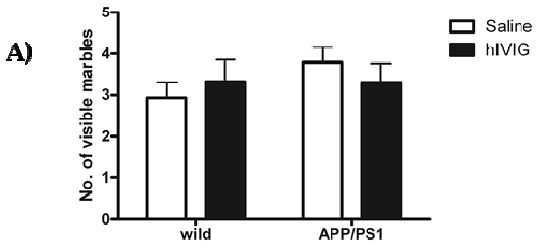
Figure 5.1.4a. **(A)** Development of anti-human antibodies in mice treated hIVIG for 8 months. Circles represent wild and triangles represent APP/PS1 mice. Titires > 200 are only considered significant.**(B)** Comparision of anti-human IgG stained mouse brain sections with anti-human IgG titres in sera. Scale bar denotes 100μm. **(C)** Anti-human IgG stained section from mouse 389 shows putative human IgG deposits in cortex (red arrows) and hippocampus (black arrows). Scale bar denotes 500μm. **(D)** High magnification image from serial sections stained with congo red (black arrows)and anti-human IgG immunostain (red arrows). Scale bar denotes 50μm. **(E)** Anti-human IgG staining on a mouse brain section treated with saline. This control section is completely devoid of human IgG signal. **(F)** Human IgG gradient present across rostral-caudal axis of hippocampus. **(G)** Triple flourescencent staining of mouse section with Aβ, huma hIgG and microglia.

In contrast, brain sections of saline treated control mice were devoid of any immunoreactivity to human IgG (figure 5.1.4a-E). This indicates that the human IgG immunoreactivity signal in mice cannot be an artefact of the human *APP* transgene. Furthermore, one can clearly appreciate the existence of a human IgG gradient within the hippocampus across rostro-caudal axis (figure 5.1.4a-F), which indicates accumulation or sequestration of hIVIG within the hippocampus as a consequence of chronic treatment. Finally, a triple immunofluorescent labelling showed amyloid plaque, human IgG and microglia all located within the same microenvironment (figure 5.1.4a-G).

Finally, we tried to correlate the important measured outcome parameters with titres of anti-human IgG antibodies. Serum Aβ40 (Pearson r = 0.035, p = 0.91), hippocampal soluble Aβ40 (Pearson r = - 0.11, p = 0.68), hippocampal CD45 immunoreactivity (Pearson r = - 0.29, p = 0.27), TNF (Pearson r = 0.01, p = 0.98) and Doublecortin positive cells in the dentate gyrus (Pearson r = - 0.11, p = 0.76) all yielded statistically nonsignificant correlations with anti-human IgG titres. Among the 16 mice that received hIVIG only 7 mice produced significant titres of neutralizing antibodies (titres >200). After removing data from these mice, the outcome of the study remained unchanged.

5.1.4b Effect of 8-month hIVIG treatment on behaviour of APP/PS1 mice

In the marble burying test for neophobia, the number of visible marbles was counted 24 h after placing them inside the home cage. Two-way ANOVA showed no statistically significant effect (figure5.1.4b-A) of either genotype ($F_{(1, 56)}$ = 0.89; p = 0.35) or treatment ($F_{(1, 56)}$ = 0.007; p = 0.93). Next, in exploratory activity as assessed by ambulatory distance (figure 5.1.4b-B), we noticed a significant genotype effect ($F_{(1, 56)}$ = 5.33; p = 0.025), but no significant treatment effects ($F_{(1, 56)}$ = 0.71; p = 0.40) or their interaction ($F_{(1, 56)}$ = 0.26; p = 0.61). This was consistent with our previous findings that APP/PS1 mice are hyper-active. In the novel object recognition test, APP/PS1 mice made significantly more exploratory visits to the objects than their wild-type-type littermates ($F_{(1, 56)}$ = 5.21; p = 0.026). However, neither a treatment effect ($F_{(1, 56)}$ = 0.91; p = 0.36) nor an interaction between the genotype and treatment were found ($F_{(1, 56)}$ = 0.18; p = 0.67). As for novelty preference, there was no treatment ($F_{(1, 56)}$ = 0.31; p = 0.58) or genotype ($F_{(1, 56)}$ = 0.64; p = 0.43) effect, or an interaction between the genotype and treatment ($F_{(1, 56)}$ = 0.02; p = 0.89). In Morris water maze test for spatial memory, there was no genotype or treatment effect on the swimming speed. Unfortunately, escape latencies (figure 5.1.4b-C) to reach the platform over the five test days did not reveal any genotype or treatment effects (genotype: $F_{(1, 56)}$ = 1.01; p = 0.32; treatment: ($F_{(1, 56)}$ = 0.1; p = 0.89). Only when considering learning within day 1, APP/PS1 mice proved to be inferior to their wild-type littermates ($F_{(1, 56)}$ = 4.1; $p < 0.05$), but no treatment effect was found on this initial learning, either (p = 0.46).



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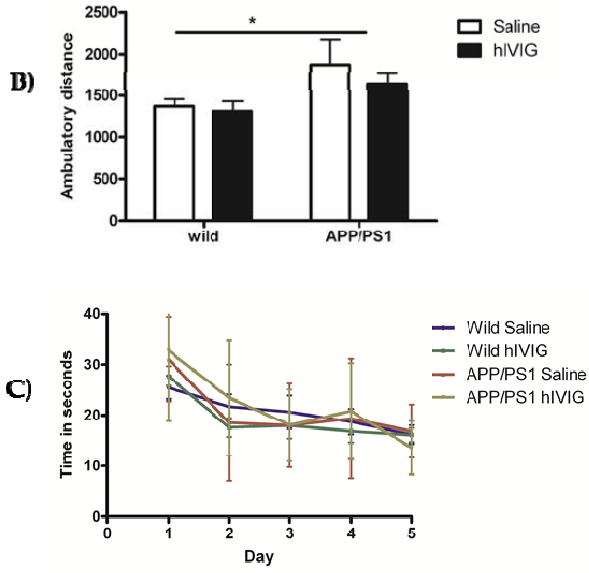


Figure 5.1.4b. Mice were subjected to a battery of behavioural tests assessing **(A)**neophobia (marble test), **(B)** locomotor activity (TrueScan) and **(C)** spatial memory (Morris water maze). * indicates significant difference between wild and APdE9 genotypes, $p < 0.05$

5.1.4c Effects of 8-month hIVIG treatment on amyloid pathology

Interestingly, when compared to saline group, chronic hIVIG treatment resulted in significant elevation of soluble Aβ40 ($t_{(29)} = 2.32$; $p = 0.03$) and Aβ42 levels ($t_{(29)} = 2.78$; $p = 0.009$) by 36.3 % and 50.3 % respectively (figure5.1.4c-A, B). After removing data from mice that produced significant titres of anti-human antibodies, elevation of soluble Aβ42 levels still remained significant ($t_{(22)} = 2.2$; $p = 0.038$), displaying a 49.4 % increase compared to saline group. However, soluble Aβ40 levels, even though displaying a 29.5 % increase, did not reach statistical significance. The levels of insoluble Aβ40 and Aβ42 in hIVIG treated group were elevated by 11 % and 10 %, respectively, when compared to saline groups. This change did not reach statistical significance (insoluble Aβ40: $t_{(29)} = 1.28$; $p = 0.21$ and insoluble Aβ42: $t_{(29)} = 1.85$; $p = 0.07$) (figure5.1.4c-C, D). Despite a significant increase in the biochemical levels of Aβ, the volume of diffuse amyloid plaques (figure 5.1.4c-E) and dense-core amyloid deposits (figure 5.1.4c-F) in the hippocampus did not differ significantly between saline and hIVIG groups (diffuse plaques: $t_{(29)} = 0.91$; $p = 0.37$ and dense-core plaques: $t_{(28)} = 0.73$; $p = 0.47$). Similarly, serum levels of Aβ40 (figure 5.1.4c-G) did not show significant differences between saline and hIVIG treated groups ($t_{(26)} = 0.09$; $p = 0.92$). Furthermore, hippocampal levels of Aβ40 and Aβ42 showed no correlation with serum Aβ40 levels (Pearson $r_{(12)} = 0.29$, $p = 0.31$; Pearson $r_{(12)} = 0.32$, $p = 0.26$, respectively).

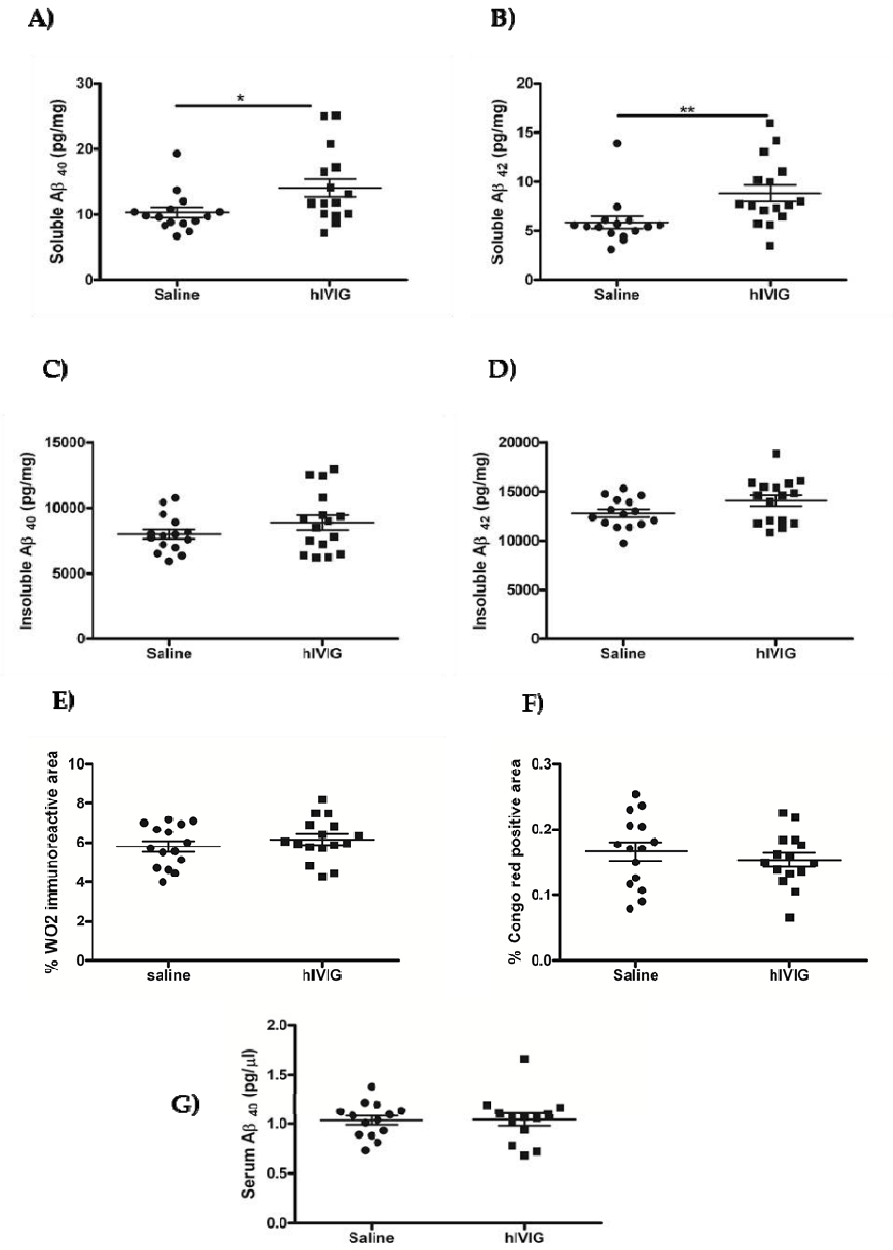
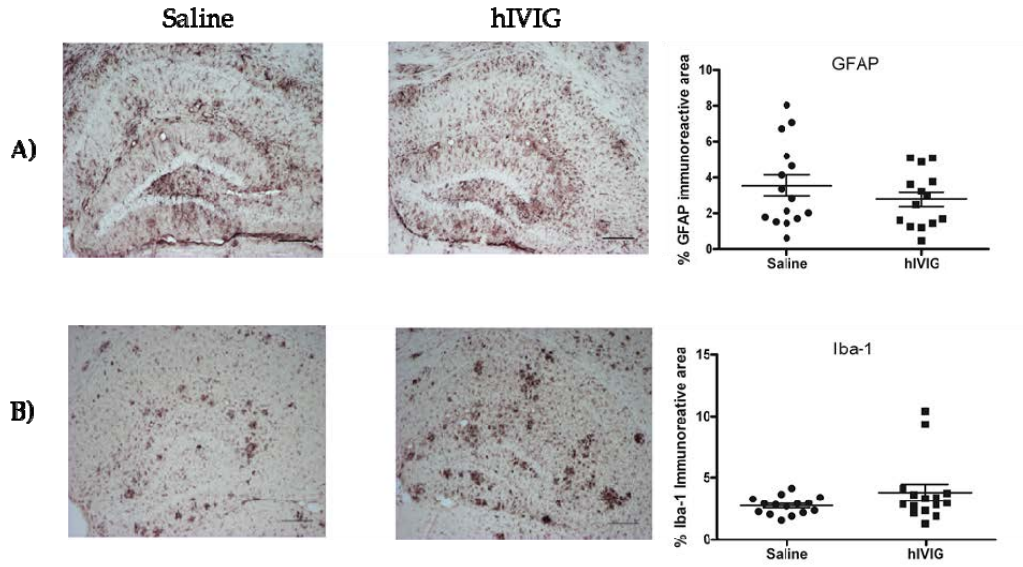


Figure 5.1.4c. Chronic hIVIg treatment (hIVIG-6b study) elevated hippocampal **(A)** soluble Aβ40 and **(B)** $A\beta_{42}$ levels. **(C)** Insoluble $A\beta_{40}$ and **(D)** $A\beta_{42}$ levels **(E)** Aβ plaque burden as measured by WO2 immunohistology, **(F)** amyloid burden as measured by congo red staining and **(G)** serum $A\beta_{40}$ levels did not show statistically significant changes due to chronic hIVIG treatment. * indicates $p<0.05$, ** indicates $p<0.01$. Mice were 12 month old at the time of sacrifice.

5.1.4d Effects of 8-month hIVIG treatment on glial pathology

We next went on to assess chronic 8-month hIVIG treatment effects on astrocytes and microglia. Astrocyte reactivity in the hippocampus as detected by GFAP immunostaining (figure 5.1.4d-A) did not differ between saline and hIVIG treated animals ($t_{(27)} = 1.03$; $p = 0.31$). Microglial reactivity was assessed by three different markers, Iba-1, CD68 and CD45. Iba-1, a structural marker for microglia, displayed a 39.2 % elevation in hIVIG treated mice compared to saline controls (figure 5.1.4d-B), but this difference did not reach statistical significance ($t_{(28)} = 1.54$; $p = 0.13$). Macrophage lysosomal marker CD68 immunohistochemistry (figure 5.1.4d-C) as well did not differ between saline and hIVIG treatments ($t_{(27)} = 0.41$; $p = 0.68$). In contrast, CD45, a marker for activated microglia (figure 5.1.4d-D) revealed a statistically significant difference between saline and hIVIG treated groups ($t_{(28)} = 3.57$; $p = 0.001$). CD45 immunoreactive area of hIVIG group (2.79 ± 0.22, mean ± SEM) was 36.4% lower than the mean of the saline group (4.39 ± 0.40). To rule out the possibility that this suppression of CD45 immunoreactivity was a consequence of development of anti-human IgG antibodies in hIVIG treated mice, we excluded data from hIVIG treated mice that developed anti-human IgG antibodies (titres > 200). However, suppression of CD45 immunoreactivity (by 38 %) still remained statistically significant ($t_{(22)} = 3.01$; $p = 0.006$). Furthermore, a moderate inverse correlation was detected between CD45 immunoreactive areas and soluble $A\beta_{42}$ levels (Spearman $r_{(28)} = -0.43$, $p = 0.02$).



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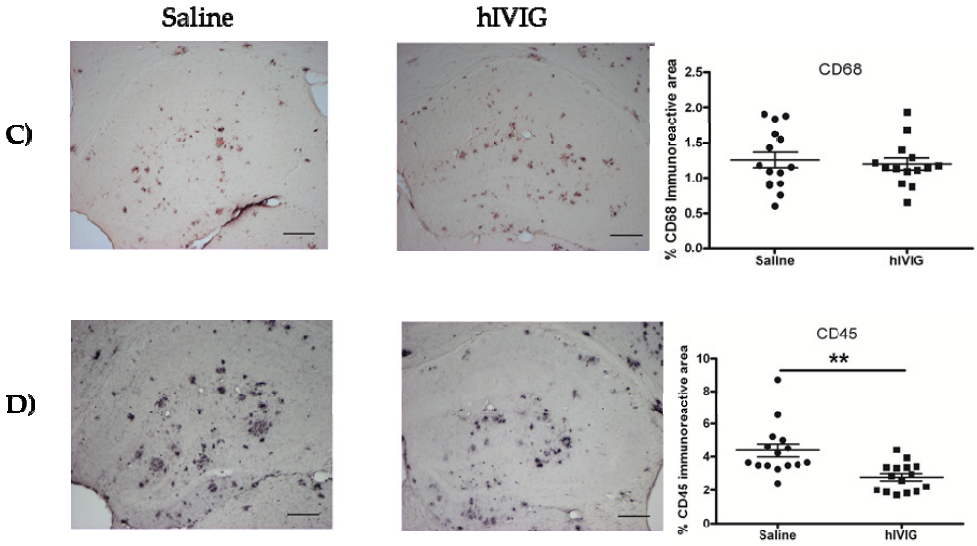


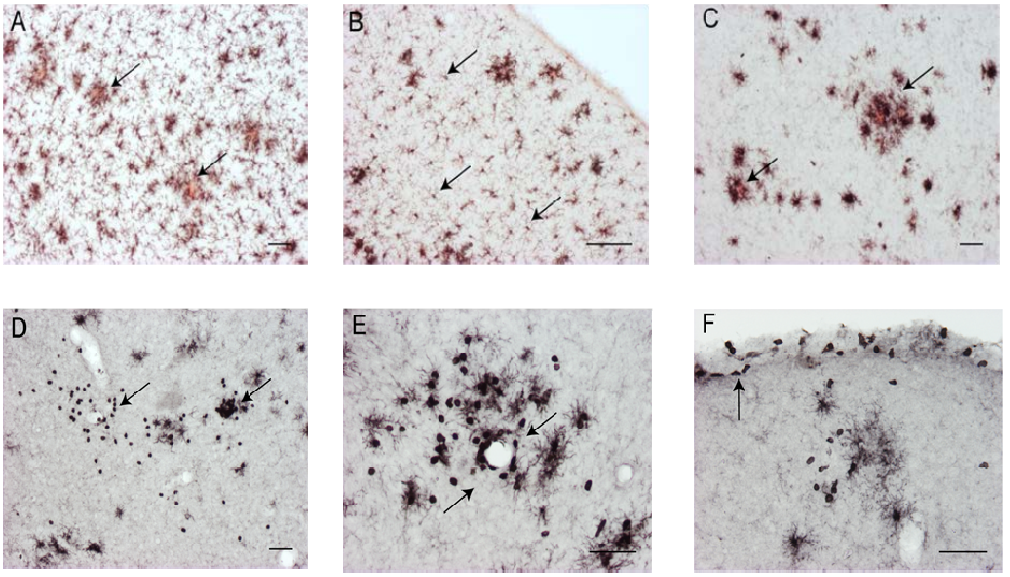
Figure 5.1.4d. Scale bars represe 200µm. Astrocytes reactivity was assessed by **(A)** GFAP imuunostaining and microglia were quantified by **(B)** Iba-1, **(C)** CD68 and **(D)** CD45 immunohistology. ** indicates $p<0.01$. All quantifications were in hippocampal region and mice were 12 month old at end of treatment period.

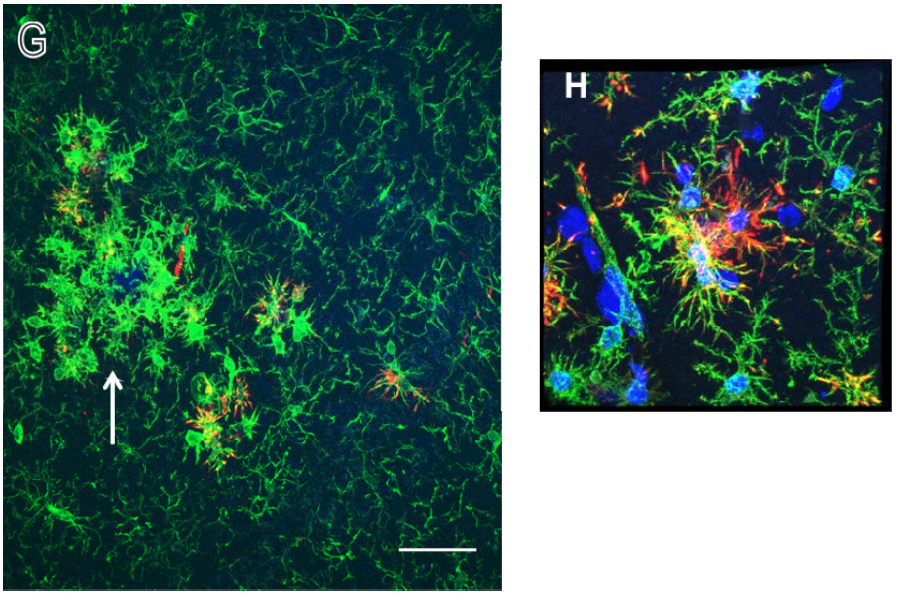
5.1.4e Chronic 8-month hIVIG treatment brings subtype specific changes to microglia

In our brain sections, microglia residing both far and near the Aβ deposits were stained by Iba-1 marker, although with different staining intensities (figure 5.1.4e-A & B). Microglia near Aβ plaques (activated) were much more intensely stained than those away from plaques (quiescent). Additionally, microglia near amyloid plaques exhibited larger cell bodies with short, thick processes, whereas those away from plaques display cell bodies that are smaller in size with relatively much thinner processes. Quiescent (resting) microglia could be found scattered ubiquitously in brain parenchyma that is devoid of any Aβ plaques whereas activated microglia can be found clumped together as clusters in the immediate vicinity of amyloid plaque microenvironment, resembling a barricade of cells contiguous to Aβ deposits. In short, Iba-1 antibody identified two distinct populations of microglia, activated and quiescent (resting) microglia (figure 5.1.4e-A, B). Another microglia marker, CD45, identified specifically activated microglia. One can easily notice intensely immunostained CD45 microglia surrounding Congo red positive amyloid deposits (figure 5.1.4e-C). Under light microscope, these usually appear as clusters of large spider-like cells with short and thick processes. Microglia of this kind of phenotype were not found in areas devoid of plaques or even in normal wild-type type mice. Additionally, CD45 marker identified small round cells (figure 5.1.4e-D, E, F) with extremely intense CD45 immunoreactive signal (many-fold higher than spider-like microglia), largely in clusters or sometimes singly scattered in the cortex, hippocampus and white matter. These cells were found in large numbers surrounding fimbria, and in areas adjoining choroid plexus, ventricles and meninges. These seemingly migratory cells were also frequently found in close proximity to blood vessels. In short, CD45

identifies two morphologically dissimilar phenotypes of activated microglia. Taken together, both Iba-1 and CD45 antibody markers label different sub-populations of microglia with distinct morphologies and activation phenotypes. This largely conforms to previously reported microglial heterogeneity (Lawson et al., 1990).

In order to separate treatment effects in different cell populations, we immunostained microglia with CD45 and Iba-1 fluorescent markers then, measured individual microglial cells on confocal Z-stacks for their CD45 and Iba-1 intensities (figure 5.1.4e-G, H). The mean integrated intensities of CD45 and Iba-1 markers across the entire Z-axis of each microglia were plotted against the circularity (morphological parameter). This 3-D scatter plot identified three different sub-populations of microglia as shown in figure 5.1.4e-I**.** Statistically, two-step cluster analysis as well revealed three different clusters of cells with an average silhouette equal to 0.7. In addition, the nonparametric Kruskal-Wallis H-test rejected the null hypothesis ($p < 0.001$) that there are no sub-populations of microglia. We call these three clusters of cells simply as type A, B and C (figure 5.1.4e-I)**.** Type A cells seen in the plot have a round soma with no processes, and exhibit very high CD45 immunostaining intensity but negligible Iba-1a staining. These cells correspond to round cells illustrated in figure 5.1.4e-D, E, F.Their number increased dramatically from 4 to 12 months of age, and much faster in APP/PS1 mice than in wild-type-type control mice (figure 5.1.4e-J). *Prima facie*, they might be infiltrating immune cells, although it is difficult to ascertain their exact peripheral origin in this particular experimental setup. Chronic hIVIG treatment did not significantly affect CD45 expression in type A cells ($U = 2117$, $p = 0.99$, Mann-Whitney U test). Type B cells were characterized by large oval cell bodies and thick branching processes, and exhibit substantial CD45 and Iba-1 signal. These cells were located in the immediate microenvironment of amyloid plaques and likely represent activated microglia around Aβ deposits (Naert and Rivest, 2011). Mann-Whitney U-test revealed significantly reduced expression of CD45 ($U = 15605$, $p < 0.02$) and elevated expression of Iba-1 ($U = 13972$, $p < 0.0001$) in type B cells as a result of chronic hIVIG treatment. Lastly, Type C cells showed a much more variable cell morphology. The cell bodies were relatively small and the processes much thinner than in type B cells. Type C cells exhibited substantial Iba-1a expression but practically no CD45 signal. These cells were found equally often around Aβ deposits and in plaque-free tissue. Those away from the plaques likely represent cells referred to in the literature as quiescent microglia (Kettenmann et al., 2011; Naert and Rivest, 2011). Iba-1a expression was significantly elevated in Type C cells as a consequence of chronic hIVIG treatment ($U = 12795$, $p = 0.0001$). Especially, the cells appeared to possess much longer processes. Collectively, these observations indicate that chronic hIVIG treatment brings about specific changes in subpopulations of microglia/macrophages.





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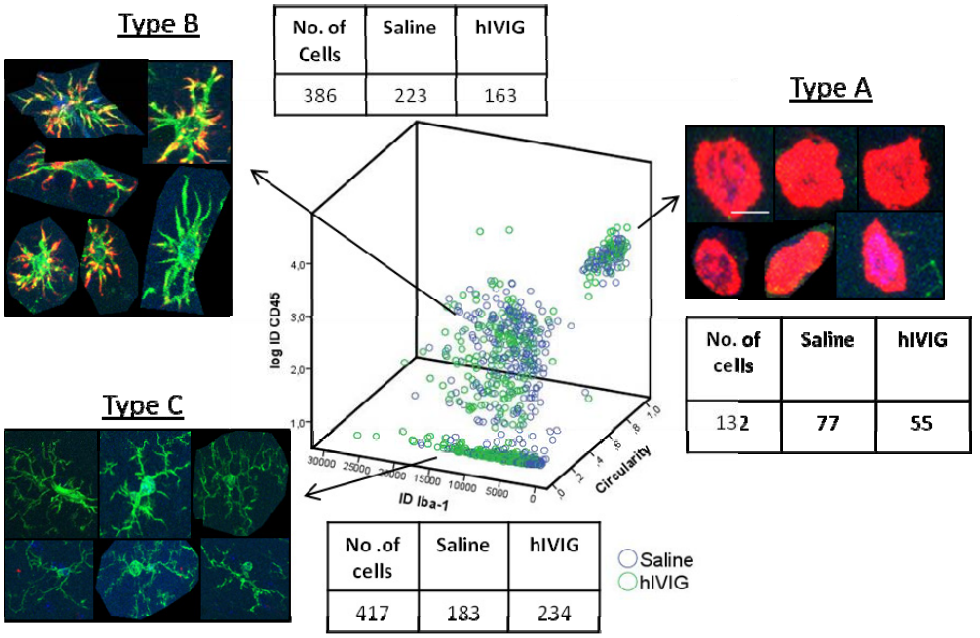


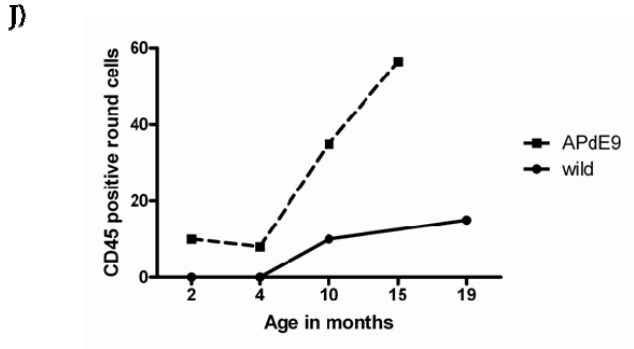
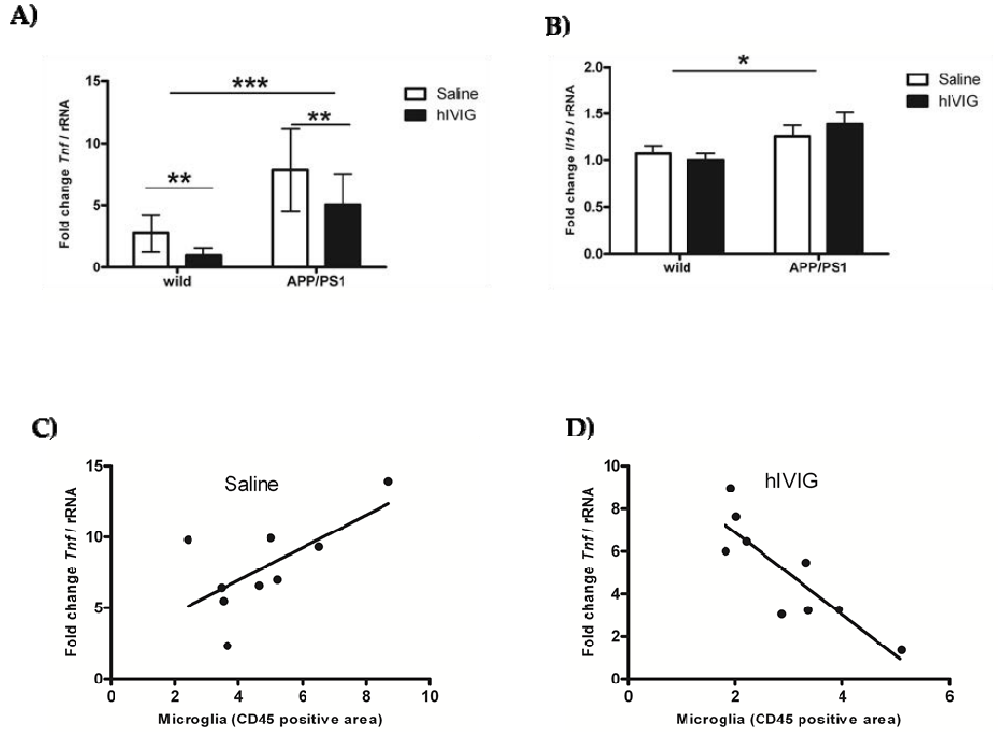


Figure 5.1.4e. Scale bars represent 500 µm unless otherwise stated. Iba-1 marker for microglia stains both **(A)** activated and **(B)** non-activated microglia phenotypes. See black arrows. **(C)** Only activated spider-like phenotype of microglia are identified by CD45 marker. In addition, CD45 also identifies a phenotype of round microglial cells **(D,E,F)** near blood vessels, meninges and ventricles. Double immunoflourescent stainings **(G, H,** Scale bar 50 µm**)** with both Iba-1 (green) and CD45 (Red) markers reveal all the above microglia phenotypes simultaneously. Quantification of complete single cell confocal images across the Z-axis for integrated intensties and plotting them on a 3-D scatter plot **(I,** Scale bar = 5 µm**)** reveals three different sub-populations of microglia namely, Type A, Type B and Type C cells. **(J)** Age dependent increase of Type A cells in both wild and APdE9 mice.

5.1.4f Chronic hIVIG treatment suppresses *Tnf* gene expression

Having uncovered a subpopulation specific effect of hIVIG on microglia, which are important cellular source of inflammatory cytokines, we utilised RT-PCR technique to assess the gene expression of two most important pro-inflammatory cytokines, *Tnf* and *Il1b*. Two-way ANOVA revealed a significant genotype ($F_{(1, 30)} = 34.3$; $p < 0.0001$) and treatment effects ($F_{(1, 30)} = 8.57$; $p = 0.0065$) for *Tnf* (figure 5.1.4f-A); however no significant interaction between the factors ($F_{(1, 30)} = 0.46$; $p = 0.5$) was seen. For *Il1b* (figure 5.1.4f-B), a significant genotype effect ($F_{(1, 31)} = 7.24$; $p = 0.01$) was found but no significant treatment effect ($F_{(1, 31)} = 0.07$; $p = 0.79$) or interaction between the factors ($F_{(1, 31)} = 0.86$; $p = 0.36$). Interestingly, in saline treated mice a trend for positive correlation (figure 5.1.4f-C) was seen between CD45 microglia (immunopositive areas) and *Tnf* expression (Pearson $r_{(7)} = 0.66$, $p = 0.05$). In contrast, chronic hIVIG treatment flipped this to a negative correlation (figure 5.1.4f-D) which was statistically significant (Pearson $r_{(7)} = -0.85$, $p = 0.003$). Similarly for *Il1b*, a trend for positive correlation (Pearson $r_{(8)} = 0.59$, $p = 0.09$) in saline treated mice changed to a statistically significant negative correlation (Pearson $r_{(8)} = -0.76$, $p = 0.01$) in hIVIG treated mice (figure 5.1.4f-E, F).



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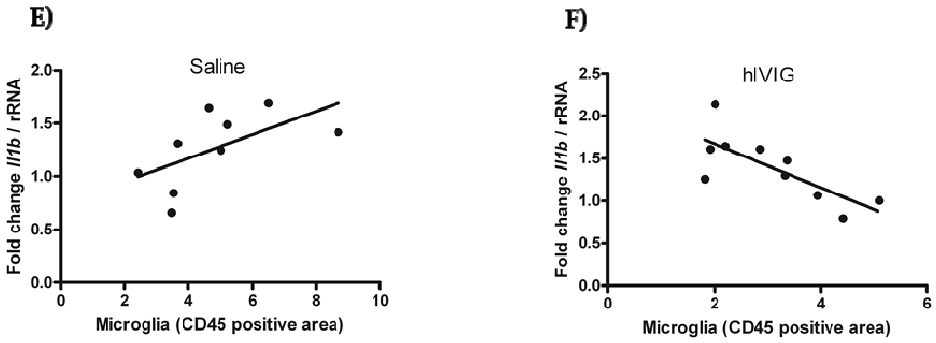


Figure 5.1.4f. Quantitative gene expression analysis by RT-PCR of **(A)***Tnf* and **(B)** *Il1b*. Positive correlation in saline treated mice **(C)** between TNF-α expression and CD45 microglia flips towards a negative correlation in hIVIG treated mice **(D)**. Similarly, positive correlation in saline treated mice **(E)** between IL-1β gene expression and CD45 microglia flips towards a negative correlation in hIVIG treated mice **(F)**. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$

5.1.4g Chronic hIVIG treatment increases the number of newborn cells in the dentate gyrus

We immunostained mouse brain sections with doublecortin, a marker for newborn neurons. Two-way ANOVA revealed statistically significant genotype ($F_{(1, 34)} = 18.9$, $p = 0.0001$) and treatment effects ($F_{(1, 34)} = 10.0$, $p = 0.003$) in the number of doublecortin positive neurons (figure 5.1.4g-A). However, the interaction between the factors ($F_{(1, 34)} = 1.18$, $p = 0.3$) was not significant. A 8-month high-dose hIVIG treatment resulted in nearly 60 % increase in the number of doublecortin positive neurons in both APP/PS1 and wild-type mice to a similar extent, indicating that this strong neurogenic effect is independent of *APP* or *PSEN1* transgenes or associated amyloid deposition. Furthermore, there was a significant inverse correlation (figure 5.1.4g-B) between hippocampal CD45 microglia (immunoreactive area) and the number of doublecortin positive cells in the dentate gyrus (Spearman $r_{(18)} = -0.50$, $p = 0.02$), suggesting that these two variables are associated with each other. In other words, a high number of activated microglia associates with a low number of newborn neurons.

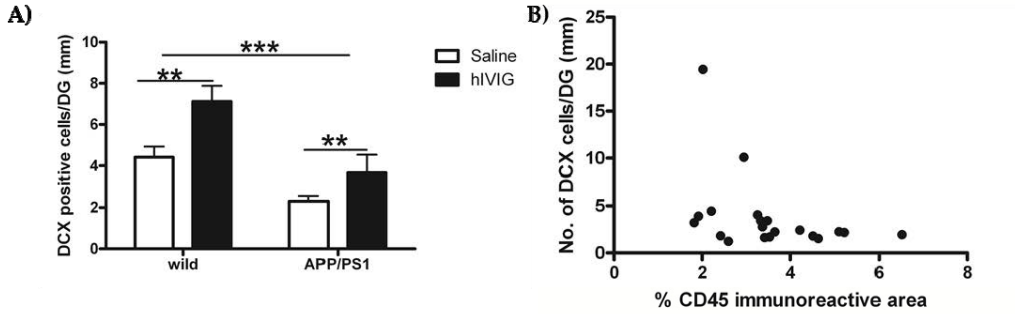


Figure 5.1.4g. **(A)** Number of new born neurons as identified by doublecortin antibody marker. **(B)** A negative correlation can be seen between CD45 positive microglia and the number of new born cells as marked by doublecortin immunohistochemistry. ** indicates $p<0.01$ and *** indicates $p<0.001$

5.2 ROLE OF *Nfkb1* GENE ON PROGRESSION OF AMYLOID AND INFLAMMATORY PATHOLOGY (NFKB STUDY)

In this study we crossed NFκB gene knockout (*Nfkb1*$^{-/-}$) mice with APP/PS1 mice, thereby creating a mouse line deficient in *Nfkb1* gene on the APP/PS1 background. This allowed us to study the effects of *Nfkb1* gene on the development of AD pathology. Since *Nfkb1* gene is ubiquitously knocked out from all cells, it will be hard to ascribe the effects solely either to neurons or microglia. In addition, NFκB regulates a number of functions in astrocytes, endothelial cells and the peripheral organs that regulate systemic immune reactions and inflammation. Moreover, there also exists considerable functional redundancy among the various subunits of NFκB transcription factors. Lastly, beneficial or detrimental effects of NFκB activation depend on a variety of factors, such as spatial and temporal relationships of the activating stimuli, cell type, and the presence of molecular mediators which might result in signalling cross-talks. Table 5.2 summarizes all key findings of NFκB study. *Nfkb1* gene deficiency in resulted in a distinct behavioural phenotype. *Nfkb1*$^{-/-}$ mice displayed smaller body weights, better motor coordination and were less anxious than their controls. However, gene deletion did not worsen or improve spatial memory deficits associated with APP/PS1 genotype. Effects of *Nfkb1* gene deletion had very limited effects on development and progression of amyloid pathology. Interestingly, at 12 month age, *Nfkb1* gene deficiency caused a dramatic decrease in CD45 microglia activation with concominant increase in *Tnf* gene expression.

Table 5.2. Bird's eye view of key NFκB study findings.

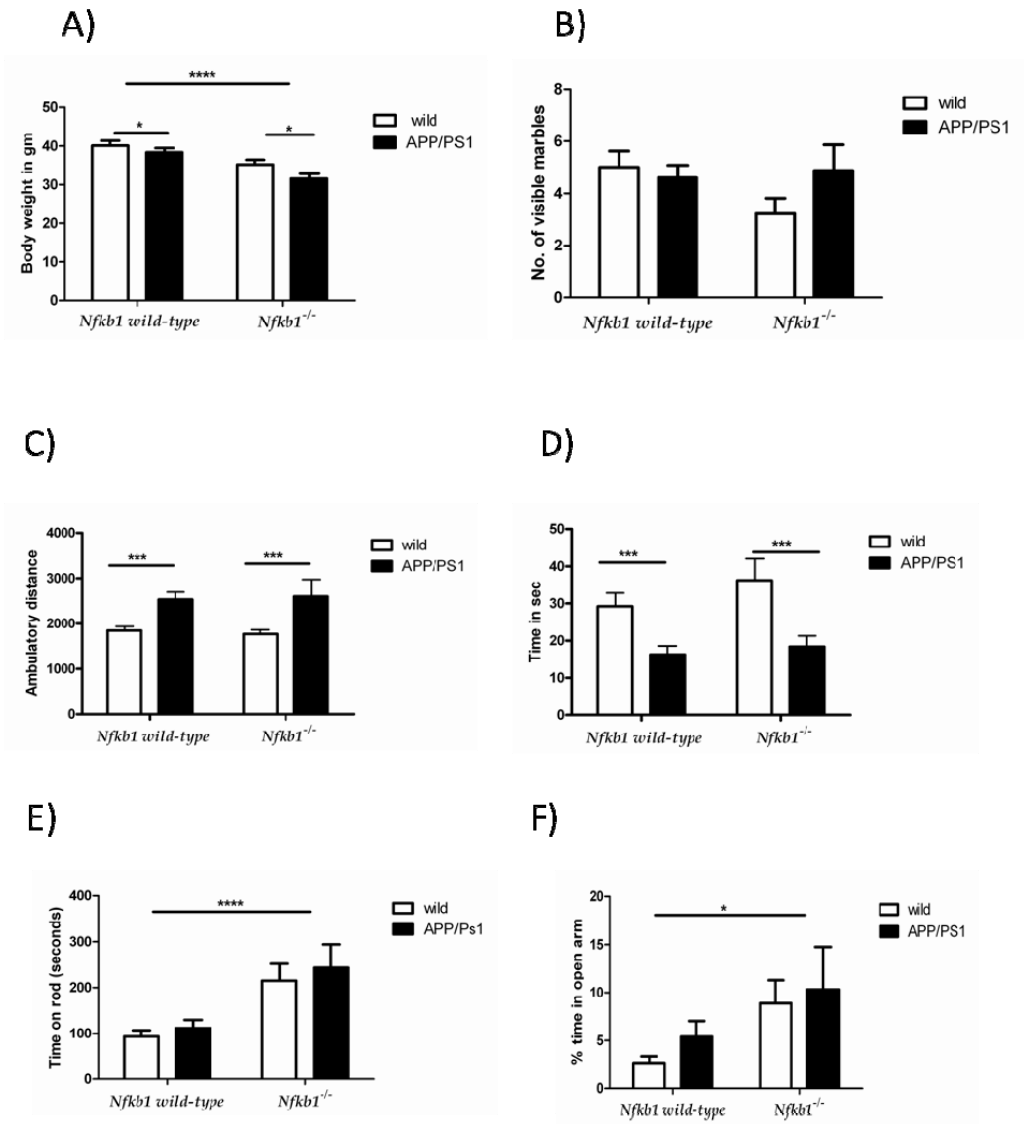
Experiment	Mouse behavioural phenotype	Amyloid pathology	Microglial pathology	Proinflammatory gene expression
3.5 month	Normal	No changes	--	--
8 month	Normal	1. Female APP/PS1 mice show more Aβ pathology. 2. No effect of *Nfkb1*$^{-/-}$ genotype.	1. Female APP/PS1 mice show more CD45 microglial activation. 2. No effect of *Nfkb1*$^{-/-}$ genotype.	--
12 month	1. Normal behaviour. 2. Both APP/PS1 and *Nfkb1*$^{-/-}$ genotypes resulted in lesser body weights. 3. APP/PS1 mice more active. 4. *Nfkb1*$^{-/-}$ genotype display better motor cordination on	1. Insoluble $A\beta_{42}$ decreased in *Nfkb1*$^{-/-}$ genotype by 14%. 2. No changes in Aβ plaques.	1. CD45 microglial activation decreased by 56% in *Nfkb1*$^{-/-}$ genotype. 2. No change in CD68 microglia marker. 3. Iba-1 more in APP/PS1 mice. 4. No *Nfkb1*$^{-/-}$ effect on Iba-1 microglial	1. Both *Nfkb1*$^{-/-}$ and APP/PS1 genotype elevated *Tnf* gene expression. Significant interaction between genotypes. 2. *Nfkb1*$^{-/-}$ genotype displayed

	rotating rod. 5. *Nfkb1*$^{-/-}$ genotype display decreased anxiety. 6. *Nfkb1*$^{-/-}$ genotype did not improve or worsen spatial memory deficits of APP/PS1 mice.		marker.	significant interaction with APP/PS1 genotype.
LPS experiment	Higher mortality in *Nfkb1*$^{-/-}$ genotype	LPS reduced diffuse Aβ but no effect of *Nfkb1*$^{-/-}$ genotype.	1. Significant decrease in Iba-1 signal due to LPS injection in *Nfkb1*$^{-/-}$ genotype. 2. No changes to CD68 microglia marker in *Nfkb1*$^{-/-}$ mice.	--

5.2.1a Behavioral phenotype of *Nfkb1* gene knockout

Breeding of *Nfkb1*$^{-/-}$ and APP/PS1 mice was successful, and pups were viable. These mice appeared normal, although they were much leaner than their wild-type-type controls. Indeed at 12 months of age, two-way ANOVA displayed a significant effect on body weight (decrease) of both APP/PS1 ($F_{(1, 42)} = 4.42$, $p = 0.042$) and *Nfkb1*$^{-/-}$ genotypes ($F_{(1, 42)} = 20.67$, $p < 0.0001$; figure 5.2.1a-A). However, there was no interaction between the two genotypes ($F_{(1, 42)} = 0.56$, $p = 0.46$).

Spontaneous motor/exploratory activity of mice was determined by automated activity monitor based on infrared detection (TrueScan). Two-way ANOVA revealed hyperactivity (figure 5.2.1a-C) due to the APP/PS1 genotype ($F_{(1, 41)} = 15.18$, $p < 0.0001$) but not effect of the *Nfkb1* genotype (APP/PS1: ($F_{(1, 41)} = 0.50$, $p = 0.48$). As seen in figure 5.2.1a-D APP/PS1 genotype was also associated with less time engaged in stereotypic movements ($F_{(1, 41)} = 19.5$, $p < 0.0001$) and decreased rearing times ($F_{(1, 41)} = 7.76$, $p = 0.008$), while the *Nfkb1*$^{-/-}$ genotype had no effect on either parameter (Stereotypy: $F_{(1, 41)} = 0.12$, $p = 0.73$ & rearing time: $F_{(1, 41)} = 2.1$, $p = 0.15$). Motor coordination and balance were evaluated in the rotarod test. The *Nfkb1*$^{-/-}$ genotype was associated with a longer time ($F_{(1, 42)} = 20.84$, $p < 0.0001$) on the rotating rod figure 5.2.1a-E, while the APP/PS1 genotype had no effect on the performance of this test (APP/PS1: $F_{(1, 42)} = 0.70$, $p = 0.40$). Also, there was no interaction between the genotypes ($F_{(1, 42)} = 0.03$, $p = 0.86$).



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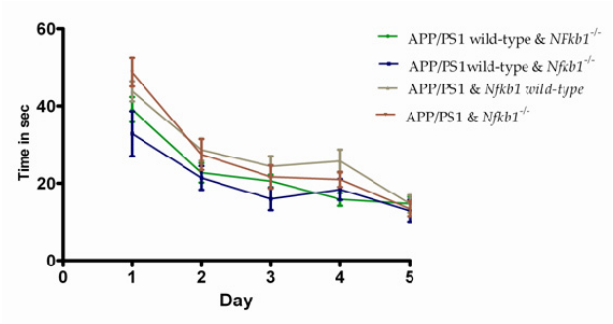


Figure 5.2.1a. Behavioural assessment of *Nfkb1* gene deficiency in APP/PS1 mice. **(A)** Body weights of male mice at 12-month age. **(B)** Marble test for neophobia. Locomotor activity of mice as indicated by **(C)** ambulatory distance travelled and **(D)** time spent on stereotypy. **(E)** Motor coordination as evaluated by Rotarod Test. **(F)** Anxiety evaluated by elevated plus maze test. **(G)**Evaluation of spatial memory by Morrs water maze.

Anxiety-like behaviour was assessed with a simple marble burying test that relies on innate behaviour of mice to cover potentially dangerous novel objects with the cage bedding. The numbers of visible or untouched marbles overnight were counted. There was no effect figure 5.2.1a-B of APP/PS1 ($F_{(1, 42)} = 0.89$, $p = 0.35$) or *Nfkb1*$^{-/-}$ ($F_{(1, 42)} = 1.280$, $p = 0.26$) genotype nor any interaction between the genotypes ($F_{(1, 42)} = 2.39$, $p = 0.13$). In elevated plus maze, another test for anxiety, the *Nfkb1*$^{-/-}$ genotype was associated with increased time on the open arm ($F_{(1, 42)} = 6.42$, $p = 0.015$), indicating decreased anxiety (figure 5.2.1a-F). In contrast, the APP/PS1 genotype was without effect on this parameter ($F_{(1, 42)} = 0.9$, $p = 0.35$).

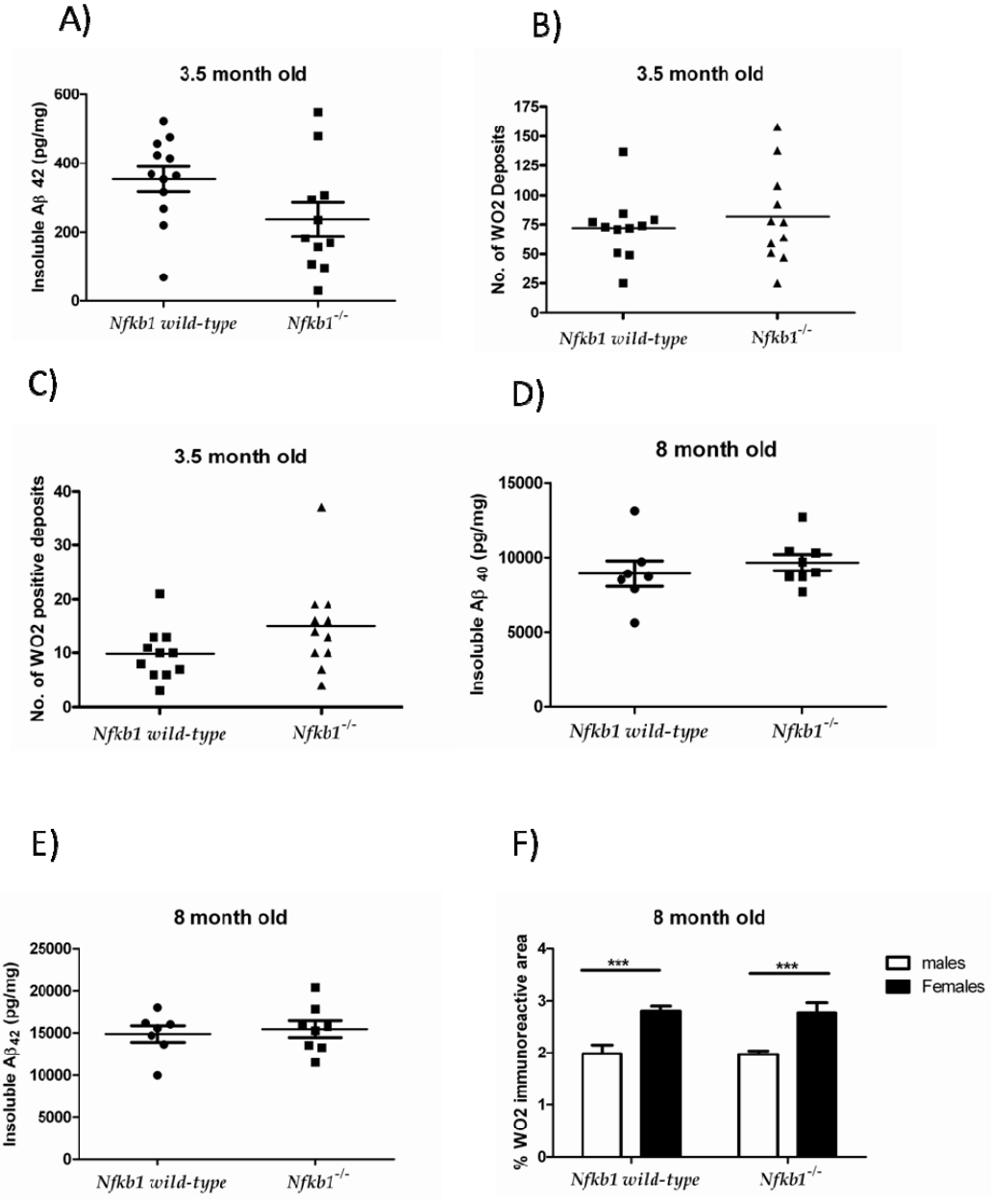
In Morris water maze test for spatial learning and memory figure 5.2.1a-G, as expected the APP/PS1 genotype was associated with significantly increased escape latencies ($F_{(1, 42)} = 11.79$, $p = 0.001$), indicative of impaired learning. In contrast, the *Nfkb1*$^{-/-}$ genotype did not affect the escape latency ($F_{(1, 42)} = 0.002$, $p = 0.96$), and there was no interaction between the two genotypes ($F_{(1, 42)} = 0.5$, $p = 0.49$). Based on this behavioural test battery, knocking down the *Nfkb1* gene did induce a characteristic phenotype (decreased anxiety and improved motor coordination and balance). However, the effect of *Nfkb1*$^{-/-}$ on behavioural was independent of APP/PS1 transgene effects.

5.2.1b Impact of *Nfkb1* gene knockout on amyloid pathology

Mice with *APP/PS1* transgene deposit their first Aβ plaques around 3-4 months of age (Garcia-Alloza et al., 2006). In order to see whether knocking down the *Nfkb1* gene had any impact on early plaque deposition, fresh brain samples from mice at 3.5 months of age were processed with routine biochemical and immunohistochemistry procedures. At this age, brain levels of insoluble $A\beta_{42}$ from females mice showed no statistically significant genotype differences (figure 5.2.1b-A), although there was a trend towards decreased levels in *Nfkb1*$^{-/-}$

mice ($t_{(21)} = 1.97$; $p = 0.06$). Counting the number of W02 positive Aβ plaques in the cortex and hippocampus of APP/PS1 mice (figure 5.2.1b-B&C) revealed no statistically significant differences between *Nfkb1*$^{-/-}$ mice and *Nfkb1* wild-type carriers (cortex: $t_{(20)} = 0.65$; $p = 0.52$; hippocampus: $t_{(20)} = 1.72$; $p = 0.1$). Fibrillar Aβ as detected by Congo red staining was not performed for mice of this age group. Overall,this indicates that lack of *Nfkb1* gene may have a very limited role in early Aβ plaque deposition.

We next went on to check amyloid pathology at 8 months of age. Levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ (figure 5.2.1b-D&E) in the hippocampus of male APP/PS1 mice revealed no statistically significant differences due to the *Nfkb1* genotype ($A\beta_{40}$: $t_{(13)} = 0.75$; $p = 0.46$; $A\beta_{42}$: $t_{(13)} = 0.42$; $p = 0.68$). Cortical and hippocampal Aβ plaque burden in male and female APP/PS1 mice was assessed with W02 immunohistology (figure 5.2.1b-F&G). Two-way ANOVA revealed a significant main effect of sex ($F_{(1, 28)} = 40.08$, $p < 0.0001$) but no main effect of *Nfkb1* genotype ($F_{(1, 28)} = 0.04$, $p = 0.85$) or an interaction between sex and *Nfkb1* genotype ($F_{(1, 28)} = 0.008$, $p = 0.93$) on amyoid plaque burden. Interestingly, hippocampal W02 burden revealed significant main effects of both sex ($F_{(1, 28)} = 11.32$, $p = 0.002$) and *Nfkb1* genotype ($F_{(1, 28)} = 6.41$, $p = 0.02$), but there no interaction between these factors ($F_{(1, 28)} = 0.12$, $p = 0.73$). Fibrillar Aβ as detected by Congo red staining was not performed for mice of this age group.



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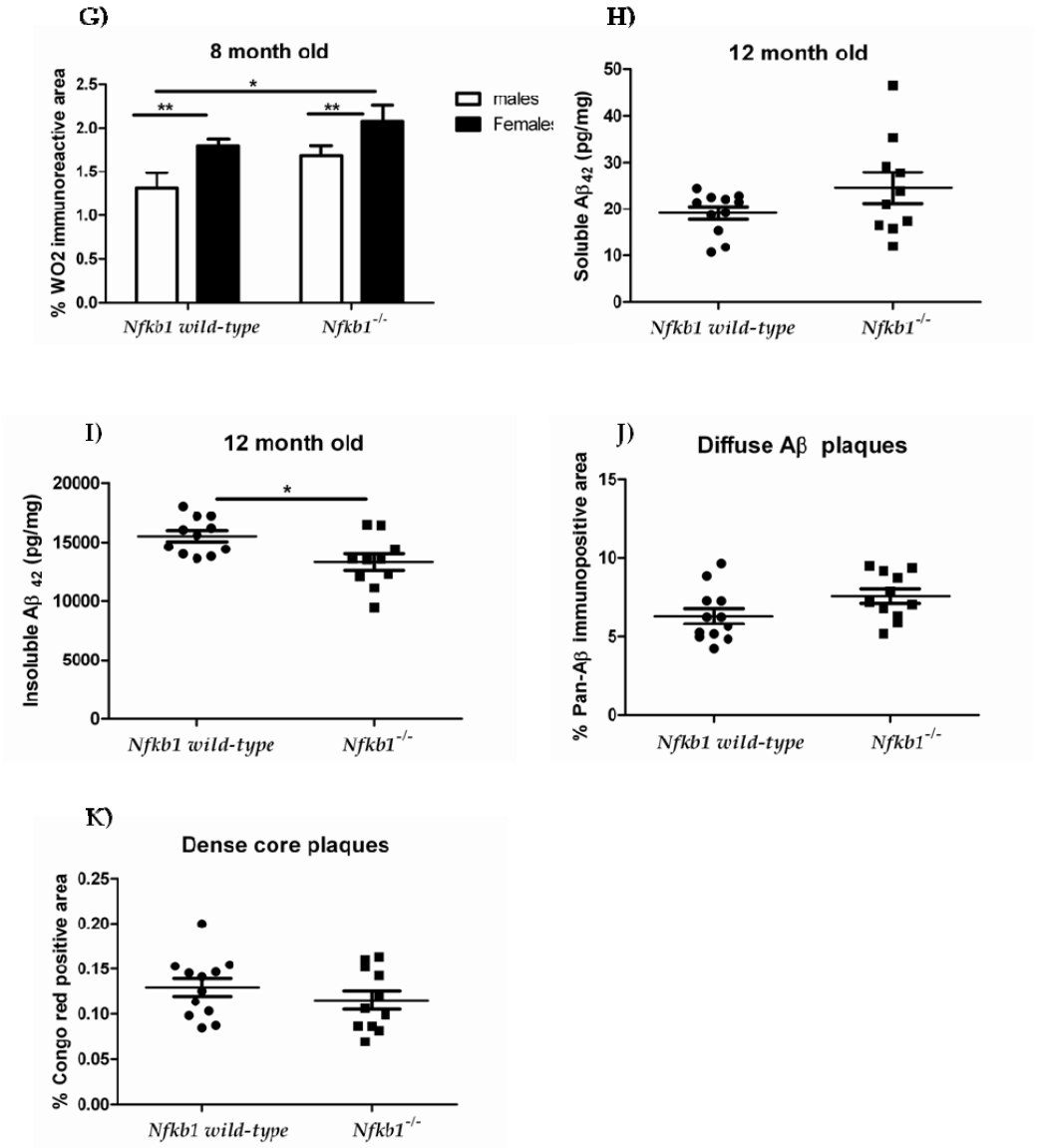

Figure 5.2.1b. Age dependent progression of amyloid pathology. Biochemical levels of **(A)** insoluble $A\beta_{42}$ levels and **(B)** WO_2 positive deposits in cortex and **(C)** hippocampus of 3.5 month old female APdE9 mice. Insoluble levels of hippocampal **(D)** $A\beta_{40}$ and **(E)** $A\beta_{42}$ from 8-month old male mice. Quantification of Aβ plaques from **(F)** cortex and **(G)** hippocampus of 8-month old mice. ELISA measurements of hippocampal **(H)** soluble and **(I)** insoluble $A\beta_{42}$ levels from 12-month old mice. Similarly, quantification of Aβ plaques at 12-month detected by **(J)** pan-Aβ immunostaining and **(K)** amyloid deposits by congo red histological stain. * indicates $p < 0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

At the age of 12 months (figure 5.2.1b-H), hippocampal soluble Aβ42 in male APP/PS1 mice did not differ significantly between *Nfkb1*$^{-/-}$ and *Nfkb1* wild-type carriers ($t_{(19)} = 1.56$; $p = 0.14$). Interestingly, insoluble Aβ42 (figure 5.2.1b-I) showed a 14 % drop in *Nfkb1*$^{-/-}$ mice, which was statistically significant ($t_{(19)} = 2.70$; $p = 0.01$). However, immunohistochemical quantification of plaque burden (figure 5.2.1b-J&K) in these mice showed no statistically significant difference between the *Nfkb1* genotypes (Pan Aβ antibody: $t_{(21)} = 1.96$; $p = 0.07$; Congo red amyloid staining: $t_{(21)} = 0.99$; $p = 0.33$). In conclusion, there was only a slight and age-dependent effect of *Nfkb1* deficiency on brain amyloid levels.

5.2.1c Impact of *Nfkb1* gene knockout on microglia and pro-inflammatory cytokine gene expression

To assess if microglial activation was influenced by absence of *Nfkb1* gene, we immunostained free-floating brain sections with microglia specific CD45, Iba-1a and CD68 primary antibodies. Two-way ANOVA of CD45 immunoreactivity in the cortex and hippocampus of 8-month-old APP/PS1 mice (figure 5.2.1c-A&B) revealed no statistically differences between *Nfkb1*$^{-/-}$ and *Nfkb1* wild-type carriers (cortex, $F_{(1, 28)} = 1.33$, $p = 0.26$; hippocampus, $F_{(1, 28)} = 2.62$, $p = 0.12$), but revealed a significant gender effect, with females exhibiting elevated CD45 immunoreactivities (cortex, $F_{(1, 28)} = 11.74$, $p = 0.001$; hippocampus $F_{(1, 28)} = 11.64$, $p = 0.002$). Also, there was no interaction between gender and *Nfkb1* genotype (cortex: $F_{(1, 28)} = 1.25$, $p = 0.27$; hippocampus: $F_{(1, 28)} = 0.32$, $p = 0.57$).

A)

B)

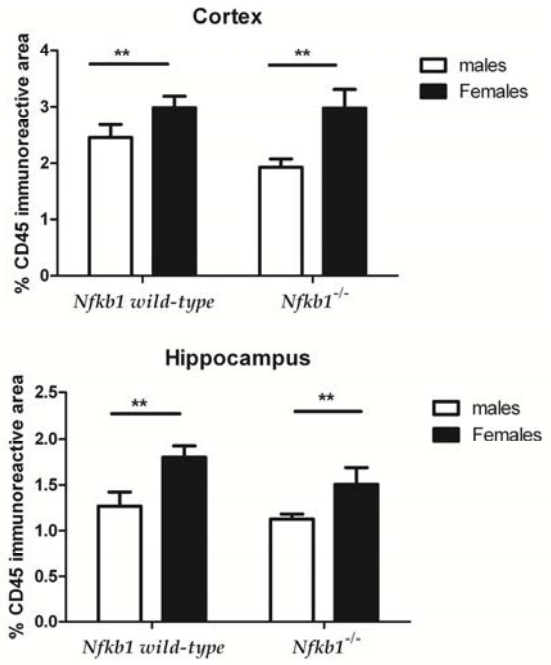

Figure 5.2.1c. Microglial reactivity in 8-month old APP/PS1 mice from NFκB age series experiment as measured by CD45 immunoreactivity in **(A)** cortex and **(B)** hippocampus. ** indicates $p<0.01$.

In 12-month-old mice (figure 5.2.1c-C), quantification of the immunostained sections revealed a statistically significant 56 % decrease in the CD45 marker for activated microglia in *Nfkb1*$^{-/-}$ mice ($t_{(21)} = 3.34$; $p = 0.003$). In contrast, the lysosomal macrophage marker CD68 (figure 5.2.1c-E), did not differ between the *Nfkb1* genotypes ($t_{(21)} = 1.26$; $p = 0.24$). Two-way ANOVA on 12-month-old APP/PS1 and wild-type mice showed a significantly higher Iba-1 signal (figure 5.2.1c-D) in APP/PS1 mice ($F_{(1, 36)} = 51.66$, $p < 0.0001$), but no effect of the *Nfkb1* genotype ($F_{(1, 36)} = 1.05$, $p = 0.31$). Also, there was no significant interaction between the APP/PS1 and *Nfkb1* genotypes ($F_{(1, 36)} = 0.02$, $p = 0.87$). A strong drop in CD45 immunopositive signal prompted us to check pro-inflammatory gene expression (*Tnf* and *Il1b*) in these mice. Two-way ANOVA for *Tnf* gene expression (figure 5.2.1c-F) showed a statistically significant increase due to both APP/PS1 ($F_{(1, 20)} = 19.07$, $p = 0.003$) and *Nfkb1* ($F_{(1, 20)} = 14.17$, $p = 0.001$) genotypes. Moreover, there was a significant interaction between these factors ($F_{(1, 20)} = 5.00$, $p = 0.03$). Similarly, *Il1b* (figure 5.2.1c-G) showed a significant increase associated with the APP/PS1 ($F_{(1, 20)} = 5.24$, $p = 0.03$), but the *Nfkb1* genotype showed no significant effect ($F_{(1, 20)} = 2.43$, $p = 0.13$). However, there was a significant interaction between the genotypes ($F_{(1, 20)} = 5.06$, $p = 0.04$).

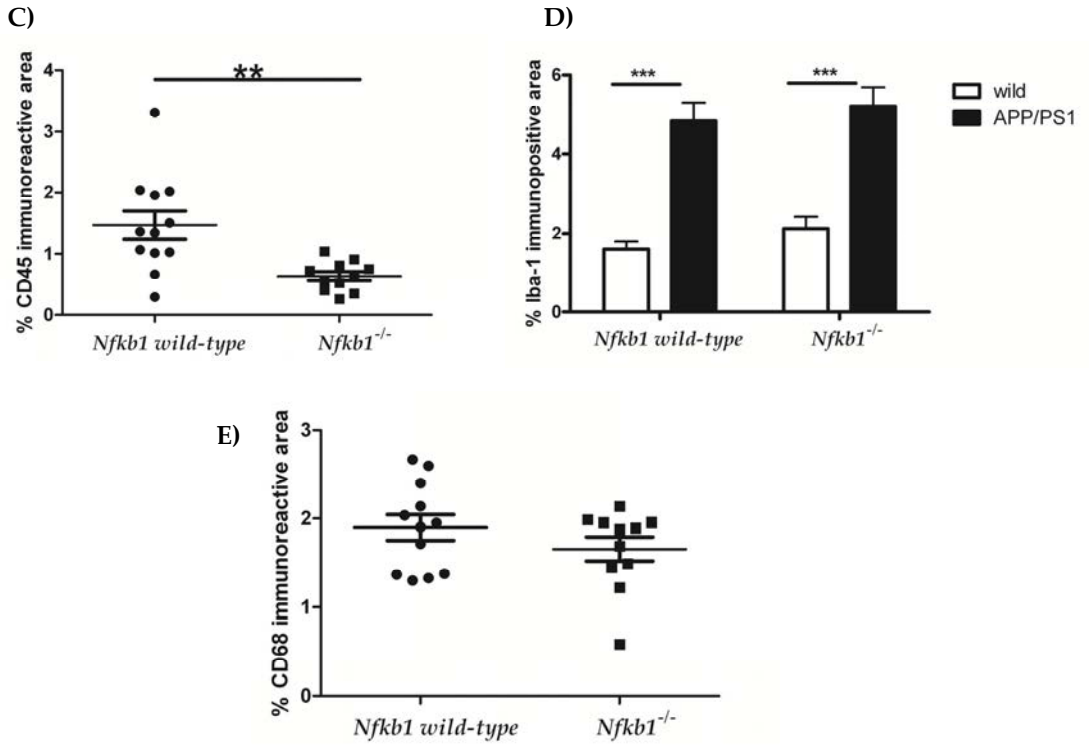

Figure 5.2.1c. Microglial reactivity in 12-month old mice from NFκB age series experiment as measured by **(C)** CD45, **(D)** Iba-1 and **(E)** CD68 immunoreactivity in 12-month old mice. ** indicates p<0.01 and *** indicates p<0.001.

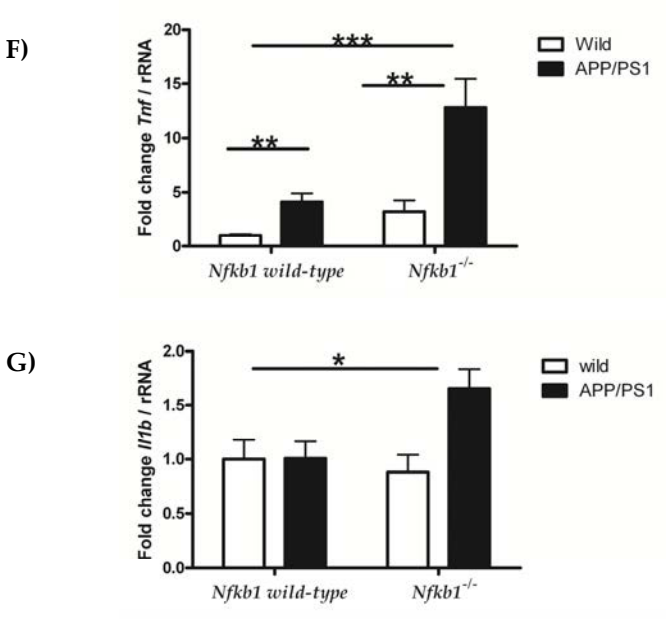

Figure 5.2.1c. Quantitative RT-PCR of proinflammatory cytokines **(F)** *Tnf* and **(G)** *Il1b*. * indicates p< 0.05, ** indicates p<0.01 and *** indicates p<0.001.

In conclusion, *Nfkb1*$^{-/-}$ at an early stage of amyloid pathology (or at an adult age) did not significantly alter activation of microglia. However, at the age of 12 months, *Nfkb1*$^{-/-}$ had a significant effect on microglia activation as measured by CD45 immunoreactivity as well as on the brain levels of pro-inflammatory cytokines. Importantly, we also observed significant interactions between the APP/PS1 and *Nfkb1*$^{-/-}$ genotypes, indicating that knocking out the p50 NFkB subunit may modify the progression of neuroinflammation in AD.

5.2.2 Impact of intrahippocampal injection of LPS on APP/PS1 mice with *Nfkb1* deficiency

We injected saline in the left hippocampus and LPS (4 μg) in the right hippocampus, and evaluated amyloid load and microglia activation 7 days after the injection (figure 5.2.2A). ANOVA for repeated measures (linear mixed model) was used to test within-subject (saline vs. LPS) and between-subject differences (*Nfkb1* wild-type and *Nfkb1*$^{-/-}$). Quantification of amyloid plaque load with 6E10 primary antibody revealed a significant decrease due to LPS injection ($F_{(1, 13.4)} = 48.1$, $p < 0.001$). However, *Nfkb1* deficiency had no significant impact on 6E10 signal in either saline ($F_{(1, 25.3)} = 0.89$, $p = 0.35$) or LPS ($F_{(1, 25.1)} = 0.14$, $p = 0.71$) treated hemibrains (figure 5.2.2B). Microglial reactivity as measured by Iba-1 significantly increased due to LPS injection ($F_{(1, 14.1)} = 10$, $p = 0.007$). Furthermore, *Nfkb1* deficiency significantly decreased Iba-1 signal from LPS hemibrains ($F_{(1, 26.4)} = 4.5$, $p = 0.04$) but not from saline treated hemibrains ($F_{(1, 26.2)} = 0.54$, $p = 0.47$) (figure 5.2.2C). Similarly, lysosomal marker CD68 indicative of phagocytosis, showed significant elevation due to LPS injection ($F_{(1, 13.5)} = 20.5$, p

= 0.001) but revealed no *Nfkb1* genotype effects in either saline ($F_{(1, 17.6)} = 0.001$, $p = 0.98$) or LPS ($F_{(1, 17.6)} = 0.14$, $p = 0.71$) treated hemibrains (figure 5.2.2D).

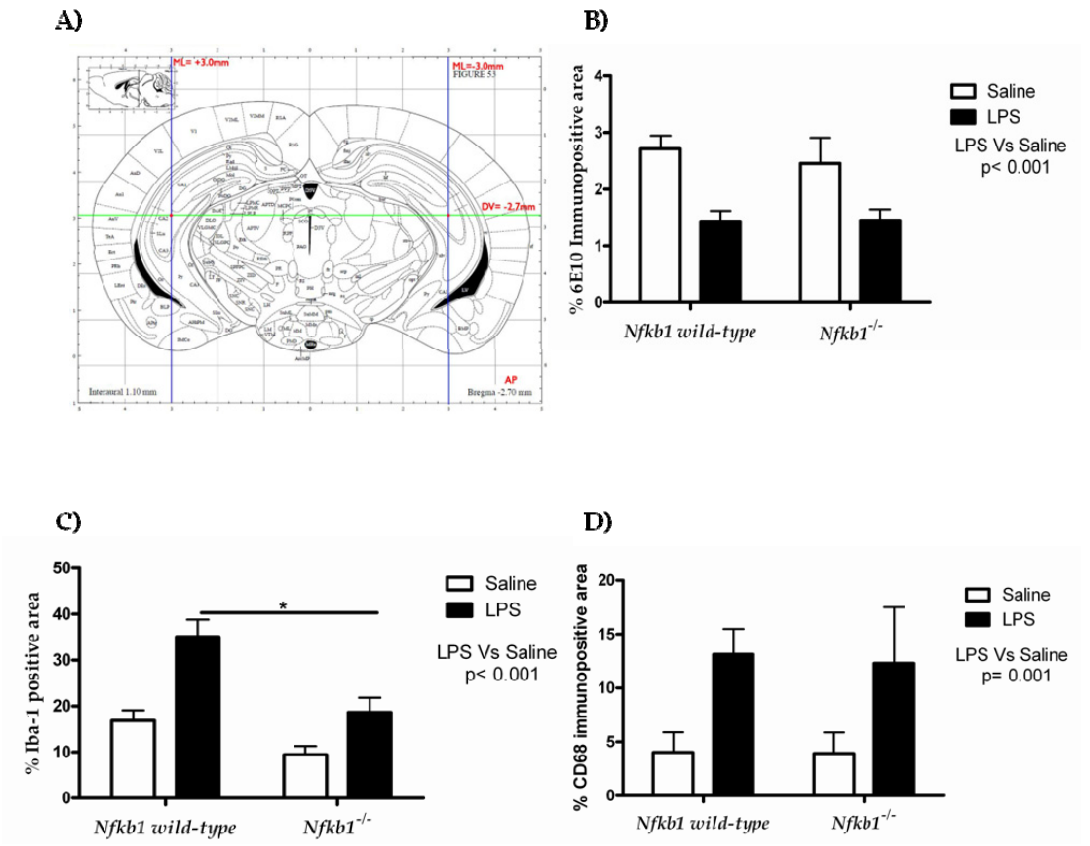

Figure 5.2.2. ***(A)*** Intrahippocampal coordinates for LPS/saline injection. Quantitative analysis of **(B)** 6E10 , **(C)** Iba-1 and **(D)** CD68 immunostainings. * indicates p< 0.05

6 Discussions

6.1 EFFECTS OF HUMAN INTRAVENOUS IMMUNOGLOBULIN IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

To our knowledge, this is the first study which systematically investigates the efficacy and safety of hIVIG therapy in a transgenic mouse model of Alzheimer's disease (AD). At present, a phase-3 clinical trial is testing hIVIG in mild to moderate AD patients (Mangialasche et al., 2010). Even though mechanisms of immunotherapy for AD are well documented (Wilcock et al., 2004a), there are hardly any reports that delve on *in vivo* effects and mechanisms of hIVIG in AD. Previous studies, including our own, tested various potential mechanisms for hIVIG in a variety of *in vitro* and *ex vivo* experimental setups (Istrin et al., 2006; Magga et al., 2010). As of now, mechanisms of action for hIVIG still remain elusive. Table 5.1 summarized the key findings of this study. Importantly, in these experiments, we show that development of anti-human IgG antibodies may not prevent us from using mouse models to test hIVIG. We report clear penetration and accumulation of human IgG across rostro-caudal axis of hippocampus in APP/PS1 mice after chronic hIVIG treatments. Further, we also noticed evidence for *in vivo* Aβ plaque decoration with human IgG. Eventhough, we could not notice any congnitive benefits with hIVIG treatment, we saw a remarkable elevation of soluble Aβ levels with out any concominant changes to diffuse and fibrillar Aβ deposits in hippocampus. Despite limited changes to Aβ pathology, we noticed profound changes in microglial activation and *Tnf* gene expression. Finally, we report neurogenic properties of human intravenous immunoglobulins which further correspond to changes in microglial activation.

6.1.1 IMPACT OF DOSE AND DURATION OF hIVIG TREATMENTS

We used highly concentrated hIVIG preparation (Gammagard Liquid,10%) to maximize the dose of putative anti-Aβ antibodies in chronic experiments. In a human study on patients with mild AD, a lower dose (0.4 g/kg) of hIVIG was found to be associated with higher MMSE scores than the higher 1 g/kg dose, which points to an inverted dose-response for *in vivo* hIVIG effects (Relkin et al., 2009). Therefore, it appears logical to expect more robust effects at a lower dose of hIVIG also in an AD mouse model. However, in IVIG-1 experiment either a low dose (0.1 g/kg) or shorter treatment durations (1-3 weeks) yielded no effects on amyloid or glial pathology. In addition, our data indicate that either a lower dose and or a shorter treatment duration likely yielded insufficient brain penetration of hIVIG. Furthermore, the brain bioavailability of hIVIG is likely to be much higher in elderly humans than in young adult mice, and with intravenous administration as used in human trials, one may end up with a dose comparable to intraperitoneal administration of the high dose used in this study. Owing to limitations of brain penetration and miniscule proportions of Aβ reactive antibodies in hIVIG (O'Nuallain et al., 2008), we stretched treatment durations to 3 and 8 months in IVIG-6a and IVIG-6b respectively.

6.1.2 IMPACT OF ANTI-HUMAN ANTIBODIES ON hIVIG STUDY

Mouse immune system reacts to intraperitoneal injections by mounting an immune response against the injected human antibodies (Loeffler et al., 2012). Development of these anti-human antibodies (also referred as neutralising antibodies) may seriously compromise

the circulating amount of antibodies and impede brain penetration as well. From our initial experiments (IVIG-1 and IVIG-3), we assumed that aged mice develop higher titres of neutralising antibodies than young mice. Our assumption was vindicated by the results of the IVIG-6a experiment, where we initiated treatments at 4months of age (in order to overcome a cross-species immune response) and noticed development of immune tolerance in all except three mice. This is consistent with pilot studies carried out by Elisabeth Maier at Baxter Innovation (Vienna, Austria) showing that anti-human IgG titres reach their peak around 3 weeks after hIVIG injections and subside thereafter. Data so far suggested that young adult mice develop immune tolerance after a prolonged hIVIG treatment and importantly age of mice was very critical for the development of neutralising antibodies. However, in IVIG-6b (8-month treatment) we came to know that this was actually not the case. Mice in this experiment developed highly variable titres of neutralising antibodies. This is in agreement with a recent report describing murine anti-human antibody response of natural human antibodies in a mouse model of Alzheimer's disease (Dodel et al., 2011).

The formation of anti-human antibodies is a serious concern in the interpretation of the present results. However, all available data in our study indicate that their role is of little significance. We offer the following explanations to support our inference. Firstly, not all mice developed anti-human antibodies. In IVIG-6a only three mice and in IVIG-6b only 44% of mice treated with hIVIG developed neutralizing antibodies. From Figure 5.1.4a-B on can appreciate that human IgG immunoreactivity in the brain does not correspond to serum titres of anti-human antibodies. As an extreme example, one mouse that developed the highest anti-human antibody titres (Fig. 5.1.4a-C) clearly demonstrated cortical and hippocampal localization of human IgG. These observations support the assumption that neutralising antibodies are not hampering brain penetration of human IgG. Secondly, none of the test parameters in this study showed a significant correlation with anti-human antibodies, indicating that these neutralising antibodies may not be associated with the primary outcomes of the present study. Lastly, to ensure that those anti-human antibodies are not confounding the results we removed data from all mice that produced significant titres of neutralizing antibodies and reperformed statistical analysis. In IVIG-6b, among the 16 mice that received hIVIG only 7 mice produced significant titres of neutralizing antibodies (titres >200). Even after data from these 7 mice were removed, the main outcome, suppression of CD45 still remained statistically significant ($t_{(22)} = 3.0$, $p = 0.0064$). In contrast, the data from these 7 mice with high titres alone did not show a statistically significanct effect on CD45 immunoreactivity ($t_{(20)} = 1.7$, $p = 0.10$). Furthermore, outcomes related to amyloid pathologies did not change, either. Data provides ample evidence that development of neutralising antibodies did not affect brain penetration of human IgG and also did not influence the outcome of our experiments.Therefore one can reasonably infer that development of cross-species immune response is a serious limitation but at the same time, anti-human antibodies did not hamper hIVIG penetration nor counfound the results of the present study.

6.1.3 BRAIN PENETRATION OF hIVIG IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

Immunoglobulins have traditionally been known to have limited entry into CNS. Only less than 1% of total injected protein can be traced into the brain (O'Nuallain et al., 2008). In order to ascertain brain localization of human IgG, we carried out anti-human IgG immunostaining

on free floating coronal sections of mice treated with hIVIG. Control sections from saline treated mice were devoid of any immunoreactivity whereas mouse sections from hIVIG treated mice showed clear human IgG immunoreactivity in the hippocampus. Anti-human IgG immunoreactivity signal in APP/PS1 mice was persistent upon changing fixation method. It was also noticed in hIVIG treated wild-type-type mice albeit to a lesser extent. In addition, strong immunoreactivity was also noticed in ventricles, and occasionally, within ill perfused blood vessels, indicating that human IgG was indeed present in brain vasculature but had restricted entry into brain parenchyma. Interestingly, penetration of human IgG was dependent on the duration of treatment. Very faint human IgG signal was noticed in experiments with durations up to 3 weeks (IVIG-1 and IVIG-3). In the 3-month experiment (IVIG-6a), there was not only a detectable hIVIG signal within the hippocampus, but also a gradient of immunoreactivity across the rostro-caudal axis, with the highest intensity of human IgG signal coming from the septal end adjoining choroid plexus. Vasculature in the anterior wall of choroid plexus might be one of the entry routes for human IgG into mouse brain. Apart from occasional presence of human IgG aggregates or deposits in the hippocampus, there was limited evidence for cortical localization. However, in the 8-month experiment (IVIG-6b), in addition to antero-posterior gradient of human IgG, one can clearly see human IgG deposits decorating amyloid plaques in the hippocampus and cortex. Double staining with Congo red shows that these amyloid deposits were covered by stippled aggregates of human IgG deposits and further fluorescent triple staining clearly showed Aβ, human IgG and microglia within the same microenvironment. We thus conclusively show evidence for brain penetration of human IgG in spite of development of anti-human antibodies. To our knowledge this is the first study systematically reporting in *in vivo* brain penetration of immunoglobulins in a transgenic mouse model of AD.

6.1.4 EFFECT OF hIVIG ON BEHAVIOR OF APP/PS1 MICE

Mice tolerated intraperitoneal injections of hIVIG well and did not exhibit any adverse effects in their general behaviour or well-being. Mice that had prolonged treatment durations showed tenderness around the injection site. Otherwise, mice were no different from those treated with saline. We assessed the behaviour of hIVIG treated mice with a neurological test battery in each experiment. Consistent with our previous studies (Kemppainen et al., 2012) APP/PS1 mice showed increased locomotor activity, which was not alleviated by the treatment. Morris water maze is a sensitive test to reveal hippocampal based spatial learning in mice. In spite of robust accumulation of hIVIG in the hippocampus, we could not conclusively detect any cognitive benefits of hIVIG treatment in APP/PS1 mice. Certain inherent limitations of our study design might have contributed for this poor show on Morris water maze test. In IVIG-1 and IVIG-6a, APP/PS1 mice were too young to show any impairment. In IVIG-3 even though mice were older, the test paradigm did not work as expected because a change in platform position did not always lead to prolonged escape latencies. Further, mere six injections of hIVIG might be too little to influence robust Aβ plaque pathology in APP/PS1 mice at this age. So, at hindsight only IVIG-6b may have had reasonably good chance to reveal any effects of hIVIG on spatial learning and memory. Consistent with our previous reports (Kemppainen et al., 2012), our transgenic mice developed cognitive deficits around 12 months of age (age series experiment). However, even in IVIG-6b experiment Morris water maze did not reveal any changes in spatial learning and memory. This limitation might be in part due to the stress associated with repeated

intraperitoneal injections for months. In addition, very low concentrations of Aβ reactive antibodies in hIVIG preparations together with no reduction of brain Aβ levels due to treatment might be responsible for the lack of cognitive treatment effects.

6.1.5 EFFECTS OF hIVIG TREATMENT ON AMYLOID PATHOLOGY OF APP/PS1 MICE

Human IVIG has been reported to possess antibodies reactive not only to Aβ epitopes but also to Aβ conformation (O'Nuallain et al., 2008). Furthermore, both epitope and conformation specific Aβ antibodies have been reported in sera from healthy humans irrespective of age or gender (O'Nuallain et al., 2010). These antibodies present in hIVIG are hypothesized to augment the clearance of Aβ from the brains of APP/PS1 mice by various established mechanisms, such as antibody-mediated disaggregation of Aβ deposits, antibody mediated microglial phagocytosis of Aβ deposits, and antibody-mediated efflux of Aβ protein from CNS into peripheral circulation due to peripheral sink. Notably, all these mutually exclusive mechanisms should ultimately result in decrease in brain total Aβ levels, and the peripheral sink effect in addition to an increase in serum Aβ levels (Solomon, 2007). Development of anti-human IgG antibodies in IVIG-1 and IVIG-3 might have hindered any changes to brain Aβ levels. However, after 3 months of treatment with hIVIG (IVIG-6a) we noticed no change in either brain or serum Aβ levels. This might be due to very low concentration of anti-Aβ antibodies of the total IgG (~ 0.1 %, (O'Nuallain et al., 2008)) and insufficient accumulation in the brain with a short duration of treatment. More surprisingly, we noticed an elevation of soluble brain Aβ levels with the present chronic 8-month treatment(IVIG-6b). The underlying mechanisms for the selective elevation of brain Aβ levels is not clear at present, but interestingly, microglia may play some role in regulating brain Aβ levels. Microglia ablation in APP transgenic mice resulted in similar elevation in soluble Aβ levels (Grathwohl et al., 2009). Furthermore, APP transgenic mice with CD45 deficiency also exhibited elevation of soluble Aβ levels (Zhu et al., 2011) and then in our hIVIG study, chronic 8-month hIVIG treatment caused suppression of the CD45 microglia marker by 30%, which correlated with elevated soluble Aβ levels. This may be an emerging evidence for the role of microglia in modulating the soluble levels of Aβ. Nonetheless, this observation together with no changes in plaque-associated Aβ burden clearly points to an inefficient disintegration of pre-existing Aβ deposits due to hIVIG treatment. Immunostainings with N-terminal specific antibodies or Congo red staining for fibrillar Aβ deposits also revealed no treatment effects. Our 8-month study (IVIG-6b) had an 80% power to detect a 19% change in mean Aβ immunopositive area and 30% change in mean Congo red positive area with a significance level (alpha) of 0.05 (two-tailed). This together with no changes in CD68 immunostaining speaks against enhanced Aβ phagocytosis by microglia due to hIVIG treatment.

According to the peripheral sink hypothesis, bidirectional transfer of Aβ between CNS and periphery would lead to elevated serum Aβ levels (DeMattos et al., 2001). However, irrespective of the dose used or treatment duration, we noticed no significant elevation of serum Aβ levels in our studies. Furthermore, there was no correlation between brain Aβ and serum Aβ levels. Statistically, our chronic 8-month study (IVIG-6b) had an 80% power to detect a 22% change in mean serum $A\beta_{40}$ levels with a significance level (alpha) of 0.05 (two-tailed). Furthermore, in IVIG -1 and IVIG -3, no difference in amyloid plaque burden or in

biochemical levels of Aβ in brain and serum was noticed after three weekly hIVIG injections in 3-month-old APP/PS1 mice or after bi-weekly hIVIG treatment for 3 weeks in 15-month-old APP/PS1 mice. These findings indicate that the outcome of Aβ related measurements was not dependent on age or amyloid plaque burden at the onset of hIVIG treatment. A recent *in vivo* study reported that natural Aβ-antibodies (Nabs-Aβ) isolated from hIVIG reduced amyloid plaque burden in 3-month-old mice but not in 12-month-old APP transgenic mice (Dodel et al., 2011). This reduction was ascribed to the peripheral sink mechanism. However, even in that study elevated plasma Aβ levels were not statistically significant outcome of the treatment, and in the absence of correlations between brain/CSF and plasma levels of Aβ, it will be difficult to acknowledge the presence of a peripheral mechanism. Moreover, mere increase in plasma Aβ due to antibody treatment might also be a consequence of stabilization of Aβ-antibody complexes in plasma (Winkler et al., 2010; Levites et al., 2006). Interestingly, a recently published report on anti-Aβ monoclonal antibody (bapineuzumab) indicates that in spite of antibodies engaging brain Aβ, it may not necessarily be reflected in CSF or serum (Blennow et al., 2012).

Even though we found no amyloid reduction with hIVIG, a recent *in vivo* study reported that natural Aβ-antibodies (Nabs-Aβ) isolated from hIVIG confer protection from Aβ toxicity along with beneficial effects on cognition in an APP transgenic mouse model of AD (Dodel et al., 2011). However, in that report absence of hIVIG treated group makes it difficult to delineate the effects of constituent anti-Aβ antibodies and non-specific polyclonal IgG antibodies from those arising from extraction procedure itself. As expected, four weeks of treatment with natural Aβ-antibodies did not alter plaque pathology in 12-month-old mice but reduced amyloid plaque burden in 3-month-old mice. However, the plaque reduction effect in young animals did not differ significantly between low and high doses of Nabs-Aβ. In addition, a recent meeting abstract also reported no significant changes in PIB retention in brains of small number of human patients treated with hIVIG (Kondo et al., 2011). Collectively, these findings suggest that hIVIG unlikely would substantially affect brain Aβ deposition or clearance.

6.1.6 EFFECTS OF hIVIG TREATMENT ON ASTROCYTES AND MICROGLIA OF APP/PS1 MICE

After finding no evidence for amyloid lowering mechanisms for hIVIG in our study, we shifted our focus to anti-inflammatory actions of hIVIG. Anti-inflammatory properties of hIVIG are well known and polyclonal antibodies of hIVIG are reactive to a wide array of inflammatory proteins including cytokines and chemokines (Nimmerjahn and Ravetch, 2008). Mean GFAP immunopositive area for astrocyte reactivity was lower in hIVIG treated mice than in the saline group, but failed to reach statistical significance. Similarly, increase in Iba-1 due to hIVIG treatment was not statistically significant inspite of increase in mean Iba-1 immunopositive area in hIVIG treated mice. A change in microglia phenotype (or activation state) was indicated by increased expression of microglial markers around the Aβ microenvironment, with CD45 measuring only activated microglia (Masliah et al., 1991) and Iba-1 measuring both activated and quiescent microglia. In addition, the CD68 marker has been associated with phagocytosis (Morgan, 2009). The lack of significant changes in CD68 immunopositive microglia confirmed limited effects of hIVIG treatment on Aβ phagocytosis. Whilst 8-month chronic hIVIG treatment suppressed CD45 expression, Iba-1 expression

increased in a subset of microglial cells as demonstrated by confocal analysis. The fact that different subtypes of microglia cells responded differently, strongly indicates that hIVIG has a highly specific immunomodulatory effect and not just a general suppression of the brain inflammatory reaction. Notably, we measured Iba-1 signal from individual microglial cells across the entire z-stack along the section thickness, so there was no interference from microglia in other activation levels that could confound quantification of this signal. In contrast, with nickel-DAB developed sections, the immunopositive Iba-1 signal from microglia often were influenced by neighbouring microglial cells present in various levels of activation. Therefore, this approach allowed us to measure integrated intensities of more than one activation marker in a single individual microglial cell and relate this information to the morphological features (in this case, circularity). To our knowledge, this is the first study to report segregation of microglial cells based on both morphology and activation markers. A change in microglial activation as a consequence of hIVIG treatment has been reported in one previous *in vitro* study (Istrin et al., 2006). In this study, BV-2 microglia cell line displayed a more ramified morphology and increased expression of CD45 in response to hIVIG treatment. In contrast, our *in vivo* study found decreased CD45 expression (only in Type B microglial cells) and increased Iba-1 expression (in Type B and Type C microglial cells) along with a shift towards more ramified microglia morphology.

6.1.7 EFFECTS OF hIVIG TREATMENT ON PRO-INFLAMMATORY CYTOKINE GENE EXPERESSION

After finding clues for differential activation of microglial cells, we focused further on classic inflammation regulatory actions of hIVIG. Many *in vitro* and *in vivo* studies have indicated the ability of hIVIG preparations to modulate cytokine induction and release (Murakami et al., 2012; Toungouz et al., 1995; Andersson and Andersson, 1990; Yang et al., 2011; Wu et al., 2006)(Toyoda et al., 1994; Aukrust et al., 1994; Ling et al., 1993). In addition to direct cytokine neutralizing antibodies in hIVIG, there are also other plausible mechanisms (Wadhwa et al., 2000; Andersson et al., 1996; Le Pottier et al., 2007). Since pro-inflammatory cytokines like TNF and IL-1β are important contributors of neuroinflammation, there is an opportunity for hIVIG to cease, decrease or suppress amyloid induced cytokine release by microglia. *Tnf* gene expression has been reported to be increased in various transgenic mouse models of AD, including the APP/PS1 mouse (Ruan et al., 2009). In our study, hIVIG treatment caused a significant reduction in *Tnf* gene expression (and a similar trend in *Il1b* expression). Moreover, this suppression of pro-inflammatory gene expression due to hIVIG treatment was not genotype specific or an artifact of the *APP* transgene as it was also present in wild-type-type littermates. One would expect a straightforward correlation between the expression levels of pro-inflammatory cytokines and CD45 marker of activated microglia. Indeed, in saline treated mice, a trend towards positive correlation was found between CD45 microglial marker and *Tnf* gene expression. However, chronic hIVIG treatment shifted this correlation to opposite direction, and a similar shift after hIVIG treatment was observed in the correlation between CD45 and *Il1b* gene expression. This inverse relationship between pro-inflammatory cytokine and CD45 expression suggests that hIVIG treatment may change the brain immune response, so that microglia may maintain a high activity despite a decrease in generalized inflammatory response mediated through pro-inflammatory cytokines. This profile of immunomodulation works against the profile associated with aging (Hickman et al., 2008) and may confer neuroprotection in the AD brain. Pro-inflammatory cytokine

regulatory effects of hIVIG are further supported by a report, where circulating levels of TNF and IL-1β cytokines dropped upon hIVIG treatment in Guillian-Barre syndrome patients (Sharief et al., 1999). More detailed experiments are still needed to fully elucidate the mechanisms involved and judge the therapeutic potential of cytokine regulatory effects of hIVIG in the context of AD and other neurodegenerative pathologies.

6.1.8 EFFECTS OF hIVIG TREATMENT ON NEUROGENESIS

Paucity of any previous studies pointing towards neurogenesis by hIVIG compelled us to discuss this novel finding indirectly within the context of microglia and inflammation. In fact, to our knowledge this is the first scientific evidence of hIVIG therapy augmenting hippocampal neurogenesis. In diverse experimental setups, several studies connect suppression of microglia and pro-inflammatory markers to various stages of neurogenesis (Zunszain et al., 2012; Seguin et al., 2009; Monje et al., 2002). Inflammation has been long known to be a negative regulator of neurogenesis (Monje et al., 2003; Ekdahl et al., 2003). Cellular mediators of neuroinflammation, astrocytes and microglia, may impact neurogenesis negatively via HPA axis mediated release of glucocorticoids that supress neurogenesis or by decreasing neurotrophin support or by excessive release of reactive oxygen species and pro-inflammatory cytokines (Song and Wang, 2011; Cameron and Gould, 1994). In addition, pro-inflammatory cytokines released by microglia are known to play role in *in vitro* differentiation of hippocampal progenitor cells (Mehler et al., 1993). Furthermore, in diverse animal models of disease pro-inflammatory cytokines TNF (van der Borght et al., 2011; Iosif et al., 2006), IL-1β (Goshen et al., 2008; Koo and Duman, 2008), IFN-α (Kaneko et al., 2006a) and IL-6 (Vallieres et al., 2002) have been shown to suppress adult neurogenesis, while in one study blockade of IL-6 alone restored it (Monje et al., 2003). On the other hand, a recent report provides stronger links between microglia and neurogenesis (Vukovic et al., 2012). In this study, microglia isolated from mice that had undergone running exercise for two weeks, mediated enhanced neural precursor cell activity in neurosphere preparations from sedentary aged mice. This study also revealed a novel CX_3CL1–CX_3CR1 axis capable of changing microglia phenotype towards one that supports neurogenesis in ageing brains (Vukovic et al., 2012).

In the context of AD, neurogenesis has been extensively studied in various transgenic AD mouse models, and Aβ itself has been shown to suppress adult neurogenesis in the hippocampus. Passive immunization with Aβ antibodies has been previously shown to modulate microglial phenotype (Wilcock et al., 2004a; Wilcock et al., 2011) and very recently Aβ immunotherapy has also been linked to neurogenesis as well (Biscaro et al., 2009). Similar to other models of experimental neuroinflammation, suppression of activated microglia in transgenic AD mouse models was also found to be sufficient to maintain hippocampal neurogenesis (Biscaro et al., 2012). Strikingly, even in our AD mouse model, chronic hIVIG treatment not only altered activation status of microglia and suppressed pro-inflammatory TNFα gene expression, but also significantly enhanced the number of doublecortin positive cells in the dentate gyrus irrespective of the genotype of mice. This effect may be selectively beneficial for aged mice in which neurogenesis is about to fade out. Moreover, the negative correlation between CD45 microglia and number of doublecortin positive cells in our study also points towards an inverse relationship between activated microglia and neurogenesis. While microglial cells activated by TNF-α and IL-6 inhibited neurogenesis, microglial cells

activated by IL-4 and T-cells have also been reported to contribute to hippocampal neurogenesis (Butovsky et al., 2006; Ziv et al., 2006). Such alternatively activated microglia were shown to express MHCII proteins and co-localize with IGF-1, a growth factor known for neuroprotection and neurogenesis (Carro et al., 2001; Trejo et al., 2001). Therefore, modulation of microglia by hIVIG and subsequent suppression of proinflammatory cytokines might restore and foster hippocampal neurogenesis, thereby representing a mechanism to compensate for the loss of neurons observed during AD progression. However, it is also possible that fostering neurogenesis might be a direct exclusive property of hIVIG, irrespective of other indirect mechanisms discussed here.

6.1.9 MECHANISM OF ACTION FOR HUMAN INTRAVENOUS IMMUNOGLOBULIN THERAPY

Our study was designed to test amyloidocentric mechanisms for hIVIG; in fact we began our experiments hoping to find evidence for reduction of brain Aβ protein by hIVIG treatment. But we clearly present evidence for hIVIG effects independent of Aβ clearance. This is the first passive immunotherapy data in AD transgenic mice that describes modulation of microglial activation, suppression of pro-inflammatory cytokine gene expression, and enhancement of neurogenesis as a consequence of hIVIG treatment independent of amyloid clearance in the brain. However, it is unclear whether these are mutually exclusive and independent effects. This combination of effects makes hIVIG a unique treatment candidate that targets multiple inflammatory antigens and immunomodulatory factors associated with neurogenesis (Schnabel, 2011). At the onset of our studies, Aβ (or toxic assemblies) was putative target for hIVIG, but towards the end of our experiments, our data consistently defied this and brought in "microglia and inflammation" as potential targets of hIVIG therapy. It will be interesting to investigate if mere modulation of CD45 microglial protein expression could as well change cytokine profiles and support neurogenesis. A recent report described alterations in plasma cytokines levels in mild to moderate AD patients treated chronically with hIVIG, and further that these changes correlated with clinical outcomes, suggesting that immunomodulation by cytokines may be one of the therapeutic mechanisms for hIVIG (Relkin et al., 2011). While Aβ (oligomers) delivers the kiss of death by a thousand tiny blows to neurons (Selkoe, 2011), hIVIG reserves the potential to deliver relief by plethora of mechanisms (Ballow, 2011) such as influencing the activation state of microglia, suppressing proinflammatory gene expression, immunomodulation, regulating cytokine networks, and fostering neurogenesis in a manner independent of constituent anti-Aβ antibodies. Therefore, enriching hIVIG preparations with anti-Aβ antibodies may not be necessary to unmask the beneficial effects of hIVIG. In view of the limited supply of hIVIG (Bayry et al., 2007) it will be of enormous benefit to further explore the anti-inflammatory, immunomodulatory and neurogenic mechanisms of hIVIG in the context of Alzheimer's pathology. Changes observed in microglia phenotype and their inverse correlation to proinflammatory gene expression advocate anti-inflammatory properties of hIVIG in AD mice. It would be tempting to check if these anti-inflammatory effects of hIVIg are related to recently discovered novel Th-2 pathway (Anthony et al., 2011). A small fraction of sialylated IgG antibodies in hIVIG interact with SIGN-R1 or hDC-SIGN receptors on spleenic macrophages to release IL-33 thereby, initiating a IL-4 driven Th-2 response (Anthony et al., 2012).

6-1.10 IMMUNOTHERAPY FOR ALZHEIMER'S DISEASE - PROSPECTS AND CAVEATS

Immunotherapy for AD has been at the forefront of experimental AD therapeutics. bapineuzumab and solaneuzumab both have failed in their pivotal phase 3 clinical trials. Gammagard Liquid (hIVIG) is currently in the phase-3 clinical trial and is expected to report its outcome by 2013. The first two are monoclonal antibodies whereas the last one is a polyclonal IgG antibody. Interestingly, all the three antibody candidates were supposedly expected to halt or slow down cognitive decline in AD patients by reducing brain amyloid protein. Treatment with high dose has already been discontinued for bapineuzumab because of vascular bleedings and amyloid related-imaging abnormalities (Sperling et al., 2012). As for hIVIG, lower doses were found to be associated with beneficial MMSE scores (Relkin et al., 2009), whereas higher doses of hIVIG are needed for its immunomodulatory effects (Nimmerjahn and Ravetch, 2008). Therefore, selecting a right dose which engages the target without precipitating any serious side effects will be a critical factor for the ongoing trials. Clinical data from bapineuzumab phase-2 trials provided the first evidence that getting rid of brain amyloid may not be sufficient to offer cognitive benefits. This might be an indication that either our target (Aβ) is wrong or we are too late to rescue already degenerated brain (Lemere and Masliah, 2010a). Clinical data from a small hIVIG trial, however, indicates that at hIVIG therapy slows down cognitive deterioration (Relkin et al., 2009). Based on our rodent experiments, we know that hIVIG may not be able to reduce brain amyloid but may show profound neurogenic immunomodulatory effects in and around amyloid plaque microenvironment. Therefore, drugs may confer benefits in AD even without removing brain amyloid.

Even if we happen to choose the right target, and an effective drug and its right dose, we may still fail if we start the treatment at a wrong time (Sperling et al., 2011a). There is evidence to postulate that outcomes of many clinical trials would have been better if the therapy had been started at a very early stage, well before massive neuronal loss ensued. In fact, researchers are now contemplating to begin large therapeutic preventive trials in cognitively healthy people. A sensitive issue here is how early one should begin treatment, and in the absence of a sufficient evidence proving the causality of Aβ protein in Alzheimer's disease, it will be potentially dangerous to immunize otherwise healthy people. Arguments for testing AD drug candidates in early stage patients or at mild cognitive impairment stage is reasonable, but again when the drug target is elusive, it will be unethical to expose prospective AD patients until the drug target is pinpointed and validated by multiple independent studies. In any case, immunotherapy trials may be regarded as ultimate test for the amyloid cascade hypothesis, only when treatment starts in early presymptomatic phases of AD.

In addition, one seriously undermined issue that will eventually pop up once hIVIG clears phase 3 trials is of significant concern. The issue is about long-term viability of hIVIG as treatment option for AD patients. It takes decades for AD to develop and thus it is very reasonable to expect longer treatment regimens for hIVIG in order to see some benefits. Equity research report by RBC Capital Markets® (Henry Brandon, January 23rd, 2012), suggests a conservative estimate of 45000 dollars per annum per AD patient in USA alone. This clearly indicates a very expensive and economically unviable treatment option.

Furthermore, as more than 10000 donations from healthy people are required to enrich single hIVIG lot, one can extrapolate how many healthy donations will be needed to meet global demand by AD patients. Eventually, as the production demand continues to rise, immunodefecient and immunocompromised patients who rely critically on availability of hIVIG infusions for their survival will also face its acute shortage. For instance, mortality rates were reduced significantly in CVID patients receiving replacement immunoglobulin therapy and further such intervention added 30-40 years of productive life to patients (Chapel et al., 2008). This might also open a moral and ethical dilemma for those who advocate hIVIG therapy to AD patients because mostly these patients are in their last years of their lives. If the production issues are not addressed now, one might end up with acute shortage thereby not only affecting patients with AD but also patients suffering from primary immunodeficiency disorders (Bayry et al., 2007). However, as the numbers of patients with conditions like CVID are much smaller when compared to AD, it would be also possible to secure supplies of hIVIG to patients critically in need for their survival.

One naive possible solution to meet this increasing demand is to collect plasma from people when young and then use their own antibodies when they need during later part of their lives. Recombinantly manufacturing sialylated IgG antibodies is also an interesting avenue to proceed. Therefore, in view of these scientific, technical, economical and commercial limitations associated with hIVIG usage, it is in the best interest of everyone to investigate the inherent mechanisms underlying hIVIG effects with an objective to identify novel drug targets.

6.2 ROLE OF NUCLEAR FACTOR KAPPA-B IN PATHOGENESIS OF ALZHEIMER'S DISEASE

Nuclear factor kappa-B plays a major role in regulating inflammatory responses of brain with AD (Granic et al., 2009). NFκB activation in neurons and glial cells can be both beneficial and detrimental. We established a novel mouse line expressing APP/PS1 transgene under *Nfkb1* null background to see whether *Nfkb1* gene deficiency in APP/PS1 alleviates age-related memory impairment by reducing levels of inflammatory modulators that might have negative effect on learning and memory. On the other hand, we hypothesized that suppression of microglia, and thereby, phagocytosis in *Nfkb1* knockout mice may result in higher brain amyloid burden. Table 5.2 summarizes key findings from this study. To our knowledge this is the first transgenic AD mouse line that is deficient in a key component of NFκB signalling. One cautionary note while interpreting the phenotype of *Nfkb1*$^{-/-}$ mice is that roughly one-third of p105 protein (precursor protein of *Nfkb1*) acts as scaffold for MEK kinase Tpl2 protein (Beinke and Ley, 2004). In *Nfkb1*$^{-/-}$ mice, the absence of p105 results in rapid degradation of Tpl2. Therefore, deficient NFκB signalling (via p105 deficiency) is also reflected as deficiency in ERK MAP kinase pathway (via deficiency of Tpl2). This compound signalling defect makes it difficult to attribute the observed phenotypes of *Nfkb1* deficient mice to either loss of *Nfkb1* function or Tpl2, or both (Gerondakis et al., 2006).

We performed two experiments with these mice. In the first age series experiment we followed these mice at 3.5, 8 and 12 months of age. Both APP/PS1 and *Nfkb1*$^{-/-}$ mice displayed significantly lower body weights than controls, which was consistent with previous report (Jhaveri et al., 2006)(Lu et al., 2006). Our data from behavioural test battery show that these mice do not show any deleterious effects of *Nfkb1* gene deletion. Interestingly, decreased

anxiety and increased motor coordination as noticed in *Nfkb1*$^{-/-}$ mice can be viewed as beneficial outcome in the context of Alzheimer's patients. Although both the APP/PS1 and *Nfkb1*$^{-/-}$ genotypes had significant manifestation in behavioural phenotypes none of the outcomes of APP/PS1 genotype interacted with *Nfkb1* knockout genotype, indicating that their phenotypes did not influence each other. Like *Nfkb1* knockout mice reported earlier (Kassed and Herkenham, 2004), *Nfkb1*$^{-/-}$ mice under APP/PS1 background as well displayed reduced anxiety-like behaviour. Our data from Morris water maze test shows that at the age of 12 months age APP/PS1 mice exhibited impaired spatial learning , but *Nfkb1* gene deletion neither increased nor decreased latencies to reach the hidden platform. Thus, we accept our null hypothesis that there is no effect of *Nfkb1* gene on memory impairments of APP/PS1 mice in current experimental setup. In light of a recent report (Lu et al., 2006), detailing neurodegenerative changes in the hippocampus, cortex and optic nerve of *Nfkb1*$^{-/-}$ mice, it becomes interesting to see whether our new *Nfkb1*-/- mouse line under APP/PS1 background exhibits neuronal loss as they age. However, due to breeding problems we could not accumulate enough mice to carry out a systematic stereological assessment of neuronal number in the hippocampus and cortex.

6.2.2 IMPACT OF *Nfkb1* GENE KNOCKOUT ON AMYLOID PATHOLOGY

NFκB activity has been shown to mediate APP processing and BACE-1 expression (Chen et al., 2011). Modulating NFκB pathway also resulted in decreased Aβ burden in transgenic *APP* mice (Sung et al., 2004). In our experiments, we used *Nfkb1*$^{-/-}$ mice to inhibit NFκB pathway. Neuronal inhibition of NFκB signalling, via regulation of BACE-1 expression can be hypothesized to cause lower Aβ burden in *Nfkb1* null mice with APP/PS1 background. On the other hand, inhibition of NFκB signalling in microglia may also result in defective Aβ clearance via phagocytosis, thus resulting in higher Aβ burden. So we set out to test both hypotheses with our current model. Across three ages, we evaluated brain amyloid burden in *Nfkb1* wild-type and *Nfkb1* knockout mice under APP/PS1 background. Our data from 3.5-month-old mice do not support any significant role of *Nfkb1* gene in early stages of Aβ plaque pathology, albeit a trend towards lower insoluble levels of $A\beta_{42}$ in *Nfkb1*$^{-/-}$ mice was noted (Fig. 5.2.1b-A). BACE-1 modulation by neuronal NFκB (Chen et al., 2011) could have contributed to this effect, although we did not check BACE-1 expression. A larger sample size for ELISA measurements might have revealed whether this trend (p = 0.06) would reach statistical significance. At 8 months APP/PS1 mice exhibited moderate amounts of Aβ plaque pathology in both cortex and hippocampus. In addition, gender differences become apparent, with females exhibiting more severe pathology. Importantly, at this age, *Nfkb1* gene deficiency did not bring about any differences in biochemical levels of $A\beta_{40}$ or $A\beta_{42}$ between *Nfkb1*$^{-/-}$ and wild-type-type carriers. Quantification of Aβ burden by immunohistochemistry revealed a significant gender differences in both cortical and hippocampal plaque load but interestingly only the hippocampus showed elevated Aβ burdens in *Nfkb1*-/- mice. Variable, non-uniform, local deposition of Aβ plaques in different anatomical subregions in the cortex as evaluated in coronal sections might be responsible for seeing no overall *Nfkb1* gene effect here. In contrast, this variation is comparatively small in hippocampus. At 12 months of age, both the cortex and the hippocampus are fully engrossed in severe Aβ plaque pathology. However, Aβ ELISA assays and amyloid plaque quantification revealed no*Nfkb1*$^{-/-}$ effects. Our sample had an 80% power to detect a 33% change in mean Aβ immunopositive area and 35% change in mean Congo red positive area with a significance level (alpha) of 0.05 (two-

tailed). So, it is unlikely that our sample was underpowered to detect a clinically meaningful change in Aβ pathology. However, insoluble $A\beta_{42}$ levels in ELISA in *Nfkb1* knockout mice were significantly lower than *Nfkb1* wild-type mice (Fig. 5.2.1b-I).

Overall, there was a trend for decreased Aβ at an early (3.5 months) and a late (12months) stage of amyloid pathology in *Nfkb1*$^{-/-}$ mice. Only during intermediate stages of amyloid pathology (8 months), *Nfkb1*$^{-/-}$ mice showed increased Aβ plaque immunoreactivity. Basal expression levels of *APP* transgene did not differ significantly between *Nfkb1* wild-type and *Nfkb1*$^{-/-}$ groups at 12 months, indicating that changes in *APP* transgene expression might not be the reason for this variability (data not shown). If we ignore the suppression of insoluble $A\beta_{42}$ levels at 3.5 and 12months of age and the elevation of Aβ plaques at 8 months of age as random effect due to a small sample size, then one can conclude that *Nfkb1* gene does not have any significant role in the development and progression of Aβ pathology. However, it should be noted that the ages of 3.5 and 12 months represent opposite ends of the rapid amyloid accumulation process in APP/PS1 mice and that many changes associated with ageing take place in a mouse between these age point. Thus, microglia at 3.5 and 12-month age might be totally different with respect to their activation and functions like phagocytosis, which may account in part for the age dependency of these findings. One can also consider giving an extra LPS stimulus to 3.5-month-old mice, in order to delineate *Nfkb1* gene dependent functions in microglia. During intermediate stages of amyloid pathology (8 months of age), insufficient phagocytosis by *Nfkb1* deficient microglia might have caused elevation of Aβ plaques in the *Nfkb1*$^{-/-}$ group. However, this is in stark contrast to our data from *ex vivo* assays where *Nfkb1* deficient microglia exhibited enhanced phagocytosis (data not shown). At the age of 12 months, combined influences of ageing, severe plaque burden, and elevated levels of TNF and IL-1β in APP/PS1 mice might have influenced microglial *Nfkb1* associated functions. In addition, recent evidence suggests that mechanisms responsible for phagocytosis of Aβ plaques might be different from those regulating soluble/insoluble levels of Aβ (Zhu et al., 2011; Grathwohl et al., 2009). This provides explanation for decreased levels of insoluble $A\beta_{42}$ in 12-month-old *Nfkb1*$^{-/-}$ mice without any changes in amyloid plaque load. The data from the age series experiment show that the age of mice as well as quality of stimulus (plaque severity and cytokine milieu) influence biochemical and neuropathological readouts of *Nfkb1* gene functions.

We further provided an acute LPS stimulus (4μg) in addition to chronic stimuli (Aβ deposits) to 12-month-old mice and examined their brains for changes in Aβ plaque pathology by immunohistochemistry. Due to increased mortality of *Nfkb1*$^{-/-}$ mice after acute LPS injections (Duckworth et al., 2006), we were left with only 5 mice compared to 10 mice in the corresponding *Nfkb1* wild-type group. As expected and previously reported (Malm et al., 2005a), LPS injection reduced Aβ plaque burden when compared to saline injections on the contralateral side. However, there was no difference in plaque burden between the *Nfkb1* wild-type and knockout APP/PS1 mice. All the above data provide evidence for a limited role of *Nfkb1* gene in the development and progression of amyloid pathology in APP/PS1 mice. Cell type specific inhibition of NFκB signalling should be used in future studies to prevent confounds of simultaneous neuronal and microglial NFκB inhibition as happens in *Nfkb1* knockout mice. In this direction, one can employ lentiviral system to express dominant negative IKK-2 (Inhibitor of nuclear factor kappa-B kinase-β), in order to effectively block canonical NFκB pathway in defined spatial and temporal patterns.

6.2.3 IMPACT OF *Nfkb1* GENE KNOCKOUT ON MICROGLIA AND INFLAMMATORY PATHOLOGIES

Microglia are activated by fibrillar Aβ deposits of APP/PS1 mice (Morgan et al., 2005)(Morgan, 2009). This increase in microglial reactivity can be demonstrated by elevation of microglial CD45 expression. We employed CD45 immunohistochemistry and examined brain sections from *Nfkb1* wild-type and *Nfkb1*$^{-/-}$ mice under APP/PS1 background at 8- and 12–month age points. At the age of 8 months, we saw no changes in CD45 immunoreactivity due to *Nfkb1* gene deletion. However, at 12 months of age we noticed a significant 56% drop in CD45 immunoreactivity in APP/PS1 mice deficient in *Nfkb1* gene compared with *Nfkb1* wild-type carriers. NFκB p65 DNA-binding activity was also significantly enhanced in *Nfkb1* wild-type but not in *Nfkb1*$^{-/-}$ APP/PS1 mice (data not shown), indicating that NFκB upregulation was prevented in the absence of functional *Nfkb1* subunit. In addition, there was a significant elevation *of Tnf* and *Il1b* gene expressions in *Nfkb1*$^{-/-}$ mice. This is consistent with a previous report, where CD45 deletion in *APP* mice as well resulted in elevation of *Tnf* and *Il1b* cytokine gene expression (Zhu et al., 2011). This particular study and data from current study suggest that substantial suppression of CD45 (> 50% to total ablation) is associated with a pro-inflammatory microglial phenotype. In stark contrast, in our hIVIG study, a meagre 36% suppression of CD45 immunoreactivity was associated with robust decrease in *Tnf* gene expression and enhanced neurogenesis, indicating an anti-inflammatory microglial phenotype. However, Iba-1 and CD68 quantifications revealed no significant *Nfkb1* genotype effects. Our data indicate that *Nfkb1* gene deficiency in APP/PS1 mice enhanced Aβ induced neuroinflammation, indicating that *Nfkb1* plays an important role in inhibiting M1 pro-inflammatory phenotypes of microglia (Porta et al., 2009). In our LPS experiment, acute injections of LPS resulted in a significant decrease in Iba-1 positive microglia in *Nfkb1*$^{-/-}$ mice. This decrease was evident in contralateral saline injected side as well, indicating an overall reduction of basal Iba-1 expression in *Nfkb1*$^{-/-}$ mice. CD68 immunoreactivity, however, did not show *Nfkb1* genotype specific changes due to LPS injection. Overall, both our age series experiment and LPS experiment indicate specific changes in microglial phenotypes accompanied by elevated *Tnf* and *Il1b* pro-inflammatory gene expression in *Nfkb1*$^{-/-}$ mice under APP/PS1 background. Importantly, this pro-inflammatory microglial phenotype did not worsen memory impairments in APP/PS1 mice.

6.2.4 TARGETING NFKB PATHWAY IN ALZHEIMER'S DISEASE - PROSPECTS AND CAVEATS

NFκB pathway plays a vital regulatory role in initiation, maintenance and resolution of inflammation within the AD brain. Even modest immunomodulation may have profound effects on the progression of disease. In such a scenario, targeting various components of NFκB pathway seems explicit. Mouse models lacking specific components of NFκB pathway are excellent tools for validating the targets in NFκB pathway in spite of certain limitations. Knockout mice have "inhibition" of the particular gene function starting from early development, while when applied as a therapy the reduction is sudden. Knockout mice are likely to adapt and develop compensatory mechanisms. Deletion of various components of the NFκB pathway can be lethal during embryonic development or postnatal, so one may never know the true extent of gene knockout in adulthood. Furthermore, genetic deficiencies in mouse models need not necessarily be translated to humans, who frequently have certain

residual activity of targeted component of NFκB activation (McDonald et al., 2006). Moreover, while interpreting the phenotype of knockout mice, NFκB independent activities of respective proteins need to be taken into account. Presence of functional redundancy among NFκB proteins can sometimes partly compensate for their loss in knockout mice (Pasparakis et al., 2006). Existence of cross-talk between canonical and alternative pathways at the level of IKKs and transcription factors also confounds the interpretation of transgenic phenotypes. Lastly, loss of NFκB function may as well result in disruption of other key intracellular signalling pathway (Gerondakis et al., 2006). It is also possible that our *Nfkb1*$^{-/-}$ mice may inhibit NFκB signalling involving *Nfkb1*, however, functions of subunits, those independent from *Nfkb1*, might still remain. Also, *Nfkb1* is involved in signalling not traditionally considered as NFκB signalling. Therefore, cell specific depletion of NFκB might be needed to inhibit only pro-inflammatory NFκB signalling in microglia and astrocytes sparing beneficial NFκB signalling in neurons necessary for survival.

7 Conclusions

We ran altogether 6 experiments to see whether pharmacological and genetic immunomodulation in transgenic APP/PS1 mice can impact their amyloid and microglial pathologies. More specifically, we treated young and aged APP/PS1 mice with a single i.p injection of hIVIG for 1 day, 1 week, 3 weeks, 3 months and 8 months. Consistently, in all experiments hIVIG did not reduce or lower on brain Aβ burden. Instead, with chronic treatment soluble levels of brain Aβ levels were elevated without any concomitant change in Aβ plaque burden. We also found no evidence that suggests Aβ plaque disintegration, microglia mediated Aβ phagocytosis or peripheral sink mechanism as mode of action for hIVIG. As our sample had enough power to detect small changes in Aβ, we can conveniently conclude that treatment with hIVIG had very limited effect on the development and progression of Aβ plaque pathology in APP/PS1 mice. Chronic treatment with hIVIG also resulted in changes in specific sub-populations of microglia, suppressed *Tnf* gene expression, and fostered neurogenesis. Importantly, changes in microglia correlated with changes in *Tnf* and number of newborn neurons, indicating that hIVIG treatment effects are mutually linked and are not pro-inflammatory. Therefore, based on our data we can conclude that hIVIG may have many inter-related effects on ongoing neuroinflammation in APP/PS1 mice. Immunomodulatory and anti-inflammatory effects may mediate beneficial effects of hIVIG in human AD patients.

In our NFκB study, our objective was to see whether immunomodulation induced by genetic manipulation of the NFκB signalling pathway affects progression of amyloid and glial pathologies in APP/PS1 mice. For this, we followed our novel *Nfkb1* deficient mice under APP/PS1 background in three age points up to 12 months. Furthermore, in an independent experiment we ascertained whether an extra acute LPS stimulus may impact hippocampal Aβ burden. However, we noticed very limited and sometimes conflicting effects of NFκB *Nfkb1* on amyloid pathologies of APP/PS1 mice. Even an extra LPS stimulus did not bring about any significant changes to Aβ burden. Based on our data, we can conclude that NFκB *Nfkb1* has very a limited role in modulating amyloid pathology of APP/PS1 mice. NFκB *Nfkb1* defecient mice on APP/PS1 background showed profound changes in microglia and elevated *Tnf* and *Il1b* gene expression, indicating a pro-inflammatory microglial phenotype. Importantly, gene deletion of *Nfkb1* did not worsen age dependent memory impairments seen in APP/PS1 mice.

In conclusion, in both studies we found immunomodulatory effects on neuroinflammatory processes but very limited effects on amyloid pathology in APP/PS1 mice.

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Neuroinflammation in Alzheimer's disease (AD) may aggravate or clear amyloid plaques. In this thesis by employing transgenic mouse models of AD, we examined the impact of pharmacological and genetic immunomodulators on progression of amyloid pathology. Treatment with polyclonal human intravenous immunoglobulins as well as *Nfkb1* gene deletion both resulted in meagre effects on plaque pathology but distinctly suppressed CD45 positive activated microglia around amyloid plaques.

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