Immunomodulation As A Potential Therapeutic Approach For Alzheimer’s Disease

by

William Veljko Nikolic

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy Department of Molecular Medicine College of Medicine University of South Florida

Co-Major Professor: Jun Tan, M.D., Ph.D.
Co-Major Professor: Huntington Potter, Ph.D.
    Paula Bickford, Ph.D.
    Inge Wefes, Ph.D.
    Andreas Seyfang, Ph.D.

Date of Approval:
    June 13, 2008

Keywords: Inflammation, CD40, immunization, human umbilical cord blood cells, microglia

© Copyright 2008, William Veljko Nikolic
ACKNOWLEDGEMENTS

Thanks are due first to my major professor, Dr. Jun Tan, for his great insights, perceptiveness, and mentorship. My sincere thanks go to co-major professor, Dr. Huntington Potter and other committee members; Dr. Paula Bickford, Dr. Andreas Seyfang, and Dr. Inge Wefes. I would further like to thank my family for their continuous and overwhelming support. Lastly, I would like to recognize those individuals who have directly or indirectly made this Ph.D. possible, especially: Kavon Rezai-Zadeh, Jared Ehrhart, Dr. Huayan Hou, Dr. Yuin Bai, Dr. Takashi Mori, Dr. Terrence Town, Dr. Brian Giunta, and Jin Zeng.
# Table of Contents

## List of Tables

iii

## List of Figures

iv

## List of Abbreviations

vi

## Abstract

viii

## Chapter One: Introduction

1.1. The impact of Alzheimer’s disease  
1.2. Pathology of Alzheimer’s disease  
1.3. Microglia and central nervous system  
1.4. Classical roles of peripheral innate immune cells  
1.4.1. The macrophage: prototypical phagocyte  
1.4.2. The dendritic cell: professional antigen presenting cell  
1.5. Microglial activation after toll-like receptor stimulation: a mixed response  
1.6. Adaptive response of activated microglia in demyelinating disease via CD40-40 ligand interaction  
1.6.1. Brain inflammation in demyelinating disease  
1.6.2. CD40-40 ligand interaction in experimental autoimmune encephalitis  
1.7. Activation of microglia after CD40 ligation in Alzheimer’s disease: a shift from innate to adaptive response  
1.7.1. Alzheimer’s disease and microglial responses to Aβ  
1.7.2. Microglial response to Aβ in context of CD40 ligation  
1.8. Immunotherapy and Alzheimer’s disease

## Chapter Two: Peripherally Administered Human Umbilical Cord Blood Cells Reduce Parenchymal and Vascular B-Amyloid Deposits and Suppress CD40-CD40L Interaction

21

## Chapter Three: CD40L Disruption Enhances Ab Vaccine-Mediated Reduction of Cerebral Amyloidosis While Minimizing Cerebral Amyloid Angiopathy and Inflammation

75
CHAPTER FOUR: TRANSCUTANEOUS AB PEPTIDE IMMUNIZATION
OF TRANSGENIC ALZHEIMER’S MICE RESULTS IN REDUCED
CEREBRAL AB DEPOSITS IN THE ABSENCE OF T-CELL
INфиLTrATiON AND MiCROHEMORRHAGE

CHAPTER FIVE  DISCUSSION
  5.1. Microglia and central nervous system
  5.2. Immunotherapy for Alzheimer’s disease
    5.2.1. Immunotherapy: transcutaneous vaccination
    5.2.2. Immunotherapy: human umbilical cold blood cells

REFERENCES

ABOUT THE AUTHOR
LIST OF TABLES

Table 1 - Phagocytic and antigen presenting cell responses of immune cells. 10
LIST OF FIGURES

Figure 1. Cerebral Aβ/β-amyloid pathology is reduced in PSAPP and Tg2576 mice peripherally infused with HUCBC. 36

Figure 2. β-amyloid associated microgliosis and astrocytosis are reduced in HUCBC infused-PSAPP mice. 42

Figure 3. HUCBC infusion results in CD40-dependent increased plasma Aβ levels in PSAPP mice. 49

Figure 4. HUCBC infusion promotes anti-inflammatory/Th2 responses and decreases sCD40L in the CNS. 52

Figure 5. HUCBC modulate microglial CD40 expression and promote Aβ microglial/macrophage phagocytic activity. 56

Figure 6. Evaluation of the effects of CD40 deficiency on Aβ antibody generation and Aβ efflux in Aβ1-42-immunized mice. 91

Figure 7. Cerebral Aβ levels are significantly reduced in Aβ1-42-immunized PSAPP mice heterozygous for CD40. 95

Figure 8. β-amyloid pathology is reduced in Aβ1-42-immunized PSAPP mice heterozygous for CD40. 97

Figure 9. PSAPP/CD40−/− mice have increased anti-inflammatory IL-10 cytokine and decreased plasma soluble CD40L (sCD40L) after Aβ1-42 vaccination. 103

Figure 10. Peripheral and cerebral Aβ levels are reduced in Aβ1-42-immunized PSAPP mice treated with CD40L neutralizing antibody. 106

Figure 11. Cerebral β-amyloid deposits and cerebral amyloid angiopathy are reduced in Aβ1-42-immunized PSAPP or Tg2576 mice treated with CD40L neutralizing antibody. 111

Figure 12. CD40L blockade inhibits APC-like microglial activation in
Aβ1-42 vaccinated PSAPP mice and promotes anti-inflammatory cellular immunity. 118

Figure 13. Aβ-specific neurotoxic inflammatory responses are reduced in Aβ1-42-immunized PSAPP mice deficient for CD40. 126

Figure 14. Generation of immune responses in wild-type C57BL/6 mice t.c. immunized with aggregated Aβ1-42 peptide plus CT. 157

Figure 15. Aβ/CT t.c. immunization resulted in LC recruitment into dermal layers. 162

Figure 16. Increased systemic Aβ after Aβ1-42/CT t.c. immunization of PSAPP mice. 167

Figure 17. Reduction of cerebral Aβ/β-amyloid pathology in PSAPP mice t.c. immunized with Aβ1-42/CT. 171

Figure 18. Simultaneous analysis of plasma and brain soluble Aβ levels on a mouse-by-mouse basis. 176

Figure 19. Absence of T-cell infiltration or brain microhemorrhage in Aβ/CT t.c. immunized mice. 180

Figure 20. Apolipoprotein B protein was undetectable in brain tissue homogenates derived from both Aβ/CT and CT t.c.-immunized mice. 182

Figure 21. Model for innate versus adaptive microglial activation responses. 198
LIST OF ABBREVIATION

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper type 1 cell</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper type 2 cell</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>MΦ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>EAE</td>
<td>Autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>HUCBC</td>
<td>Human umbilical cord blood cells</td>
</tr>
<tr>
<td>HAMNC</td>
<td>Human adult mononuclear cells</td>
</tr>
<tr>
<td>CC</td>
<td>Cingulate cortex</td>
</tr>
<tr>
<td>H</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>TGF</td>
<td>Tumor growth factor</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>t.c.</td>
<td>Transcutaneous</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cells</td>
</tr>
</tbody>
</table>
Immunomodulation As A Potential Therapeutic Approach for Alzheimer’s Disease

William Veljko Nikolic

ABSTRACT

Alzheimer’s disease (AD) is the most prevalent form of progressive dementia and is characterized by the accumulation of amyloid beta (Aβ) peptide in the brain and in the cerebral vessels forming cerebral amyloid angiopathy (CAA). As previously reported, an active immunization strategy of mice with Aβ1-42 peptide results in decreased Th1 and increased Th2 cytokine responses as well as an effectively clearance of CNS Aβ. This approach has also yielded favorable results for many patients, unfortunately, a small percentage of these study participants developed severe aseptic meningoencephalitis likely secondary to CNS invasion of activated T-cells. We have previously demonstrated that disruption of CD40-40L pathway reduces Aβ plaque load, promotes Th2 response, and rescues from cognitive impairments. However, direct blockage of the CD40 pathway by passive vaccination with anti-CD40L antibody leads to immunosupression. Therefore, in its current form this therapeutic strategy poses an unacceptable risk to the recipient of treatment, aged individual. For those reasons, the identification and characterization of alternative modulators/inhibitors of CD40 signaling may be necessary for the development of safe and effective AD immunotherapy.

This proposal introduces novel immunomodulatory therapies that are based on previous vaccination strategies or cell based therapies across medial field. We showed
that transcutaneous vaccination can both be efficacious and safe, thus clearly
demonstrating that the right combination of the antigens, adjuvants, and the routes of
administration are crucial for the right vaccine. Furthermore, we demonstrated that the
effects of current Aβ vaccine strategies could be enhanced by a simultaneous blockade of
CD40-40L signaling. As an alternative approach, we explored the possibility of cell-
based therapies and showed that human umbilical cord blood cells, which are currently
used as a treatment for systemic lupus erythematosus and leukemia, and currently
investigated against stroke, amyotropic lateral sclerosis, age-related macular
degeneration, multiple sclerosis, and Parkinson’s disease, and showed that not just they
improved the AD like pathology in transgenic animals but altered both the brain and
peripheral inflammation levels. Lastly, we discussed the involvement of microglia, one of
the key players in both AD pathogenesis and Aβ clearance and suggested that microglia in
actuality has a continuum of physiological activation states that contribute to
proinflammation, antiinflammation, and phagocytosis.
CHAPTER ONE
INTRODUCTION

1.1 The impact of Alzheimer’s disease

First described in 1906 by Alois Alzheimer, the Alzheimer’s disease (AD) is the leading cause of dementia among elderly. Dementia is a collective name for progressive degenerative brain syndromes, which affect memory, behavior, emotions, and thinking. Currently, about 10 percent of Americans over the age of 65 and half of those over age of 85 have been diagnosed with AD, which equates to over 5.2 million Americans that suffer from this disease. According to 2008 Alzheimer’s Association Facts and Figures report, AD is the seventh-leading cause of death in U.S. and where every 71 seconds someone develops AD. The report also estimates that the direct and indirect costs of Alzheimer's and other dementias to Medicare, Medicaid and businesses amount to more than $148 billion each year, which makes it a third most expensive disease to threat in the U.S.

Generally speaking, Alzheimer’s disease can be divided into three basic stages. The early stage is characterized by mild cognitive decline, i.e. word- or name-finding problems, decline in ability to organize, and performance issues in social or work settings. The middle stage is characterized by severe cognitive decline (confusion, disorientation, and delusions), disruption in normal sleep/walking cycle, and increasing

1
episodes of urinary or fecal incontinence. The late and final stage of AD is characterized by the loss of capacity of recognizable speech, individuals lose the ability to work, lose the ability to walk without assistance, then the ability to sit without support, the ability to smile, the ability to hold their head up, and individuals need help with eating and toileting and there is general incontinence of urine.

1.2 Pathology of Alzheimer’s disease

Brain regions being affected early are entorhinal cortex, hippocampus, and basal forebrain. These are small specialized structures that play a crucial role in memory. Over time, the disease destroys large areas of the brain that control vital body functions and as a result AD patients typically die from pneumonia or lack of nutrition. Pathologically speaking, major AD characteristics are senile plaques (caused by amyloid beta peptide deposition), neurofibrillary tangles (caused by tau protein deposition), and neuronal loss (characterized by dystrophic neurites). Amyloid or “senile” plaque is primarily composed of 39-43 amino acid peptide amyloid beta (A\(\beta\)). A\(\beta\) is derived by a proteolytic cleavage of amyloid precursor protein (APP). There are A\(\beta\) is normally water soluble, however it does have a tendency to aggregate, ex. especially longer forms of the peptide (A\(\beta\)\(_{1-40}\) and A\(\beta\)\(_{1-42}\)) as regular \(\beta\)-plated sheet conformations and come out as insoluble amyloid plaques. These plaques are normally detergent resistant. A\(\beta\) can also be deposited as diffused deposits, often believed to be the intermediate step before the more compact amyloid plaques form. In recent years, A\(\beta\) deposition has been identified in the vascular wall. This is know as a cerebral amyloid angiopathy (CAA), a pathology that was identified in as much as 83 percent of AD patients (Ellis et al., 1996). Neurofibrilary
tangles are intraneuronal inclusions of abnormal fibers that consist of paired helical filaments. The filaments consist of microtubule-associated hyperphosphorylated tau proteins. Lastly, neuronal loss is thought to originate from amyloid plaques, inflammatory reactions due to cytokine releases, oxidative injury that disrupt neuronal metabolic and ionic homestasis, and impaired neuronal trafficking due to hyperphosphorylated tau filaments.

1.3 Microglia and central nervous system

Microglia are somewhat enigmatic central nervous system (CNS) cells that have been traditionally regarded as CNS macrophages (MΦs). They represent about 10% of the adult CNS cell population (Pessac et al., 2001). In mice, microglial progenitors can be detected in neural folds at the early stages of embryogenesis. Murine microglia are thought to originate from the yolk sac at a time in embryogenesis when monocyte/macrophage progenitors (of hematopoietic origin) are also found (Pessac et al., 2001) (Alliot et al., 1999). Based on this observation, it is now generally accepted that adult mouse microglia originate from monocyte/macrophage precursor cells migrating from the yolk sac into the developing CNS, most likely independently of CCR2 (Mildner et al., 2007). Once CNS residents, these newly migratory cells actively proliferate during development, thereby giving rise to the resident CNS microglial cell pool. More recently however, it has been shown that bone marrow-derived cells can enter the CNS and become cells that phenotypically resemble microglia in the adult mouse (Eglitis and Mezey, 1997) (Brazelton et al., 2000) (Mezey et al., 2000). Interestingly, under conditions of CNS damage such as stroke, cholinergic fiber degeneration, or motor
neuron injury, Priller and colleagues found that green fluorescent protein-labeled bone marrow cells could enter the CNS and take up a microglial phenotype (Priller et al., 2001). On other hand it was shown that bone-marrow derived cells do not contribute at all to the CD11b+/Iba-1+ population when the CNS was protected from irradiation, suggesting that proliferation of endogenous microglia is the most plausible explanation for the increase in Iba-1+ cell number (Mildner et al., 2007). This latter view could be further supported with citings that local microglia proliferate following lesioned CNS with intact blood-brain barrier (Priller et al., 2006) (Remington et al., 2007).

Microglia normally exist in a quiescent (resting) state in the healthy CNS, and are morphologically characterized by small soma and ramified processes. However, upon “activation” in response to invading viruses or bacteria or CNS injury, microglia undergo morphological changes including shortening of cellular processes and enlargement of their soma (sometimes referred to as an “amoeboid” phenotype). Activated microglia also up-regulate a myriad of cell surface activation antigens and produce innate cytokines and chemokines (discussed in detail below). As the microglial lineage originates from peripheral myeloid precursor cells, it is helpful to consider the activation states of such peripheral innate immune cells to better understand the nature of microglial activation.

1.4 Classical roles of peripheral innate immune cells

It is now widely accepted that both innate and adaptive arms of the immune system play important roles in maintaining immune homeostasis. However, little attention was paid to the evolutionarily much older innate immune system until the late Charlie Janeway proposed the involvement of innate mechanisms in vertebrate immunity.
Specifically, Janeway pioneered the idea that lymphocyte activation could be critically regulated by the evolutionarily ancient system of antigen clearance by phagocytic cells of myeloid origin. Together with Ruslan Mezhitov, they originated the concept that these phagocytic innate immune cells recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors, the most notable examples being a set of phylogenetically conserved, germ-line encoded Toll-like receptors (TLRs, currently 11-12 members, (Qureshi and Medzhitov, 2003) (Yamamoto et al., 2004) (Zhang et al., 2002) (Tabeta et al., 2004), resulting in expression of cell-surface activation molecules [for example, major histocompatibility complex (MHC) class I and II, B7.1, B7.2, and CD40] and secretion of innate cytokines (e.g., tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-12, and IL-18 (Janeway and Medzhitov, 2002) (Medzhitov and Janeway, 2000a). Once activated, the innate arm of the immune response calls adaptive immune cells into action, and both branches act in concert to promote neutralization and clearance of invading pathogens. Thus, innate immune cells are able to discriminate “non-infectious self” from “infectious non-self” and thereby form the first line of defense against invading bacteria and viruses (for review see (Medzhitov and Janeway, 2000b) (Medzhitov and Janeway, 2002) (Medzhitov and Janeway, 2002) (Iwasaki and Medzhitov, 2004).

1.4.1 The macrophage: prototypical phagocyte

Macrophages (MΦs) are quintessential phagocytes whose primary role is to engulf pathogens such as invading bacteria and to remove debris and detritus, i.e.,
remnants of apoptotic cells. Tissue MΦs develop when blood monocytes enter into the various organs and tissues and differentiate into specialized, site-specific MΦs depending on their anatomical location, such as alveolar MΦs (lung), histiocytes (connective tissue), kupffer cells (liver), mesangial cells (kidney), osteoclasts (bone), or microglia (brain) (Goldsby et al., 2002). Resting MΦs are both weak phagocytes and weak lymphocyte activators (Adler et al., 1994). Upon activation however, for example in response to TLR stimulation by PAMPs, their phagocytic potential greatly enhances (Blander and Medzhitov, 2004) and they up-regulate cell-surface co-stimulatory molecules and produce pro-inflammatory innate cytokines as mentioned above. Typically, engulfment of the pathogen by phagocytosis triggers a “respiratory burst” involving production of reactive oxygen species such as superoxide and peroxinitrite that kill the pathogen (Adler et al., 1994) (Forman and Torres, 2001). In addition, activated MΦ up-regulate cell-surface Fc receptors that aid in phagocytosis of pathogens opsonized by antibodies produced by plasma cells (Tsukada et al., 1994) (Blom et al., 2003). On the other hand, in response to debris from apoptotic cells, the MΦ mounts a phagocytic response essentially in the absence of pro-inflammatory cytokines (Gregory and Devitt, 2004). The most likely reason for this anti-inflammatory phagocytic response is that pro-inflammatory cytokines such as TNF-α promote bystander injury which may further damage tissues in which the apoptotic cells reside. Thus, MΦs are highly capable of “innate activation” characterized by a strong phagocytic response sometimes accompanied by pro-inflammatory cytokine production (for a review see (Fujiwara and Kobayashi, 2005).
1.4.2 The dendritic cell: professional antigen presenting cell

Whereas MΦs have limited ability to process and present antigen to T cells, dendritic cells (DCs) are considered professional antigen presenting cells (APCs). DCs can be found under the epithelia and in most organs where they capture and process non-self antigens, migrate to lymphoid organs, and present antigen in the context of MHC to CD4+ and CD8+ T lymphocytes. With their many finger-like cellular processes, DCs are morphologically optimized to simultaneously display antigen to many T cells. Like MΦs, DCs respond to invading pathogens by recognizing PAMPs through TLRs, and subsequently phagocytose and process antigen. DCs then up-regulate cell-surface co-stimulatory molecules and secrete innate cytokines and chemokines (typically at levels an order of magnitude higher than those secreted by MΦs) to promote recruitment and activation of CD4+ and/or CD8+ T lymphocytes. There are three generally accepted classifications of DCs in mice: plasmacytoid (p) DCs (CD11clo, CD11blo, B220+, CD8-), lymphoid (l) DCs (CD11c+, CD11b-, CD8+), and myeloid (m) DCs (CD11c+, CD11b+, B220-, CD8-; there are several subtypes,) (Banchereau et al., 2000). In humans, there are clearly two distinct subsets of DCs: pDCs (CD11c-, CD11b-, CD14-, CD45RA+) and monocyte DCs (CD11c+, CD11b+, CD14+, CD45RA-) (for a review see (Shortman and Liu, 2002). DC classes differ from each other predominately in tissue distribution, production of specific cytokines, TLR expression, and ability to promote innate versus adaptive immune responses (for a review see (Iwasaki and Medzhitov, 2004). For example, freshly isolated human pDCs express TLR7 and 9, whereas mDCs express
TLR1, 2, 3, 5, 6, and 8 (Hornung et al., 2002) (Jarrossay et al., 2001) (Kadowaki et al., 2001). Stimulation of human pDCs or monocyctic DCs with synthetic TLR7 ligands induces the secretion of interferon (IFN)-\(\alpha\) (important for anti-viral innate immunity) or IL-12 [a key inducer of the adaptive T helper (Th) type I response], respectively (Ito et al., 2002). Similarly, stimulation of TLR9 via DNA containing unmethylated CpG motifs results in IFN-\(\alpha\) secretion by pDCs and IL-12 production by murine mDCs (Hemmi et al., 2003). Despite these relative differences between DC classes, the major role of DCs on the whole remains; they act as potent APCs capable of strongly activating T lymphocytes. Their APC capacity is much stronger than that of MΦs, as DCs are able to directly activate naïve T cells whereas MΦs are not (Iwasaki and Medzhitov, 2004). Thus, by virtue of their ability to promote T cell activation responses, DCs are highly capable of “adaptive activation”. Activation markers of phagocytosis and APC responses in various innate immune cells are presented in the Table 1.

1.5 Microglial activation after toll-like receptor stimulation: a mixed response

Recent evidence indicates that microglia, like their peripheral innate immune cell counterparts, express a wide array of TLRs, including mRNA for TLRs 1-9 in mice (Olson and Miller, 2004) and in humans (Bsibsi et al., 2002). Furthermore, many of these TLRs have been shown to be functional, allowing microglial recognition of a variety of PAMPs. For example, Kielian and coworkers found that heat-killed Staphylococcus aureus and its cell wall product peptidoglycan (PGN) are able to stimulate innate activation of microglia characterized by pro-inflammatory cytokine and
chemokine production (Kielian et al., 2002). Those authors found that the effect of PGN was critically dependent on TLR2, as TLR2-deficient mice demonstrated reduced cytokine and chemokine production after PGN challenge (Kielian et al., 2005). Furthermore, murine microglia respond to the TLR9 agonist, unmethylated CpG-DNA, by secreting numerous pro-inflammatory innate cytokines (probably responsible for neurotoxicity in organotypic brain slice cultures treated with CpG-DNA (Iliev et al., 2004), by up-regulating co-stimulatory cell surface molecules, and by promoting adaptive activation by secreting IL-12 to affect T cell activation (Dalpke et al., 2002). In two recent studies, murine microglial pro-inflammatory responses to bacterial lipopolysaccharide (LPS), a known TLR4 ligand, resulted in dramatic injury to cultured oligodendrocytes (Lehnardt et al., 2002) and neurons (Lehnardt et al., 2003), further
Table 1 - Phagocytic and antigen presenting cell responses of immune cells.

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>Phagocytosis</th>
<th>APC function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD36</td>
<td>++</td>
<td>+</td>
<td>(Albert et al., 1998) (Tait and Smith, 1999) (Coraci et al., 2002)</td>
</tr>
<tr>
<td>CD40</td>
<td>ND</td>
<td>++</td>
<td>(Fischer and Reichmann, 2001) (Brawand et al., 2002) (Kaul et al., 2000)</td>
</tr>
<tr>
<td>CD80</td>
<td>ND</td>
<td>++</td>
<td>(Brawand et al., 2002) (Kaul et al., 2000) (Prilliman et al., 2002)</td>
</tr>
<tr>
<td>CD86</td>
<td>ND</td>
<td>++</td>
<td>(Brawand et al., 2002) (Kaul et al., 2000) (Prilliman et al., 2002)</td>
</tr>
<tr>
<td>MHC II</td>
<td>ND</td>
<td>++</td>
<td>(Brawand et al., 2002)</td>
</tr>
<tr>
<td>CD11c</td>
<td>ND</td>
<td>++</td>
<td>(Fischer and Reichmann, 2001)</td>
</tr>
<tr>
<td>CD40L</td>
<td>+/-</td>
<td>++</td>
<td>(Fischer and Reichmann, 2001) (Townsend et al., 2005)</td>
</tr>
</tbody>
</table>

**Cytokine**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Phagocytosis</th>
<th>APC function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>ND</td>
<td>++</td>
<td>(Quaranta et al., 2002)</td>
</tr>
<tr>
<td>IL-6</td>
<td>ND</td>
<td>++</td>
<td>(Spisek et al., 2001)</td>
</tr>
<tr>
<td>IL-12</td>
<td>ND</td>
<td>++</td>
<td>(Spisek et al., 2001) (Quaranta et al., 2002)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td>++</td>
<td>(Spisek et al., 2001) (Quaranta et al., 2002) (Townsend et al., 2005)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+/-</td>
<td>++</td>
<td>(Townsend et al., 2005)</td>
</tr>
<tr>
<td>IL-4</td>
<td>++</td>
<td>+/-</td>
<td>(Townsend et al., 2005)</td>
</tr>
<tr>
<td>IL-10</td>
<td>++</td>
<td>+/-</td>
<td>(Townsend et al., 2005)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>++</td>
<td>ND</td>
<td>(Akiyama et al., 2000)</td>
</tr>
</tbody>
</table>

Note: ND, assay not performed; +/-, weak response; +, modest response; ++, strong response
demonstrating microglial bystander injury after TLR stimulation (probably mediated by over-production of innate pro-inflammatory cytokines). It has recently been shown that microglia respond to poly I:C [a synthetic double-stranded (ds) RNA analog thought to be recognized by TLR3, (Alexopoulou et al., 2001)] by producing pro-inflammatory cytokines and chemokines (Jack et al., 2005), and microglial pro-inflammatory responses to dsRNA seem to be dependent on TLR3, as TLR3-deficient microglia have blunted innate cytokine responses in vitro and markedly reduced cell surface activation markers in brain after poly I:C stimulation (Town et al., submitted). Finally, infection with West Nile virus, a retrovirus that produces dsRNA during its life cycle, results in profound microglial activation as assessed by pro-inflammatory cytokine production in vitro and cell surface activation markers in vivo, effects that are dramatically reduced in TLR3-deficient animals (Wang et al., 2004).

In peripheral innate immune cells, TLR responses to PAMPs seem to be dependent on at least four different TLR intracellular adapter molecules: MyD88 (involved in TLR1, 2, 4, 6, 7, 8, and 9 signaling), TRIF/TICAM-1 (mediates TLR3 and 4 signaling), TIRAP/Mal (involved in TLR1, 2, 4, and 6 responses) and TIRP/TRAM/TICAM-2 (mediates TLR4 signaling). These adapter molecules bind to the intracellular leucine-rich repeat region of the TLR and promote recruitment of additional factors such as IRAKs and TRAF6 that allow for activation of transcription factors including IRF-3 and NF-κB, which are responsible for activation of numerous innate cytokines and cell-surface activation antigen genes (for review see (Vogel et al., 2003) (Hemmi and Akira, 2005). It is still unclear how different TLR responses in innate immune cells (i.e., promotion of innate versus adaptive responses) can be achieved when
many TLRs share intracellular signaling molecules. While little work has been done on intracellular signaling following TLR stimulation in microglia, it is likely that microglia utilize the same signaling cascades described for MΦs and DCs.

1.6 Adaptive response of activated microglia in demyelinating disease via CD40-CD40 ligand interaction

1.6.1 Brain inflammation in demyelinating disease

Experimental autoimmune encephalomyelitis (EAE) is a mouse model of the human disease multiple sclerosis (MS), an autoimmune disease characterized by inflammatory CNS demyelinating lesions accompanied by motor disturbances. EAE can be induced in different strains of mice by subcutaneous or intraperitoneal inoculation with adjuvant plus epitopes found in myelin such proteolipid protein, myelin basic protein, or myelin oligodendrocyte glycoprotein. The disease is critically dependent on activation of pro-inflammatory CD4+ T helper type I (Th1) cells by APCs, and these auto-aggressive Th1 cells can be adoptively transferred to non-diseased recipient mice that subsequently develop disease. EAE is characterized by paralysis, typically beginning in the tail and hind limbs and progressing to the fore limbs. In the SJL mouse strain, animals develop a relapsing-remitting form of the disease while C57BL/6 mice manifest paralysis that progressively worsens until death. Upon histopathological analysis, brains from EAE mice generally show infiltration of Th1 cells (and other lymphocytes including MΦs and DCs) and activation of microglia, typically in white matter regions where demyelinating lesions are found (for review see (Olsson, 1995))
1.6.2 CD40-CD40 ligand interaction in experimental autoimmune encephalomyelitis

Immune/inflammatory cells receiving a primary stimulus (i.e., MHC-T cell receptor interaction between APCs and T lymphocytes, respectively) typically require co-stimulatory signals via other pairs of molecules in order to become activated [for instance, the B7-CD28 and/or CD40-CD40 ligand (L) dyads in APC/T-cell activation; (van Kooten and Banchereau, 2000)]. CD40L is a key immunoregulatory molecule that plays a co-stimulatory role in the activation of immune cells from both the innate and adaptive arms of the immune system, and is typically expressed by activated CD4+ and some CD8+ T cell subsets (Grewal and Flavell, 1998). CD40 receptor, a member of TNF and nerve growth factor super-family, is expressed on many professional and non-professional APCs, including dendritic cells, B cells, monocytes/macrophages and microglial cells (O'Keefe et al., 2002) (Carson et al., 1998) (Havenith et al., 1998) (Tan et al., 1999b) (Tan et al., 1999a). Nearly 10 years ago, activated Th cells that expressed CD40 ligand (CD40L) were found in brains of MS patients, and these cells were found in close apposition to CD40-bearing cells in active demyelinating lesions (Gerritse et al., 1996). The authors determined that the CD40-expressing cells were either macrophages or microglia based on staining for acid phosphatase or CD11b.

To evaluate whether the CD40-CD40L interaction was pathogenic in EAE, Gerritse and co-workers administered a CD40L neutralizing antibody to SJL mice that were given proteolipid protein with adjuvant to induce EAE. Strikingly, EAE was prevented in a prophylactic treatment regimen of anti-CD40L, and, when EAE was
induced in another cohort of animals, CD40L antibody treatment significantly reduced
disease severity in an active treatment paradigm (Gerritse et al., 1996). It was later
shown that genetic deficiency in CD40L (Grewal et al., 1996) or antibody-mediated
blockade of CD40L (Howard et al., 1999) resulted in attenuation of Th1 differentiation
and effector function, including marked inhibition of the Th1 cytokine IFN-γ and reduced
numbers of encephalitogenic effector T cells. In an effort to further understand the nature
of the CD40-CD40L interaction responsible for promotion of EAE, Becher and
colleagues used a bone marrow reconstitution system to determine which CD40-
expressing cells were responsible for promoting EAE (Becher et al., 2001). In that
report, the authors showed that CD40 expression by parenchymal microglia was
responsible for recruitment/retention of encephalitogenic T cells in EAE. Strikingly,
treatment of microglia with a combination of granulocyte macrophage-colony stimulating
factor and CD40L has been shown to promote differentiation of these cells into cells that
(1) express the pan-DC marker CD11c, (2) morphologically resemble DCs, and (3)
secrete the Th1-promoting cytokine IL-12 p70 (Fischer and Reichmann, 2001). Such
CD11c+ CD11b+ “DC-like” microglia were found in EAE brain lesions in inflammatory
foci containing T cells, and exhibited potent stimulation of allogeneic T cell proliferation
versus CD11c- CD11b+ microglia (Fischer and Reichmann, 2001). Although their origin
was not determined, it was recently shown that “CNS DCs” (possibly “DC-like”
microglia) are responsible for activation of naïve T cells in response to endogenous
myelin epitopes (termed “epitope spreading”), and this process was initiated in the CNS
as opposed to the peripheral lymphoid organs (McMahon et al., 2005). Thus, in the
context of EAE, CD40-CD40L interaction on microglia seems to promote adaptive
function of these cells, resulting in a “DC-like” activated microglia phenotype that promotes encephalitogenic Th1 cell differentiation and effector function.

1.7 Activation of microglia after CD40 ligation in Alzheimer disease: a shift from innate to adaptive response

1.7.1 Alzheimer disease and microglial responses to β-amyloid

It has been suggested that activated microglia play a key role in AD pathogenesis as they secrete pro-inflammatory innate cytokines such as TNF-α and IL-1β, which have been shown to promote neuronal injury at high levels (Meda et al., 1995) (Barger and Harmon, 1997) (McGeer and McGeer, 1998). Furthermore, there is a large body of evidence that non-steroidal anti-inflammatory drug (NSAID) use is associated with reduced risk for AD in humans (Shapshak et al., 2004) (in t' Veld et al., 2001) (Zandi et al., 2002), for a review see (Szekely et al., 2004), and NSAID treatment of AD mice results in reduced β-amyloid plaque burden concomitant with ameliorated microglial activation (Matsushima et al., 1994) (Matsushima et al., 1994) (Lim et al., 2001). Work done in Maxfield’s laboratory showed that challenge of microglia with labeled Aβ peptides promotes phagocytosis but poor degradation of soluble or fibrillar Aβ via scavenger receptors (Paresce et al., 1996) (Paresce et al., 1997) (Chung et al., 1999). Using knockout mice, his laboratory showed that the class A scavenger receptor (type I and II) is the predominant scavenger receptor responsible for Aβ uptake by microglia, with other scavenger receptors playing a more minor role (including the class B
scavenger receptor CD36) (Chung et al., 1999).

1.7.2 Microglial responses to β-amyloid in the context of CD40 ligation

We previously showed that, while murine microglial challenge with soluble Aβ peptides alone does not elicit TNF-α secretion, co-stimulation provided in the form of CD40 ligation (either via CD40L or an agonistic CD40 antibody) results in TNF-α production being synergistically affected (Tan et al., 1999a). Further, microglia cultured from AD mice deficient in CD40L demonstrate reduced TNF-α secretion versus CD40L-sufficient AD mouse microglia. This form of microglial activation in CD40L-sufficient AD mice is pathogenic, as CD40L-deficient AD mice demonstrate reduced activated (CD11b+) microglia, an effect that is associated with mitigated abnormal hyper-phosphorylation of tau protein (a key indicator of neuronal stress) (Tan et al., 1999a). Furthermore, genetic ablation of CD40L or administration of a CD40L-neutralizing antibody markedly reduces β-amyloid plaques in mouse models of AD, effects that are associated with mitigated astrocytosis and microgliosis (Tan et al., 2002a), for review see (Town et al., 2001) (Tan et al., 2002b). More recently, overproduction of microglia-associated CD40 and of astrocyte-derived CD40L was found in and around β-amyloid plaques in AD patient brains (Togo et al., 2000) (Calingasan et al., 2002), raising the possibility that the CD40-CD40L interaction may contribute to AD pathogenesis by promoting brain inflammation.

In order to better understand the form of microglial activation affected by Aβ plus CD40L stimulation, we examined innate and adaptive activation of murine microglia challenged with Aβ in the presence or absence of CD40L co-stimulation (Townsend et
al., 2005). When microglia were challenged with fluorescent-tagged synthetic human Aβ alone, they mounted a time-dependent phagocytic response (from 15 min to 60 min) which could be enhanced by Fc receptor stimulation using an anti-human Aβ antibody. This phagocytic response to Aβ alone was not associated with production of the pro-inflammatory innate cytokines TNF-α, IL-6, or IL-1β, a result similar to that seen when microglia are challenged with apoptotic cells and mount an anti-inflammatory, pro-phagocytic innate response (Minghetti et al., 2005). Importantly, CD40L treatment opposed this phagocytic response, as determined by measuring both cell-associated Aβ and free extracellular Aβ. As mentioned above, Maxfield’s laboratory demonstrated that microglia slowly degrade phagocytosed Aβ peptides (Paresce et al., 1996) (Paresce et al., 1997) (Chung et al., 1999). We examined the ability of microglia to degrade Aβ peptides by first pulsing them with Aβ and then chasing these cells after 1 hour of culture in the presence or absence of CD40L stimulation. Using this experimental approach, we found that CD40L also retarded microglial clearance of the peptide. We further assessed putative modulation of microglial Aβ phagocytosis by cytokines known to promote effector T cell function, and found that the pro-inflammatory Th1-type cytokines IFN-γ and TNF-α inhibited Aβ phagocytosis whereas the anti-inflammatory Th2-type cytokines IL-4 and IL-10 boosted this response.

1.8 Immunotherapy and Alzheimer’s disease

Alzheimer’s disease is described by many pathological characteristics and yet the predominant theory of the disease process in the amyloid hypothesis. This
hypothesis suggests that Aβ deposition can directly through diffuse or compact plaques, or indirectly through inflammatory cascade, result in progressive synaptic and neuritic injury. This injury causes alterations in intracellular kinases functions that further contribute to tau protein hyperphosphorylation and the formation of neurofibrillary tangles. The combination of these events and their propagation is believed to contribute to neuronal dysfunction and loss which will contribute to dementia observed in Alzheimer’s disease (Hardy and Selkoe, 2002). Thus, methods developed to clear or prevent formation of Aβ in the brains of AD patients represent a possible treatment modality. One promising approach involves utilization of “active” Aβ immunization strategies, which produce dramatic reductions in Aβ pathology in animal studies (Schenk et al., 1999). However, a phase IIa clinical trial was abandoned after about 6% of Aβ-immunized AD patients developed aseptic meningoencephalitis (Nicoll et al., 2003; Orgogozo et al., 2003) that appeared to involve brain inflammatory reactions mediated by T-cells and microglia (Monsonego et al., 2001; Schenk and Yednock, 2002; Greenberg et al., 2003; Monsonego et al., 2003a). Interestingly, a 12 month post-vaccination period analysis revealed an inverse correlation between titers of amyloid plaque-reactive antibodies and rate of cognitive decline (Hock et al., 2003), suggesting clinical efficacy. Despite the discontinuation of the clinical trials, Aβ vaccination studies have continued in effort to identify an immunization approach that is both safe and effective. Current approaches have focused on minimizing T-cell mediated inflammatory responses in efforts to prevent CNS invasion of auto-aggressive T-cells, while promoting Aβ antibody-mediated clearance mechanisms (Chackerian et al., 2006; Maier et al., 2006;
Okura et al., 2006; Nikolic et al., 2007). A possible avenue to both enhance Aβ clearance and down-regulate CNS inflammatory responses (including invasion of reactive T-cells) involves modulation of the CD40 receptor (CD40)-CD40 ligand (CD40L) system. In the periphery, a variety of innate immune cells known as antigen-presenting cells (APCs) express CD40, including dendritic cells, B-cells, and monocytes/macrophages. In the CNS, CD40 is expressed by resident cells including microglia, neurons, and astrocytes, as well as by peripherally-derived APCs (Tan et al., 2002a; Town et al., 2005). Recently, the CD40-CD40L interaction was determined to play a central role in promoting and maintaining dendritic cell APC phenotype during infections (Straw et al., 2003). In the context of CNS immunity, the CD40-CD40L interaction is required for microglial maturation into functional APCs (Ponomarev et al., 2006). We have recently shown that CD40L treatment of primary cultures of microglia inhibits phagocytosis of Aβ antibody opsonized, as well as non-opsonized Aβ species (Townsend et al., 2005). A blockade of the CD40-CD40L system down-regulates T-cell/microglia-mediated injury in the context of experimental autoimmune encephalitis (EAE) (Howard et al., 1999; Howard et al., 2002). Altogether, these studies suggest that blockade of the CD40-CD40L interaction could enhance Aβ vaccination-mediated Aβ clearance mechanisms, while minimizing pro-inflammatory T-cell-mediated damage in the CNS. However, a complete blockade of CD40-40L interaction would lead to distortion of immune functioning and hence an autoimmunity would emerge. Therefore, the need for an immunomodulator that can partially block the CD40-40L interaction and yet still allow normal immune functioning would be needed. Human umbilical cord blood cells (HUCBC) have been shown to
oppose the pro-inflammatory T helper cell type 1 (Th1) response, as demonstrated in an animal model of stroke where HUCBC infusion promoted a strong anti-inflammatory T-helper 2 (Th2) response (Vendrame et al., 2004). Importantly, this effect was associated with reduced infarct volume and rescue of behavioral deficit (Vendrame et al., 2004). HUCBC infusion has also shown therapeutic benefit in other neuroinflammatory conditions including multiple sclerosis, amyotrophic lateral sclerosis, neurodegenerative macular degeneration, and Parkinson’s disease (El-Badri et al., 2006; Garbuzova-Davis et al., 2006; Henning et al., 2006). In AD preclinical models, administration of these cells to the PSAPP mouse model of AD was associated with extension of lifespan, although high doses were administered in this paradigm (Ghorpade et al., 2001). So perhaps a proper immunomodulator together with active Aβ vaccine working in tangent could be a right step towards future Alzheimer’s disease treatment.
CHAPTER TWO
PERIPHERALLY ADMINISTERED HUMAN UMBILICAL CORD BLOOD CELLS REDUCE PARENCHYMAL AND VASCULAR \( \beta \)-AMYLOID DEPOSITS AND SUPPRESS CD40-CD40L INTERACTION

2.1 Abstract

Modulation of immune/inflammatory responses by diverse strategies including amyloid-\( \beta \) (A\( \beta \)) immunization, non-steroidal anti-inflammatory drugs, and manipulation of microglial activation states has been shown to reduce Alzheimer’s disease (AD)-like pathology and cognitive deficits in AD transgenic mouse models. Human cord blood cells (HUCBC) have unique immunomodulatory potential, and we wished to test whether these cells might alter AD-like pathology after infusion into the PSAPP mouse model of AD. Here, we report a marked reduction of A\( \beta \) levels/\( \beta \)-amyloid plaques and associated astrocytosis following multiple low dose infusions of HUCBC. HUCBC infusions also reduced cerebral vascular A\( \beta \) deposits in the Tg2576 AD mouse model. Interestingly, these effects were associated with suppression of the CD40-CD40L interaction as evidenced by decreased circulating and brain soluble CD40L (sCD40L) and elevated systemic IgM levels, attenuated CD40L-induced inflammatory responses, and reduced surface expression of CD40 on microglia. Importantly, deficiency of CD40 abolishes the
effect of HUCBC on elevated plasma Aβ levels. Moreover, microglia isolated from HUCBC-infused PSAPP mice demonstrated increased phagocytosis of Aβ. Further, sera from HUCBC-infused PSAPP mice significantly increased microglial phagocytosis of Aβ1-42 peptide while inhibiting IFN-γ-induced microglial CD40 expression. Increased microglial phagocytic activity in this scenario was inhibited by addition of recombinant CD40L protein. These data suggest that HUCBC infusion confers mitigation of AD-like pathology by disrupting CD40L activity.

2.2 Introduction

Alzheimer’s disease (AD) is the most common progressive dementia, and is pathologically characterized by deposition of amyloid-β-peptide (Aβ) in the brain parenchyma. Aβ plaques are potent activators of both microglia and astrocytes, central nervous system (CNS)-resident immunocompetent cells that respond to cerebral amyloidosis by chronic, pro-inflammatory activation (Benzing et al., 1999). While it was once thought that activation of microglia and astrocytes in the AD brain was an epiphenomenon and not a pathoetiological contributor to AD, more recent studies have suggested that the Aβ-mediated inflammatory cascade is an etiological perpetrator in AD. For example, therapeutic strategies aimed at manipulating this inflammatory cascade, including Aβ immunization (Schenk et al., 1999; Bard et al., 2000; Nicoll et al., 2003), non-steroidal anti-inflammatory drugs (NSAID) (Matsushima et al., 1994; in t' Veld et al., 2001; Szekely et al., 2004), and modulation of microglial activation (Tan et al., 1999a; Town et al., 2001; Tan et al., 2002b; Todd Roach et al., 2004; Laporte et al.,
2006), are able to reduce AD-like pathology and improve behavioral impairment in Alzheimer’s transgenic mouse models and, in some cases, reduce AD pathology in humans.

We previously showed that the CD40-CD40 ligand (CD40L) interaction plays a critical role in Aβ-induced pro-inflammatory microglial activation (Tan et al., 1999a). Moreover, we have demonstrated that disruption of this signaling pathway reduces cerebral Aβ deposits in the Tg2576 mouse model of AD and improves cognitive deficits in PSAPP AD mice (Town et al., 2001; Tan et al., 2002a; Tan et al., 2002b; Todd Roach et al., 2004). The implication of CD40-CD40L interaction in AD-associated brain inflammatory process is supported from studies demonstrating increased expression of CD40 and CD40L in and around β-amyloid plaques in AD brain (Togo et al., 2000; Calingasan et al., 2002). Recently, Desideri and colleagues (Desideri et al., 2006) reported circulating soluble CD40L (sCD40L) levels are significantly increased in AD patients vs. healthy elderly controls, further supporting a role for this receptor/ligand dyad in the pathogenesis of AD.

Human umbilical cord blood cells (HUCBC) have been shown to oppose the pro-inflammatory T helper cell type 1 (Th1) response, as demonstrated in an animal model of stroke where HUCBC infusion promoted a strong anti-inflammatory T-helper 2 (Th2) response (Vendrame et al., 2004). Importantly, this effect was associated with reduced infarct volume and rescue of behavioral deficit (Vendrame et al., 2004). HUCBC infusion has also shown therapeutic benefit in other neuroinflammatory conditions including multiple sclerosis, amyotrophic lateral sclerosis, neurodegenerative macular
degeneration, and Parkinson’s disease (El-Badri et al., 2006; Garbuzova-Davis et al., 2006; Henning et al., 2006). In AD preclinical models, administration of these cells to the PSAPP mouse model of AD was associated with extension of lifespan, although high doses were administered in this paradigm (Ghorpade et al., 2001).

Based on these lines of evidence, we investigated whether multiple low-dose administration of HUCBC to AD transgenic mouse models could reduce AD-like pathology through suppression of deleterious inflammatory responses involving the CD40 pathway. To address this possibility, we infused both double transgenic PSAPP mice and Tg2576 AD mouse models with HUCBC and then examined cerebral parenchymal and vascular Aβ levels/deposits, astrocytosis, microgliosis, and CD40 pathway-associated molecules.

2.3 Materials and methods

2.3.1 Animals and administration of human umbilical cord cells

HUCBC (95-98% mononuclear cells) were provided by Saneron CCEL Therapeutics, Inc. (Tampa, FL). Transgenic PSAPP (APPswe, PSEN1dE9) and Tg2576 mice were obtained from the Jackson Laboratory (Jankowsky et al., 2001; Garcia-Alloza et al., 2006) and Taconic Inc. (Hsiao et al., 1996), respectively, and were intravenously (i.v.) treated with HUCBC (100,000 cells/mouse) or PBS bi-weekly for the first two months and monthly for the remaining four months (n = 10/group, 5♂/5♀). Mice were treated starting at 7 months of age (after appreciable Aβ deposits), and blood was
collected by sub-mandibular bleeding at 0, 2, 4 and 6 months to monitor plasma cytokines, sCD40L and Aβ levels throughout the study. We analyzed brains of these mice for Aβ deposits and gliosis at 13 months of age (when these mice manifest well-established AD-like pathology, including Aβ deposits and gliosis). For PSAPP mice deficient in CD40, we treated these mice and controls at 8 weeks of age (preliminary studies showed that we can clearly detect plasma Aβ levels at this age) with HUCBC. Blood samples were collected by sub-mandibular bleeding at the 2nd month after the treatment. Animals were housed and maintained in the College of Medicine Animal Facility at the University of South Florida (USF), and all experiments were performed in compliance with protocols approved by USF Institutional Animal Care and Use Committee.

2.3.2 Immunohistochemistry analysis

Mice were anesthetized with isofluorane and transcardially perfused with ice-cold physiological saline containing heparin (10 U/mL). Brains were rapidly isolated and quartered using a mouse brain slicer (Muromachi Kikai, Tokyo, Japan). The first and second anterior quarters were homogenized for Western blot analysis, and the third and fourth posterior quarters were used for microtome or cryostat sectioning (Tan et al., 2002a). Brains were then fixed in 4% paraformaldehyde in PBS at 4°C overnight and routinely processed in paraffin in a core facility at the Department of Pathology (USF College of Medicine). Five coronal sections from each brain (5 μm thickness) were cut with a 150 μm interval [for cingulate cortex (CC) bregma -0.10 mm to -0.82 mm; for
entorhinal cortex (EC) and hippocampus (H), bregma -2.92 mm to -3.64 mm]. Sections were routinely deparaffinized and hydrated in a graded series of ethanol before preblocking for 30 min at ambient temperature with serum-free protein block (Dako Cytomation, Carpinteria, CA). Aβ immunohistochemical staining was performed using anti-human amyloid-β antibody (4G8) in conjunction with the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, CA) coupled with diaminobenzidine substrate. Congo red staining was done according to standard practice using 10% (w/v) filtered Congo red dye cleared with alkaline alcohol. These sections were rinsed three times for 5 min each in 70% ethanol, hydrated for 5 min in PBS, and mounted in Vectashield fluorescence mounting media (Vector Laboratories). β-amyloid plaques positive for 4G8 or Congo red were visualized under bright field using an Olympus BX-51 microscope. Aβ burden was determined by quantitative image analysis. Briefly, images of five 5-µm sections (150 µm apart) through each anatomic region of interest (hippocampus and neocortex) were captured and a threshold optical density was obtained that discriminated staining from background. Manual editing of each field was used to eliminate artifacts. Data are reported as percentage of immunolabeled area captured (positive pixels divided by total pixels captured). Quantitative image analysis was performed by a single examiner (TM) blinded to sample identities.

2.3.3 Immunofluorescence analysis

Double immunofluorescence for Aβ and CD40 was performed using rat anti-mouse CD40 (1:1000; Pharmingen) and rabbit anti-pan Aβ (1:100; Biosource
International, Inc) with overnight incubation followed by incubation at ambient
temperature with goat anti-rat IgG FITC (1:50; PharMingen) and donkey anti-rabbit
Alexa Fluor555 (1:500; Invitrogen) for 45 min. Double immunofluorescence for Aβ and
activated astrocytes was performed using a biotinylated human amyloid-β monoclonal
antibody (4G8; 1:100, Signet Laboratories, Dedham, MA) and GFAP polyclonal
antibody (1:500, DAKO). Normal rabbit, normal mouse serum (isotype control), or
phosphate buffered saline (PBS, 0.1 M, pH 7.4) were used instead of primary antibody or
ABC reagent as a negative control. Quantitative image analysis was done based on a
previous method (Tan et al., 2002a) with minor modifications. Images were acquired as
digitized tagged-image format files to retain maximum resolution using an Olympus
BX60 microscope with an attached digital camera system (DP-70, Olympus, Tokyo,
Japan), and digital images were routed into a Windows PC for quantitative analyses using
SimplePCI software (Compix, Inc. Imaging Systems, Cranberry Township, PA). The
cingulate cortex region was captured from the image of the cortex adjacent to the sagittal
fissure, and the entorhinal cortex region was captured from the image of the cortex
ventral to the rhinal fissure. In images from cingulate and entorhinal regions, the cortical
dge was not included in order to capture the full anatomic region of interest. The
hippocampal region was captured from between a portion of the CA1 subfield of the
pyramidal cell layer and the lacunosum molecular layer. The anatomical locations and
boundaries of the regions analyzed were based on those previously defined (Obregon et
al., 2006). Images of five 5 µm sections through each anatomic region of interest were
captured, and a threshold optical density was obtained that discriminated staining from
background. Each anatomic region of interest was manually edited to eliminate artifacts. For “burden” analyses, data are represented as percentage of immunolabeled area captured (positive pixels) relative to the full area captured (total pixels).

2.3.4 Flow cytometric and Western blot analyses of CD40 expression

For flow cytometric analysis of microglial CD40 expression, primary cultured microglial cells were plated in 6-well tissue culture plates at 5 x 10^5 cells/well and incubated with IFN-γ (100 ng/mL) in the presence or absence of serum derived from HUCBC- or PBS-infused individual PSAPP mice. Twelve hours after incubation, microglial cells were washed with flow buffer [PBS containing 0.1% (w/v) sodium azide and 2% (v/v) FCS] and re-suspended in 250 µl of ice-cold flow buffer for fluorescence activated cell sorting (FACS) analysis, according to methods described previously (Tan et al., 1999d). Briefly, cells were pre-incubated with anti-mouse CD16/CD32 monoclonal antibody (clone 2.4G2, PharMingen) for 10 min at 4°C to block non-specific binding to Fc receptors. Cells were then centrifuged at 5,000 x g, washed 3 times with flow buffer, and then incubated in flow buffer with hamster anti-mouse CD40-FITC or isotype control antibody-FITC (1:100 dilution; PharMingen). After 30 min incubation at room temperature, cells were washed twice with flow buffer, re-suspended in 250 µL of flow buffer and analyzed by a FACScan™ instrument (Becton Dickinson). A minimum of 10,000 cells were accepted for FACS analysis. Cells were gated based on morphological characteristics using CellQuest™ software (Beckton Dickinson) such that apoptotic and necrotic cells were not accepted for FACS analysis. Percentages of positive (CD40-
expressing) cells were calculated as follows: for each treatment, the mean fluorescence value for the isotype-matched control antibody was subtracted from the mean fluorescence value for the CD40-specific antibody.

For Western immunoblotting analysis of brain CD40 expression, mouse brain homogenates were prepared from HUCBC- and PBS-infused PSAPP mice as previously described (Tan et al., 2002a). An aliquot corresponding to 100 µg of total protein of each sample was separated by SDS-PAGE and transferred electrophoretically to immunoblotting PVDF membranes. Nonspecific antibody binding was blocked with 5% nonfat dry milk for 1 hr at room temperature in Tris-buffered saline (TBS; 20 mM Tris and 500 mM NaCl, pH 7.5). Subsequently, membranes were first hybridized with rabbit anti-CD40 antibody (1:1,500 dilution; StressGen) for 2 hrs and then washed 3 times in TBS and immunoblotting was by an anti-rabbit HRP-conjugated IgG secondary antibody as a tracer. The luminol reagent was used to develop the blots. To demonstrate equal loading, the same membranes were then stripped with β-mercaptoethanol stripping solution (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) and re-probed with mouse monoclonal antibody to actin. Densitometric analysis was done as previously described (Tan et al., 2002a) using a FluorS Multi imager with Quantity One™ software (BioRad).

2.3.5 β and cytokine ELISAs

Mouse brains were isolated under sterile conditions on ice and placed in ice cold lysis buffer as previously described (Tan et al., 2002a). Brains were then sonicated on ice
for approximately 3 minutes, allowed to stand for 15 minutes at 4°C, and centrifuged at
15,000 rpm for 15 minutes. This fraction represented the detergent-soluble fraction.
Detergent-insoluble Aβ1-40, 42 species were further subjected to acid extraction of brain
homogenates in 5 M guanidine buffer (Johnson-Wood et al., 1997), followed by a 1:5
dilution in lysis buffer. Aβ1-40, 42 were detected in brain homogenates prepared with lysis
buffer or in plasma samples at a 1:10 or 1:5 dilution, respectively, in dilution buffer (PBS
+ 1% BSA + PMSF). Aβ1-40, 42 was quantified in these samples using our own Aβ1-40, 42
ELISA kits (Rezai-Zadeh et al., 2005) and further evaluated with commercially available
Aβ1-40, 42 ELISA kits (IBL-America) in accordance with the manufacturer’s instructions,
except that standards included 0.5 M guanidine buffer in some cases to facilitate Aβ
aggregation. Aβ1-40, 42 were represented as pg/mL of plasma and pg/mg of total protein
(mean ± SD).

Cell suspensions of splenocytes from individual mice were prepared as previously
described (Town et al., 2002) and passed in 0.5 mL aliquots into 24-well plates at 3
×10⁶/mL. These cells were treated for 48 hrs with concanavalin A (Con A, 5 µg/mL).
Supernatants were then collected and assayed by IL-10, TNF-α, and IL-12(p70) cytokine
ELISA kits in strict accordance with the manufacturer's instruction (R&D Systems). The
Bio-Rad protein assay (Bio-Rad) was performed to measure total cellular protein from
each of the cell groups under consideration just prior to quantification of cytokine release
by ELISA, and cytokine secretion is expressed as pg/mg total cellular protein (mean ±
SD). To verify whether stimulation of splenocytes produced any between-groups
differences on cell death that might account for altered cytokine profiles, LDH release
assay was carried out as described (Town et al., 2002), and LDH was not detected in any of the wells studied. ELISAs for IgM and IgG antibodies were carried out as previously described (Nikolic et al., 2007). Optical densities were determined by a microplate reader at 450 nm. The ratio of IgM to IgG was calculated using optical density values and then the average ratio for each group was determined (mean ± SD). Brain tissue-derived (from the detergent-soluble brain homogenate fraction) and serum-derived (plasma) samples were analyzed for sCD40L (Bener MedSystems Burlingame, CA), IL-4, IL-10, IL-2, IFN-γ, TNF-α, IL-1β, IL-12 (p70), and TGF-β cytokines by Bioplex assays (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol.

2.3.6 Microglial phagocytosis assay

Primary cultures of murine microglia were established as previously described (Tan et al., 1999a; Townsend et al., 2005). For fluorometric analysis of FITC-Aβ1-42, primary murine microglia were seeded at 1 x 10^5 cells/well (n = 6 for each condition) in 24-well tissue-culture plates containing 0.5 mL of complete RPMI 1640 medium. These cells were treated for 60 min with “aged” Aβ1-42 conjugated with FITC (Biosource International) (Townsend et al., 2005). In the presence of FITC-Aβ1-42, microglia were then co-treated with serum (1/200, 1/400, 1/800 dilution) derived from HUCBC- or PBS-infused individual PSAPP mice in the presence or absence of CD40L protein (2 µg/mL). Microglia were rinsed 3 times in Aβ-free complete medium, and media were exchanged with fresh Aβ-free complete medium for 10 min both to allow for removal of non-incorporated Aβ and to promote concentration of the Aβ into phagosomes. Extracellular
and cell associated FITC-Aβ were quantified using an MSF reader (SpectraMax®, Molecular Devices) with an emission wavelength of 538 nm and an excitation wavelength of 485 nm. A standard curve from 0 nM to 500 nM of FITC-Aβ was run for each plate. Total cellular proteins were quantified using the Bio-Rad protein assay. The mean fluorescence values for each sample were determined by fluorometric analysis. Relative fold change values were calculated as the mean fluorescence value for each experimental sample over control. In this manner, both extracellular and cell associated FITC-Aβ were quantified. To determine the extent to which cell death might have influenced phagocytic activity in the various treatment groups, we performed LDH release assay, and no significant cell death was detected over the 3 h time-frame in any of the treatment groups (p > 0.05).

Primary culture peripheral macrophages were collected from 3-month old wild type mice by infusing their peritoneal cavity with ice-cold PBS following a four day i.p. immunization with 1mL of 3% (w/v) brewer’s thyoglycollate resuspended in PBS. Cells were pooled following the isolation in order to decrease the variables. Further, they were plated in a culture medium (RPMI-1640; 10% fetal bovine serum and antibiotics) to give $1.5 \times 10^6$ cells/well in 6 well plates. Cells were incubated overnight at 37 °C under 5% CO$_2$ in a humidified incubator, and non-adherent cells were removed by washing twice with PBS at 37 °C. Following the removal of non-adherent cells, the remaining cells were tested for Aβ phagocytosis as described above with the addition of 1:200, 1:400, and 1:800 dilution of sera derived from HUCBC-treated mice, sera derived from PBS-treated mice, as well as supernatants from cultured HUCBC cells.


2.3.7 Statistical Analysis

Data are presented as mean ± SD. All statistics were calculated using one-way analysis of variance (ANOVA) for multiple comparisons. A p value of < 0.05 was considered significant. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, Illinois) was used for all data analysis.

2.4 Results

2.4.1 Cerebral parenchymal and vascular β-amyloid plaques are reduced in AD transgenic mice peripherally infused with HUCBC

Previous work in a mouse model of stroke has shown that HUCBC infusion results in significant reduction in infarct volume as well as rescue of behavioral deficits associated with decreased pro-inflammatory cytokine production (Vendrame et al., 2004). We sought to determine whether HUCBC (95-98% mononuclear cells) infusion could impact Aβ-associated pathology in PSAPP double transgenic mice. These animals were intravenously (i.v.) injected with HUCBC (100,000 cells/mouse) beginning at 7 months of age (when β-amyloid deposits have already accumulated). At 13 months of age, mice were sacrificed and evaluated for changes in AD-like pathology. We chose to administer multiple low doses of HUCBC because, in our pilot studies, we observed that this strategy was superior compared to a single high dose of HUCBC on reducing cerebral amyloidosis in Tg2576 mice (data not shown). HUCBC infusion in PSAPP mice resulted
in marked reduction of cerebral β-amyloid pathology as assayed by Aβ antibody (4G8) immunohistochemistry (Figure 1A) and Congo red histochemistry (Figure 1C). Quantitative image analysis revealed statistically significant differences for each brain region examined ($P < 0.001$) between PSAPP mice infused with HUCBC (PSAPP/HUCBC) and PSAPP mice peripherally infused with PBS (PSAPP/PBS) for both Aβ antibody (Figure 1B) and Congo red staining (Figure 1D). Furthermore, ELISA analysis of brain extracts showed that levels of both detergent-soluble and -insoluble Aβ$_{40}$, Aβ$_{42}$ peptides were reduced in PSAPP mice infused with HUCBC (by 62% and 70%, respectively; Figure 1E). A $t$-test for independent samples revealed significant between-groups differences for each group examined ($P < 0.001$).

Given that peripheral administration of HUCBC reduces cerebral parenchymal Aβ deposits and brain Aβ levels in PSAPP mice, we wished to investigate the impact of HUCBC infusion on cerebral amyloid angiopathy (CAA), which is characterized by Aβ deposits in the cerebral vasculature and is known to occur in 83% of AD patients (Ellis et al., 1996). For this analysis, we used the Tg2576 mouse model of AD, which is known to manifest copious Aβ deposits in cerebral vessels at 15 to 20 months of age (Kaul et al., 2000; Christie et al., 2001; Li et al., 2003; Friedlich et al., 2004; Robbins et al., 2006). We peripherally infused these mice with HUCBC or controls (PBS vehicle treatment or no treatment) ($n = 10$, $5♂/5♀$ per group) at 12 months of age using the identical procedure above. Six months thereafter, these mice were sacrificed for analyses of cerebral parenchymal or vascular β-amyloid deposits by Congo red histochemistry. As shown in Figure 1F, Tg2576 mice receiving HUCBC treatment demonstrated reduction...
of both cerebral parenchymal and vascular Congo red deposits compared with controls. Quantitative image analysis revealed statistically significant differences between Tg2576/HUCBC and Tg2576/PBS or non-treated control groups when examining total (78%), vascular (86%), or parenchymal (74%) Congo red staining ($P < 0.001$; Figure 1G). No significant difference was revealed between Tg2576/PBS and non-treated Tg2576 control mice ($P > 0.05$). In addition, we also analyzed cerebral Aβ levels/β-amyloid deposits by Aβ ELISA and Aβ antibody immunohistochemistry, and we obtained statistically significant results similar to those observed in HUCBC-infused PSAPP mice ($P < 0.001$; data not shown).
Fig. 1C, D


**E**

![Graph showing Aβ40,42 levels](image)

**Aβ1-40,42 (pg/mg)**

1% Triton X-100

- PSAPP/PBS: 6000 pg/mg
- PSAPP/HUCBC: 2000 pg/mg (↓62%)

**Aβ1-40,42 (pg/mg)**

5 M guanidine

- PSAPP/PBS: 12000 pg/mg
- PSAPP/HUCBC: 4000 pg/mg (↓70%)
F

Tg2576

Tg2576/PBS

Tg2576/HUCBC

10 X

G

**Total Congo red**

- 78%

**Vascular Congo red**

- 86%

**Parenchymal Congo red**

- 74%
Figure 1. Cerebral Aβ/β-amyloid pathology is reduced in PSAPP and Tg2576 mice peripherally infused with HUCBC. Mouse paraffin-embedded coronal brain sections from the cingulate cortex (CC), hippocampus (H), and entorhinal cortex (EC) were stained with monoclonal human Aβ antibody, (A) 4G8 or (C) Congo red. Percentages (plaque area/total area) of (B) Aβ antibody-immunoreactive deposits or of (D) Congo red-stained deposits were calculated by quantitative image analysis (mean ± SD; n = 10, 5♀/5♂ per group). (E) Aβ ELISA analysis was carried out for both levels of detergent-soluble Aβ1-40, 42 (top panel) or 5 M guanidine-extracted Aβ1-40, 42 (bottom panel). Data are represented as mean ± SD of Aβ1-40, 42 (pg/mg protein). Mouse paraffin-embedded coronal brain sections from hippocampal regions of Tg2576 mice were stained with (F) Congo red. Positions of the hippocampal subfields CA1, CA3, and dentate gyrus (DG) are indicated in the upper left panel. Arrows indicate Aβ deposit-affected vessels. (G) Percentages (% labeled area) of Congo red-stained plaques/vessels were quantified by image analysis (mean ± SD; n = 10, 5♀/5♂), and percentage reduction is indicated.
2.4.2 Reduced CD40-positive microglia and GFAP-positive astrocytes in PSAPP mice peripherally infused with HUCBC

It has previously been suggested that brain inflammation resulting from activated microglia and astrocytes contributes to β-amyloid plaque formation (Frackowiak et al., 1992; Potter et al., 2001), and we have previously shown that ligation of microglial CD40 enables activation in response to Aβ peptides (Tan et al., 1999a; Tan et al., 1999b; Tan et al., 1999c). To investigate whether HUCBC could inhibit brain inflammation, we examined co-localization of β-amyloid deposits with CD40-positive microglia [an in vivo microgliosis marker (Togo et al., 2000)] or reactive [glial fibrillary acidic protein (GFAP)-positive] astrocytes by immunohistochemistry and Western blot analyses in PSAPP mice. As shown in Figure 2A, CD40-positive microglial cells were reduced in the PSAPP/HUCBC-infused group. Quantitative image analysis revealed statistically significant reductions when comparing PSAPP/HUCBC-infused and PSAPP/PBS-infused groups for both Aβ and CD40 staining in hippocampal dentate gyrus and CA1 regions (**P < 0.001) (Figure 2B). Western blot analysis of CD40 expression showed a statistically significant decrease in brain homogenates from HUCBC-infused PSAPP mice (P < 0.001) (Figure 2C). Furthermore, immunohistochemistry/histochemistry and immunofluorescence analyses showed reductions in β-amyloid-associated astrocytosis in PSAPP/HUCBC mice vs. PSAPP/PBS-treated mice (Figure 2D), and morphometry revealed reductions for neocortex and hippocampus by 84% and 86%, respectively in PSAPP/HUCBC mice (P < 0.001) (Figure 2E).
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>PSAPP/PBS</th>
<th>PSAPP/HUCBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>merge</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10 X
B

Pixel intensity

Aβ  CD40  Aβ  CD40
Dentate gyrus  CA1

C

kDa

PSAPP/PBS  PSAPP/HUCBC

CD40

42

Actin

Ratio of CD40/Actin

0.77 ± 0.05  0.26 ± 0.06
E

GFAP/Aβ double positive plaques

Mean plaque number per mouse

PSAPP/PBS  PSAPP/HUCBC  PSAPP/PBS  PSAPP/HUCBC

Neocortex  Hippocampus

↓84%  ↓86%
Figure 2. β-amyloid associated microgliosis and astrocytosis are reduced in HUCBC infused-PSAPP mice.  (A) Immunofluorescence was performed on mouse brain coronal paraffin sections prepared from PSAPP mice infused with HUCBC or PBS.  Red signal indicates Aβ positive (top panels); green indicates CD40 positive (middle panels), and merged images (bottom panels) reveal co-localization of CD40 and Aβ. DAPI (blue) was used as a nuclear counterstain.  (B) Immunofluorescence intensity for Aβ and CD40 was determined.  (C) Western blot analysis shows reduced CD40 expression in brain homogenates from PSAPP/HUCBC vs. PSAPP/PBS mice as indicated (actin was used as an internal reference control).  Densitometry analysis shows the ratio of CD40 to actin as indicated below the figure.  (D) Immunohistochemistry analysis shows GFAP and Aβ double staining (top panel), and immunofluorescence (bottom panel) reveals co-localization of GFAP (red signal) and Aβ (green signal).  (E) Morphometric analysis results (mean GFAP/β-amyloid double positive plaques per mouse ± SD) are shown for the neocortex and the hippocampus of PSAPP/HUCBC vs. PSAPP/PBS mice.  Percent reduction of plaques double positive for GFAP and Aβ in PSAPP/HUCBC mice is indicated.
2.4.3 Increased plasma Aβ levels correlate with decreased CD40-CD40L interaction in HUCBC-infused PSAPP mice

We have previously shown that administration of neutralizing CD40L antibody to PSAPP mice results in increased levels of plasma Aβ concomitant with reduced cerebral Aβ/β-amyloid pathology, suggesting that depletion of CD40L promotes brain-to-blood clearance of Aβ (Tan et al., 2002a). It is well-known that the CD40-CD40L interaction promotes pro-inflammatory Th1 and opposes anti-inflammatory Th2 immune responses (Grewal and Flavell, 1998; Mackey et al., 1998). In addition, HUCBC treatment has been shown to be an immunoregulator in an animal model of stroke (Vendrame et al., 2004; Newman et al., 2006). We investigated whether reduction of cerebral Aβ levels/β-amyloid deposits in HUCBC-infused PSAPP mice might 1) result from increased brain-to-blood clearance of Aβ, and 2) be associated with suppression of the pro-inflammatory CD40-CD40L interaction. We probed individual blood samples from PSAPP mice infused with HUCBC or PBS for Aβ1-40, 42 and soluble CD40L (sCD40L). ELISA revealed increased plasma Aβ1-40, 42 levels in PSAPP/HUCBC mice which inversely correlated with decreased levels of plasma sCD40L in these animals (Figures 3A-C). One-way ANOVA followed by post hoc comparison revealed significant differences between PSAPP/HUCBC-infused and PSAPP/PBS-infused mice for plasma Aβ1−40, 42 levels and plasma sCD40L levels at each time point indicated (Figures 3A-C) (**P <0.001). It is well established that CD40-CD40L interaction on B cells is required for IgM to IgG antibody class switching. Therefore, we went on to evaluate the functional consequence of HUCBC-mediated suppression of the CD40-CD40L interaction on IgM
and IgG titers in mouse blood samples obtained at the time of sacrifice. ELISA data revealed a significantly increased ratio of IgM to IgG in PSAPP/HUCBC mice when compared to control (Figure 3D, *P* < 0.05), suggesting that the CD40 signaling pathway is functionally suppressed in HUCBC-infused PSAPP mice. It has been recently reported that CD40 deficiency in APP transgenic mice confers a decrease in Aβ/β-amyloid loads (Laporte et al., 2006). Although to a lesser extent than PSAPP/CD40+/+ mice, we also found PSAPP/CD40−/− mice do clearly manifest β-amyloid deposits (data not shown), allowing us to test whether administration of HUCBC to PSAPP/CD40−/− mice resulted in further amelioration of amyloidosis in these animals. Thus, we treated PSAPP/CD40−/− at 8 weeks of age with HUCBC or PBS (control) and assayed circulating Aβ levels, which correlate with cerebral amyloid levels in transgenic AD mice (DeMattos et al., 2002a). Results indicate no further benefit of HUCBC in PSAPP/CD40−/− mice on enhanced Aβ plasma levels (Figures 3E and F; *P* > 0.05), a presumed indicator of Aβ brain-to-blood efflux. These data suggest that HUCBC mediate beneficial effects on reduction of amyloidosis via reducing CD40 pathway bioactivity, and are consistent with our previous studies showing that genetic or pharmacologic ablation of CD40-CD40L interaction mitigates AD-like pathology in transgenic mice (Tan et al., 1999a; Tan et al., 2002a).
**Figure 3.** HUCBC infusion results in CD40-dependent increased plasma Aβ levels in PSAPP mice. ELISA analysis results are shown from blood (plasma) for (A) Aβ_{1-40}, (B) Aβ_{1-42}, (C) sCD40L, and (D) IgM/IgG. Data are presented as mean ± SD (n = 10) for Aβ_{1-40}, Aβ_{1-42} or sCD40L (pg/mL plasma). Arrows below the panels show the time for each peripheral infusion with HUCBC or PBS. (D) data are presented a ratio of IgM to IgG in blood (plasma) from mice at the 6th month following the treatment. Aβ ELISA analysis for (E) Aβ_{1-40} and (F) Aβ_{1-42} in blood (plasma) derived from PSAPP/CD40^{+/+} or PSAPP/CD40^{-/-} mice at the 2nd month following the third HUCBC infusion. Data in (E and F), are presented as mean ± SD (n = 4, 2♂/2♀) of Aβ_{1-40} or Aβ_{1-42} (pg/mL plasma).
We hypothesized that, if HUCBC mediated reduced amyloidosis by reducing CD40 pathway activity, this should be associated with a shift from pro- to anti-inflammatory cytokines in HUCBC-infused PSAPP mice. Consistent with this hypothesis, we found that plasma levels of the anti-inflammatory cytokines IL-4 and IL-10 were increased in HUCBC-infused PSAPP mice (Figure 4A, **$P < 0.001$).

Furthermore, primary splenocytes from HUCBC-infused PSAPP mice showed reduced pro-inflammatory TNF-α and IL-12 (p70) and increased anti-inflammatory IL-10 secretion. We also analyzed brain cytokine levels by ELISA, and results showed statistically significant increases in anti-inflammatory TGF-β1 and IL-10 levels in PSAPP/HUCBC-infused mouse brain homogenates (Figures 4B and C; **$P < 0.001$).

Consistent with our data showing reduction in circulating sCD40L after HUCBC treatment, we also measured sCD40L in brain homogenates and found a significant decrease in PSAPP/HUCBC mice compared to control (Figure 4D, **$P < 0.001$).
Figure 4

A. Plasma-derived Th1/Th2 cytokines

B. Splenocyte-derived Th1/Th2 cytokines

C. Brain tissue-derived Th1/Th2 cytokines

D. Soluble CD4+IL

Cytokine (pg/ml; mean ± SD)
Figure 4. HUCBC infusion promotes anti-inflammatory/Th2 responses and decreases sCD40L in the CNS. ELISA results are shown for (A) plasma-derived, (B) splenocyte culture-derived, (C) brain tissue derived cytokines, and (D) brain tissue derived sCD40L. Data are presented as mean ± SD (n = 10) values of cytokines (pg/mL plasma or medium) (D and E), or fold increase of cytokines over control (untreated) mice (C and D).
2.4.4 HUCBC inhibit microglial CD40 expression and enhance in vitro phagocytosis of Aβ peptides

We and others have previously shown that microglial CD40 expression is important for CNS inflammatory responses (Tan et al., 1999a; Tan et al., 1999b; Tan et al., 1999c; Togo et al., 2000; Calingasan et al., 2002; Tan et al., 2002a; Tan et al., 2002b), and IFN-γ is a strong inducer of microglial CD40 expression (Carson et al., 1998; Tan et al., 2000; Sokol et al., 2006). In order to investigate whether a soluble factor secreted following HUCBC infusion could modulate microglial expression of CD40, we treated primary microglial cells with serum from HUCBC- or PBS-infused individual PSAPP mice in the presence of IFN-γ (100 ng/mL) for 8 hrs. We then examined CD40 expression by FACS analysis. As shown in Figure 5A, sera derived from HUCBC-infused PSAPP mice significantly inhibited IFN-γ-induced microglial CD40 expression compared to controls \((P < 0.001)\). However, this effect was not directly mediated by HUCBC or human adult mononuclear cells (HAMNC), but was rather due to a soluble circulating factor produced by HUCBC-infused PSAPP mice (Figure 5A).

We and others have shown that stimulation of microglial CD40 results in impaired Aβ phagocytic activity (Townsend et al., 2005) and promotion of microglial neurotoxic inflammatory responses (Ponomarev et al., 2006). Thus, we wished to examine whether HUCBC could enhance microglial phagocytosis of Aβ peptide. We prepared primary cultures of adult microglia from HUCBC- and PBS-infused PSAPP mice according to previously described methods (Lue and Walker, 2002), and then subjected these cells to Aβ phagocytosis assay using native or Aβ antibody-opsonized
fluorescent-tagged Aβ1-42 (FITC-Aβ1-42) according to our previously described methods (Townsend et al., 2005). As shown in Figure 5B, when measuring FITC-tagged Aβ1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed a significant increase in Aβ uptake by microglia derived from HUCBC- vs. PBS-infused PSAPP mice (**P < 0.001). Interestingly, the presence of Aβ IgG [2.5 µg/mL (Townsend et al., 2005)] significantly enhanced Aβ uptake by PSAPP/HUCBC- vs. PSAPP/PBS-derived microglial cells (##P < 0.001). Given that sera from HUCBC-infused PSAPP mice suppressed IFN-γ-induced microglial CD40 expression, we wished to test if the sera could increase microglial Aβ phagocytosis. We incubated primary cultures of neonatal microglia with serum from individual PSAPP/HUCBC- vs. PSAPP/PBS mice at 1:200, 1:400, and 1:800 dilutions in the presence of FITC-Aβ1-42. We found that sera at the 1:200 dilution markedly enhanced microglial phagocytosis of Aβ1-42 peptide, which was opposed by the presence of recombinant mouse CD40L protein at 2 µg/mL (Figure 5C).

In addition, we wished to test if sera-derived HUCBC-treated mice could increase peripheral macrophage phagocytic activity. We incubated both sera derived from HUCBC and PBS-treated animals at 1:200, 1:400, and 1:800 dilutions with primary macrophage cells from wild-type mice in six-well tissue-culture plates in the presence of 300 nM FITC-Aβ1-42 as described above. We found that sera at the 1:200 dilution significantly enhanced macrophage phagocytosis of Aβ1-42 peptide (Figure 5D) (**P < 0.01 with n = 4 for each mouse group presented). However these effects were not observed in cultured HUCBC media (data not shown).
D

**Extracellular FITC-Aβ\textsubscript{1-42}**

**Cell associated FITC-Aβ\textsubscript{1-42}**

<table>
<thead>
<tr>
<th></th>
<th>PSAPP/ PBS-sera</th>
<th>PSAPP/ HUCBC-sera</th>
<th>Aβ IgG (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative fold mean (Fluor. change)

**Note:**

*Significance levels:*

**: p < 0.01

---

58
**Figure 5.** HUCBC modulate microglial CD40 expression and promote Aβ microglial/macrophage phagocytic activity.  (A) FACS analysis for CD40 expression in primary wild-type neonatal microglial cells treated with cultured medium from HUCBC or human adult mononuclear cells (HAMNC), or serum from individual PSAPP/HUCBC or PSAPP/PBS mice following IFN-γ challenge.  Data are presented as percentage of CD40 expressing cells (mean ± SD; n = 5).  (B,C,D) Microglial/macrophage phagocytosis assay results for extracellular and cell-associated FITC-Aβ_1-42_, which was detected using a fluorometer.  Data are represented as the relative fold of mean (± SD) fluorescence over control for each sample (n = 4 for each condition presented).  Primary microglial cells from (B) adult PSAPP/HUCBC or PSAPP/PBS mice, (C) wild-type neonatal microglia, and (D) primary peripheral macrophage.
2.5 Discussion

Based on genetic, biochemical, and post-mortem evidence, Aβ peptides are key etiological contributors to AD pathogenesis (Hardy and Selkoe, 2002). In addition to parenchymal Aβ deposits, deposition of Aβ in the cerebral vasculature (known as CAA) is a pathological feature of AD, and occurs with 83% frequency in AD patients (Ellis et al., 1996; Hardy and Selkoe, 2002; Jellinger, 2002; Green et al., 2005). Aβ has been shown to mediate pro-inflammatory and neurodegenerative changes, and oligomeric forms of the peptide are neurotoxic (Malinin et al., 2005). It is well-documented that brain inflammatory mechanisms mediated by reactive glia are activated in response to Aβ plaques (Benzing et al., 1999; Eikelenboom and van Gool, 2004; Rozemuller et al., 2005; Townsend et al., 2005). Expression profiles of two such pro-inflammatory molecules, CD40 and CD40L, are markedly increased in and around Aβ plaques in AD patients and in mouse models of the disease (Togo et al., 2000; Calingasan et al., 2002), and genetic or pharmacologic blockade of CD40-CD40L interaction reduces AD-like pathology in transgenic AD mice (Tan et al., 2002a), suggesting an etiologic role of this receptor/ligand dyad in the disease (Town et al., 2001; Tan et al., 2002b). In a recent clinical report, it was found that circulating sCD40L levels are significantly increased in AD patients (Desideri et al., 2006), suggesting that peripheral as well as brain dysregulation of the CD40 pathway occurs in AD. We have previously shown that CD40 ligation promotes pro-inflammatory activation of microglia and reduces microglial phagocytosis of Aβ peptide in vitro (Tan et al., 1999a; Townsend et al., 2005), supporting a mechanistic explanation for reduced AD-like pathology after blocking the CD40-
CD40L interaction (Tan et al., 2002b).

HUCBC have been shown to down-regulate pro-inflammatory Th1 response in an animal model of stroke (Vendrame et al., 2004), and have also shown therapeutic benefit in other neuroinflammatory/neurodegenerative conditions (El-Badri et al., 2006; Garbuzova-Davis et al., 2006; Henning et al., 2006). Based on this evidence, we sought to examine their putative therapeutic value in mitigating AD-like pathology in transgenic mice. After HUCBC infusion, treated mice exhibited diminished cerebral Aβ/β-amyloid pathology and down-regulation of pro-inflammatory responses in the brain and in the periphery. Based on the conspicuous role of CD40-CD40L interaction in mediating brain pro-inflammatory response and exacerbating AD-like pathology, we investigated whether HUCBC-mediated reduction of AD-like pathology might be associated with alteration in this receptor/ligand dyad. Our results show decreased expression of microglial CD40 and reduction in both CNS and peripheral sCD40L concomitant with HUCBC-induced diminished AD-like pathology, raising the possibility that disruption of CD40-CD40L interaction may be responsible for mitigation of AD-like pathology in this scenario. To directly address this hypothesis, we treated PSAPP mice deficient for CD40 with HUCBC and assayed circulating Aβ levels as a marker of brain-to-blood Aβ efflux, and results showed no further benefit of HUCBC in these mice.

Here, we demonstrate that infusion of the HUCBC mononuclear fraction into PSAPP and Tg2576 mice results in reduced levels of both soluble and insoluble brain Aβ1-40,42 concomitant with increased plasma Aβ1-40,42 levels. Past studies have suggested brain-to-blood clearance mechanisms that selectively remove Aβ from the brain,
potentially reducing Aβ levels in normal as well as AD patient brains (Shibata et al., 2000; DeMattos et al., 2002a; DeMattos et al., 2002b; Shiiki et al., 2004; Crossgrove et al., 2005). Experiments in rat models demonstrating clearance of Aβ₁₋₄₀ peptide from the brain via the blood-brain-barrier (BBB) support this notion (Shibata et al., 2000; Shiiki et al., 2004; Terasaki and Ohtsuki, 2005). Vascular endothelial cells, which are important BBB constituents, express CD40 (Suo et al., 1998; Tan et al., 1999d; Town et al., 2001; Sokol et al., 2006), and we now show that sCD40L is reduced in blood plasma from HUCBC-treated PSAPP mice, raising the possibility that interruption of CD40-CD40L interaction at the level of cerebrovascular endothelial cells may promote brain-to-blood clearance of Aβ. Further, reduced circulating sCD40L levels in HUCBC-treated PSAPP mice raises the possibility that inflammatory cytokines produced by CD40-CD40L interaction on endothelial cells are reduced, and this idea is consistent with our finding of a shift towards anti-inflammatory cytokines in the CNS after HUCBC infusion.

Interestingly, we also demonstrate that CAA [which is present in 83% of AD patients (Ellis et al., 1996)] is reduced by 68% after HUCBC treatment in Tg2576 AD mice. This result shows that reduction in parenchymal Aβ does not come at the cost of increased vascular Aβ deposits, unlike a model in which transforming growth factor-β₁ overexpression reduces parenchymal plaques but increases vascular Aβ deposits (Wyss-Coray et al., 1997; Wyss-Coray et al., 2001).

In vitro HUCBC studies have shown that these cells secrete soluble factors that have beneficial effects (Vendrame et al., 2005; Newman et al., 2006). For example, supernatants from cultured HUCBC promote survival of NT2 neural cells and peripheral
blood mononuclear cells cultured under conditions designed to induce cell stress and limit protein synthesis (El-Badri et al., 2006). Additionally, HUCBC have been shown to produce a number of neurotrophic factors and cytokines that modulate inflammatory responses, including nerve growth factor, colony stimulating factor-1, thrombopoietin, and IL-11 (Suen et al., 1994; McGowan et al., 1999; Vendrame et al., 2004). Previous reports have shown that HUCBC entry into the brain is not required to promote neuroprotection (Borlongan et al., 2004), and that recovery following brain injury is mediated through peripheral responses (Townsend et al., 2005). We did not detect infiltration of HUCBCs into brain parenchyma, either at 4 hrs after HUCBC administration or at the time of mouse sacrifice (data not shown), making it unlikely that these cells were directly involved in ameliorating cerebral amyloidosis. Therefore, we hypothesized that a soluble factor produced after HUCBC infusion in the periphery was responsible for reduced AD-like pathology and inflammatory response. To test this, we 1) measured cytokines in blood plasma, spleen, and brains from HUCBC- or PBS-treated PSAPP mice, 2) evaluated the impact of sera from these treated mice on IFN-γ-induced microglial CD40 expression, and 3) assayed Aβ phagocytosis in vitro in neonatal microglia treated with sera from HUCBC/PSAPP or PBS/PSAPP mice and in adult microglial cultures derived from these mice. Results generally show a shift from pro-inflammatory Th1-type cytokines towards anti-inflammatory Th2 cytokines in tissues from HUCBC-treated PSAPP mice. Further, sera from HUCBC-treated mice are able to reduce microglial CD40 expression and enhance Aβ phagocytosis by these cells. Finally, adult microglia from HUCBC-treated PSAPP mice have increased capacity to
phagocytose Aβ.

When taken together, the above results suggest that, in addition to promoting brain-to-blood Aβ efflux, HUCBC infusion promotes production of a peripheral anti-inflammatory soluble factor that is likely able to cross the BBB and affect microglial Aβ clearance. Previous reports have show that soluble factors, including heat-shock proteins and pro-inflammatory cytokines, are capable of modulating Aβ phagocytosis by microglia (Kakimura et al., 2002; Koenigsknecht-Talboo and Landreth, 2005), and our previous work has shown that microglial CD40-CD40L interaction retards Aβ phagocytosis/clearance (Townsend et al., 2005). Non-saturable BBB transport mechanisms have been described for a number of cytokines including TNF-α (which is transported via TNF receptors) and IL-1, and other soluble factors such as leukemia inhibitory factor, chemoattractant-1, and epithelial growth factor (Quan and Banks, 2007). Thus, it remains possible that soluble factors produced by the host in response to HUCBC treatment gain access to the brain via the BBB and encounter microglia. Ultimately, we propose that infused HUCBCs exert their effect on reducing cerebral amyloidosis by causing the host to secrete a soluble factor that acts by reducing sCD40L-CD40 interaction on microglia, which then promotes microglial clearance of Aβ. This mechanism is supported by our observations of 1) reduced brain levels of sCD40L in HUCBC-infused PSAPP mice, 2) reduced CD40 expression on microglia cultured in the presence of HUCBC-infused PSAPP mouse sera, 3) increased Aβ phagocytosis/removal by microglia cultured in the presence of HUCBC-infused PSAPP mouse sera or cultured from adult PSAPP/HUCBC mice, and 4) our previous observations that microglial CD40
ligation shifts these cells away from a Aβ phagocytic phenotype and towards a pro-
inflammatory response (32). Future studies designed to identify this soluble factor are
warranted, and may yield additional pharmacotherapeutic target(s). Additionally, our
observation of no further therapeutic benefit of HUCBCs when administered to
PSAPP/CD40-/- mice establishes a CD40 pathway-dependent mechanism for HUCBC
therapeutic benefit on reduction of cerebral amyloidosis. These results dovetail with our
previous studies showing that CD40-CD40L interaction mitigates AD-like pathology in
transgenic mice (Tan et al., 1999a; Tan et al., 2002a).

It was recently shown that peripheral macrophages are able to infiltrate the brain
and limit cerebral amyloidosis in AD mice after irradiation, suggesting that
hematogenously-derived macrophages are efficient at phagocytosing and clearing Aβ
deposits (Simard et al., 2006). However, earlier studies have shown that brain-resident
microglia are also able to phagocytose/clear Aβ (Paresce et al., 1996; Paresce et al., 1997;
Chung et al., 1999). We did not detect the presence of brain infiltrating macrophages in
the current experimental paradigm. Specifically, we stained for CD40 (a marker for both
macrophages and microglia), and noted the presence of process-bearing cells that
morphologically resembled microglia in and around Aβ plaques (see Figure 2A). Also,
we did not observe vascular “cuffing” that would suggest the presence of infiltrating
macrophages that are frequently observed in other CNS inflammatory conditions such as
experimental autoimmune encephalomyelitis (Imrich and Harzer, 2001). Furthermore,
our results provide evidence that both primary culture microglia and macrophages posses
the ability for enhanced Aβ phagocytosis following in vitro stimulation with sera derived
from HUCBC-treated animals. This too is consistent with peripheral immunomodulation of CD40-CD40L interaction by HUCBC treatment. Additionally, given the difficulties inherent to discriminating macrophages from microglia, and the ability of peripheral macrophages to engraft into the CNS and take up a microglial phenotype after brain injury (Priller et al., 2001), it remains possible that peripheral macrophages may contribute to reduced cerebral amyloidosis after HUCBC treatment.

In this report, we have shown that HUCBC infusion ameliorates AD-like pathology, including reductions in 1) cerebral Aβ levels/β-amyloid pathology, 2) CAA, and 3) brain inflammation including CD40-positive activated microglia and GFAP-positive activated astrocytes. These effects of HUCBCs were associated with increased brain-to-blood efflux of Aβ and a shift from pro-inflammatory Th1 to anti-inflammatory Th2 cytokines both in the brain and in the periphery, similar to what we observed after Aβ immunization (Town et al., 2002; Town et al., 2005a; Town et al., 2005b). Further, HUCBC infusion of PSAPP mice reduces both CNS and circulating sCD40L levels, and sera from these mice is able to promote microglial Aβ phagocytosis. When taken together, our results provide the basis for a novel immunomodulatory strategy for AD using HUCBC.
2.6 References


Aging.


Tan, J., Town, T., Crawford, F., Mori, T., DelleDonne, A., Crescentini, R., Obregon, D.,


CHAPTER THREE

CD40L DISRUPTION ENHANCES AB VACCINE-MEDIATED REDUCTION OF CEREBRAL AMYLOIDOSIS WHILE MINIMIZING CEREBRAL AMYLOID ANGIOPATHY AND INFLAMMATION

D. Obregon¹, H. Hou¹, Y. Bai¹, W.V. Nikolic¹, T. Mori¹,³, Deyan Luo¹, J. Zeng¹,
J. Ehrhart¹, F. Fernandez¹, D. Morgan², B. Giunta¹, T. Town¹,⁴, and J. Tan¹,²,³

¹Neuroimmunology Laboratory, Institute for Research in Psychiatry, Department of Psychiatry and Behavioral Medicine; ²Department of Molecular Pharmacology and Physiology, University of South Florida, 12901 Bruce B. Downs Blvd, Tampa, Florida 33613

³Institute of Medical Science, Saitama Medical School, 1981 Kamoda, Kawagoe, Saitama 350-8550, Japan

⁴Department of Immunobiology, Yale University School of Medicine, 300 Cedar Street, New Haven, Connecticut 06520-8011
3.1 Abstract

Amyloid-β (Aβ) immunization efficiently reduces amyloid plaque load and memory impairment in transgenic mouse models of Alzheimer’s disease (AD) (Schenk et al., 1999; Morgan et al., 2000). Active Aβ immunization has also yielded favorable results in a subset of AD patients (Hock et al., 2003). However, a small percentage of patients developed severe aseptic meningoencephalitis associated with brain inflammation and infiltration of T-cells (Nicoll et al., 2003; Orgogozo et al., 2003). We and others have shown that blocking the CD40-CD40 ligand (L) interaction mitigates Aβ-induced inflammatory responses and enhances Aβ clearance (Tan et al., 2002b; Townsend et al., 2005). Here, we utilized genetic and pharmacologic approaches to test whether CD40-CD40L blockade could enhance the efficacy of Aβ1-42 immunization, while limiting potentially damaging inflammatory responses. We show that genetic or pharmacologic interruption of CD40-CD40L interaction enhanced Aβ1-42 immunization efficacy to reduce cerebral amyloidosis in the PSAPP and Tg2576 mouse models of AD. Potentially deleterious pro-inflammatory immune responses, cerebral amyloid angiopathy (CAA) and cerebral microhemorrhage were reduced or absent in these combined approaches. Pharmacologic blockade of CD40L decreased T-cell neurotoxicity to Aβ-producing neurons. Further reduction of cerebral amyloidosis in Aβ-immunized PSAPP mice completely deficient for CD40 occurred in the absence of Aβ immunoglobulin G (IgG) antibodies or efflux of Aβ from brain to blood, but was rather correlated with anti-inflammatory cytokine profiles and reduced plasma soluble CD40L. These results suggest CD40-CD40L blockade promotes anti-inflammatory cellular immune responses,
likely resulting in promotion of microglial phagocytic activity and Aβ clearance while precluding generation of neurotoxic Aβ-reactive T-cells. Thus, combined approaches of Aβ immunotherapy and CD40-CD40L blockade may provide for safer and more effective Aβ vaccine.

3.2 Introduction

Amyloid-β (Aβ), a proteolytic product of amyloid precursor protein (APP), is a key molecule in the pathogenesis and progression of Alzheimer’s disease (AD) (Blennow et al., 2006). Overproduction of soluble and aggregated Aβ species drives cerebral amyloidosis including β-amyloid plaque formation, a hallmark pathological feature of AD. Thus, methods developed to clear or prevent formation of Aβ in the brains of AD patients represent a possible treatment modality. One promising approach involves utilization of “active” Aβ immunization strategies, which produce dramatic reductions in Aβ pathology in animal studies (Schenk et al., 1999). However, a phase IIa clinical trial was abandoned after about 6% of Aβ-immunized AD patients developed aseptic meningoencephalitis (Nicoll et al., 2003; Orgogozo et al., 2003) that appeared to involve brain inflammatory reactions mediated by T-cells and microglia (Monsonego et al., 2001; Schenk and Yednock, 2002; Greenberg et al., 2003; Monsonego et al., 2003).

Interestingly, a 12 month post-vaccination period analysis revealed an inverse correlation between titers of amyloid plaque-reactive antibodies and rate of cognitive decline (Hock et al., 2003), suggesting clinical efficacy. Despite the discontinuation of the clinical trials, Aβ vaccination studies have continued in effort to identify an immunization
approach that is both safe and effective. Current approaches have focused on minimizing T-cell mediated inflammatory responses in efforts to prevent CNS invasion of auto-aggressive T-cells, while promoting Aβ antibody-mediated clearance mechanisms (Chackerian et al., 2006; Maier et al., 2006; Okura et al., 2006; Nikolic et al., 2007).

A possible avenue to both enhance Aβ clearance and down-regulate CNS inflammatory responses (including invasion of reactive T-cells) involves modulation of the CD40 receptor (CD40)-CD40 ligand (CD40L) system. CD40 is a ~ 45-50 kDa cell surface molecule, which is a member of the tumor necrosis factor-α (TNF-α)/nerve growth factor (NGF) receptor super-family. In the periphery, a variety of innate immune cells known as antigen-presenting cells (APCs) express CD40, including dendritic cells, B-cells, and monocytes/macrophages. In the CNS, CD40 is expressed by resident cells including microglia, neurons, and astrocytes, as well as by peripherally-derived APCs (Tan et al., 2002a; Town et al., 2005). CD40L (also known as CD154), is expressed as a membrane-anchored molecule by activated T-cells and astrocytes, and can also be secreted as a smaller soluble protein (van Kooten and Banchereau, 2000). The CD40-CD40L interaction acts as an accessory co-stimulatory pathway involved in key immune cell processes including: activation, maturation/differentiation, growth/proliferation, and regulation of apoptosis (Town et al., 2001a).

We have previously shown that CD40 ligation is a molecular trigger for pro-inflammatory microglial activation in response to Aβ peptides (Tan et al., 1999). Further, genetic or pharmacologic blockade of the CD40-CD40L interaction reduces β-amyloid pathology in the brains of transgenic mouse models of AD (Tan et al., 1999; Tan
et al., 2002a). Increased CD40 and CD40L immunoreactivity has been found in and around β-amyloid plaques in AD brain (Togo et al., 2000; Calingasan et al., 2002), further suggesting that CD40-CD40L interaction may contribute to Aβ and β-amyloid plaque pathology.

Recently, the CD40-CD40L interaction was determined to play a central role in promoting and maintaining dendritic cell APC phenotype during infections (Straw et al., 2003). In the context of CNS immunity, the CD40-CD40L interaction is required for microglial maturation into functional APCs (Ponomarev et al., 2006). We have recently shown that CD40L treatment of primary cultures of microglia inhibits phagocytosis of Aβ antibody opsonized, as well as non-opsonized Aβ species (Townsend et al., 2005). Associated with reduced Aβ phagocytic capacity, CD40L treatment up-regulated cell surface markers indicative of an APC phenotype including CD45, CD86, MHC II, and promoted the release of pro-inflammatory molecules including interleukin (IL)-1β and TNF-α (Townsend et al., 2005). Thus, CD40-CD40L interaction may act as a molecular switch necessary to drive pro-inflammatory microglial APC phenotype maturation at the cost of reducing phagocytosis (Townsend et al., 2005; Ponomarev et al., 2006).

Additionally, blockade of the CD40-CD40L system down-regulates T-cell/microglia-mediated injury in the context of experimental autoimmune encephalitis (EAE) (Howard et al., 1999; Howard et al., 2002). Altogether, these studies suggest that blockade of the CD40-CD40L interaction could enhance Aβ vaccination-mediated Aβ clearance mechanisms, while minimizing pro-inflammatory T-cell-mediated damage in the CNS.

To investigate this hypothesis, we studied transgenic “PSAPP” mice overexpressing
mutant human presenilin-1 (DeltaE9), and “Swedish” mutant human APP (APP_{Swe}), which develop AD-like pathology (Jankowsky et al., 2001). We took a genetic approach to CD40 blockade by crossing these mice with CD40^{−/−} mice to yield: CD40 wild-type (PSAPP), CD40 heterozygous deficient (PSAPP/CD40^{+/−}), and CD40 homozygous deficient (PSAPP/CD40^{−/−}) animals. We then vaccinated these mice over a course of 4 months utilizing aggregated Aβ_{1-42} peptide or vehicle. We also took a pharmacologic approach by administering CD40L neutralizing antibody to Aβ_{1-42}-vaccinated PSAPP mice. Results from both strategies showed enhanced reduction of cerebral amyloidosis as evidenced by reductions in Aβ load, β-amyloid plaque burden, and cerebral amyloid angiopathy (CAA). Moreover, we report reduced cerebral microhemorrhage and inflammatory immune responses as measured by cytokine analysis and T-cell induced neurotoxicity to Aβ producing neurons. These results were associated with inhibition of microglial APC phenotype. Interestingly, homozygous CD40 deficient Aβ_{1-42}-immunized PSAPP mice displayed reduced cerebral amyloidosis in the absence of immunoglobulin G (IgG) antibodies or efflux of Aβ from brain to blood. These effects were correlated with reduced plasma CD40 ligand (CD40L) and increased anti-inflammatory cytokine levels. Altogether these data suggest that disruption of the CD40-CD40L interaction enhances Aβ_{1-42}-immunization mediated Aβ clearance mechanisms by promoting anti-inflammatory cellular immunity to support microglial clearance of Aβ.

3.3 Materials and methods
3.3.1 Reagents

Anti-human Aβ monoclonal antibody (4G8) was purchased from Signet Laboratories. Aβ1-42 peptide was obtained from Biosource International (Camarillo, CA). Aβ1-42 peptide was added to 0.9% saline (4 mg/mL), vortexed, and incubated for 24 h at 37°C. This solution was aliquoted, frozen and stored at −80°C. Immediately prior to use, Aβ1-42 aliquots were thawed and then mixed with adjuvant or PBS at 1:1 (v/v). Complete and incomplete Freund’s adjuvant were purchased from Sigma. DuoSet™ enzyme-linked ELISA kits (including TNF-α, transforming growth factor-β1 (TGF-β1), IL-1β, and IL-10) were obtained from R&D Systems (Minneapolis, MN). Purified rat anti-mouse MHC class II antibody was obtained from PharMingen (San Diego, CA). Congo red and concanavalin A (Con A) were obtained from Sigma. Aβ1-40 and Aβ1-42 ELISA kits were purchased from IBL-America (Minneapolis, MN). Murine IgG and HRP-conjugated goat anti-mouse IgG were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Goat anti-mouse IgM peroxidase conjugate antibody (A8786) was obtained from Sigma.

3.3.2 Mice

Wild-type C57BL/6, PSAPP mice (APP<sup>Swe</sup>, PSEN1dE9) and CD40 deficient (CD40<sup>−/−</sup>) mice were all obtained from Jackson Laboratories (Bar Harbor, ME). We crossed CD40<sup>−/−</sup> mice with PSAPP mice and characterized offspring by polymerase chain reaction-based genotyping for the mutant APP construct and mutant presenilin1 (PS1) gene (to examine PSAPP status) and neomycin selection vector (to type for CD40
deficiency), followed by Western blot for brain APP and splenic CD40 protein, respectively. Animals were housed and maintained under specific pathogen-free conditions in the College of Medicine Animal Facility at the University of South Florida, and all experiments were in compliance with protocols approved by the University of South Florida Institutional Animal Care and Use Committee. The animals that we studied were PSAPP/CD40\(^{+/+}\), PSAPP/CD40\(^{+/-}\), PSAPP/CD40\(^{-/-}\), CD40\(^{-/-}\), and their littermates. All of the mice included did not develop infections or neoplasms during the duration of this study.

3.3.3 Immunization strategies

For our genetic approach to CD40-CD40L blockade, we crossed PSAPP and CD40\(^{-/-}\) mice and, at 8 months of age, divided them into groups consisting of A\(\beta\)\(_{1-42}\) or vehicle (PBS)-vaccinated CD40\(^{-/-}\) and wild-type mice (n = 8 for each group, 4\(\circ\)/4\(\varnothing\)), or PSAPP/CD40\(^{+/+}\) (PSAPP/CD40\(^{+/+}\)/A\(\beta\)\(_{1-42}\), PSAPP/CD40\(^{+/+}\)/PBS), PSAPP/CD40\(^{+/-}\) (PSAPP/CD40\(^{+/-}\)/A\(\beta\)\(_{1-42}\), PSAPP/CD40\(^{+/-}\)/PBS), or PSAPP/CD40\(^{-/-}\) (PSAPP/CD40\(^{-/-}\)/A\(\beta\)\(_{1-42}\), PSAPP/CD40\(^{-/-}\)/PBS) mice (n = 16 for each group, 8\(\circ\)/8\(\varnothing\)). Immunization of these mice was performed at regular time intervals in a similar fashion to the methods described by Schenk et al. (Schenk et al., 1999). Briefly, 8 month-old mice were injected with A\(\beta\)\(_{1-42}\) (100 \(\mu\)g/mouse) or PBS emulsified in monophosphoryl lipid A (detoxified endotoxin) from \textit{S. minnesota} (MPL) and synthetic trehalose dicorynomycolate (TDM) biweekly until 9 months of age, and monthly injection with A\(\beta\)\(_{1-42}\) or PBS alone was performed thereafter.
For our pharmacologic approach to CD40-CD40L blockade, we studied 8 month-old PSAPP mice divided into five groups (n = 16 for each group, 8♂/8♀) as follows: PBS-treated Aβ1-42 immunized PSAPP mice (PSAPP/Aβ1-42/PBS), CD40L antibody-treated Aβ1-42 immunized PSAPP mice (PSAPP/Aβ1-42/CD40L antibody), Isotype control IgG-treated Aβ1-42 immunized PSAPP mice (PSAPP/Aβ1-42/IgG antibody), or CD40L antibody-treated non- Aβ1-42 immunized PSAPP mice (PSAPP/CD40L antibody). We immunized these mice with Aβ1-42 as described above and treated them with CD40L antibody (200 µg/mouse) based on our previous report (Tan et al., 2002a). For all mice, blood samples were collected from the sub-mandibular vein just before immunization and then on a monthly basis thereafter 1-2 days prior to the succeeding monthly injection (except the final collection, which was taken one month after the final injection) throughout the course of immunization, and mice were sacrificed at 12 months of age.

3.3.4 Measurement of plasma IgG and IgM Aβ antibodies by ELISA

Aβ antibodies in individual mouse plasma and brain homogenates were measured in duplicate according to previously described methods (Maier et al., 2005). Briefly, human Aβ1-42 peptide was coated at 1 µg/mL in 50 mM carbonate buffer, pH 9.6 (coating buffer) on 96-well immunoassay plates overnight at 4º C. The plates were washed with 0.05% Tween 20 in PBS (washing buffer) five times and blocked with blocking buffer (PBS with 1% BSA, 5% horse serum) for 2 hrs at room temperature. Murine IgG or IgM was serially diluted in coating buffer (1,000-0 µg/mL) to generate a standard curve. Mouse plasma and brain homogenate samples were diluted in blocking buffer at
concentrations ranging from 1:400 to 1:102,400, added to the plates, and incubated for 2 hrs at room temperature. After 3 washes with washing buffer, a detection antibody (HRP-conjugated goat anti-mouse IgG, or HRP-conjugated goat anti-mouse IgM was diluted at 1:4,000), added to the plates and incubated for 1 hr at 37º C. Following 4 washes, tetramethylbenzidine substrate was added to the plates and incubated for 15 min at room temperature. Fifty µL of stop solution (2 N H₂SO₄) was added to each well of the plates. The optical density of each well was immediately determined by a microplate reader at 450 nm. Aβ antibody data are reported as µg per mL of plasma (mean ± SD).

3.3.5 Measurement of Aβ species from plasma and brain homogenates by ELISA

Mouse brains were isolated under sterile conditions on ice and placed in ice-cold lysis buffer (containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 µg/mL leupeptin, 1 mM PMSF) as previously described (Rezai-Zadeh et al., 2005). Brains were then sonicated on ice for approximately 3 min, allowed to stand for 15 min at 4ºC, and centrifuged at 15,000 rpm for 15 min. Aβ₁₋₄₀ and Aβ₁₋₄₂ species were detected by a 2-step extraction protocol, similar to previously published methods (Johnson-Wood et al., 1997; Rezai-Zadeh et al., 2005). Detergent-soluble Aβ₁₋₄₀ and Aβ₁₋₄₂ were directly detected in plasma and brain homogenates prepared with lysis buffer described above by a 1:4 or 1:10 dilution, respectively. Total Aβ₁₋₄₀ and Aβ₁₋₄₂ species were detected by acid extraction of brain homogenates in 5 M guanidine buffer, followed by a 1:10 dilution in lysis buffer. Aβ₁₋₄₀ and Aβ₁₋₄₂ were quantified in individual
samples in duplicate using Aβ_{1-40} and Aβ_{1-42} ELISA kits in accordance with the manufacturer’s instructions (IBL-America), except that standards included 0.5 M guanidine buffer in some cases. Aβ_{1-40} and Aβ_{1-42} are represented as pg per mL of plasma or pg per mg of total protein (mean ± SD).

3.3.6 Brain and plasma cytokine analysis

Enzyme-linked immunoabsorbance assay (ELISA) for detection of IL-1β, IL-10, TGF-β1, or TNF-α was carried out for measurement of cytokines in mouse blood plasma, or brain. Tissues were obtained at the time of sacrifice, and were diluted in PBS and assayed using the kits described above in strict accordance with the manufacturer's instruction (R&D Systems). The Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) was performed to measure total cellular protein from each sample prior to quantification of cytokine release by ELISA, and cytokine secretion is expressed in pg/mg total protein.

3.3.7 Histology

Mice were anesthetized with isofluorane and transcardially perfused with ice-cold physiological saline containing heparin (10 U/ml). Brains were rapidly isolated and quartered using a mouse brain slicer (Muromachi Kikai, Tokyo, Japan). The first and second anterior quarters were homogenized for Western blot analysis, and the third and fourth posterior quarters were used for microtome or cryostat sectioning as previously described (Tan et al., 2002a). Brains were then fixed in 4% paraformaldehyde in 0.9%
saline at 4°C overnight and routinely processed in paraffin in a core facility at the Department of Pathology (University of South Florida College of Medicine). Five coronal sections from each brain (5 µm thickness) were cut with a 150 µm interval. Paraffin sections were routinely deparaffinized and hydrated in a graded series of ethanol. All sections were pre-blockading for 30 min at ambient temperature with serum-free protein block (Dako Cytomation, Carpinteria, CA).

Aβ immunohistochemical staining was performed using anti-human amyloid-β antibody (clone 4G8; 1:100; Signet Laboratories) in conjunction with the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, CA) coupled with the diaminobenzidine substrate. For microglia/macrophage immunostaining (MHC II, Iba, and CD45), sections were prepared as described above. Following pre-blocking, sections were treated overnight with anti-mouse MHC II (1:500) Iba (1:100) or CD45 (1:500) antibodies diluted in PBS (obtained from Santa Cruz (O'Keefe et al., 2002)), incubated with HRP-conjugated anti-mouse IgG, and developed. For congo red histochemistry, sections were routinely deparaffinized and rinsed in 70% (v/v) ethanol before staining with fresh-filtered 1% (w/v) congo red diluted in 70% ethanol for 5 min. These sections were rinsed three times for 5 min each in 70% ethanol, hydrated for 5 min in 0.9% saline, and mounted. β-amyloid plaques and reactive microglia were visualized using an Olympus BX-51 microscope (Olympus, Tokyo, Japan).

Quantitative image analysis was performed for 4G8 immunohistochemistry and congo red histochemistry. Images were obtained using an Olympus BX-51 microscope and digitized using an attached MagnaFire™ imaging system (Olympus, Tokyo, Japan).
Briefly, images of five 5-µm sections (150 µm apart) through each anatomic region of interest (hippocampus or cortical areas) were captured and a threshold optical density was obtained that discriminated staining from background. Manual editing of each field was used to eliminate artifacts. Data are reported as percentage of immunolabeled area captured (positive pixels) divided by the full area captured (total pixels). Quantitative image analysis was performed by a single examiner (TM) blinded to sample identities.

3.3.8 Splenocyte cultures

Cell suspensions of splenocytes from individual mice were prepared as previously described (Town et al., 2001b; Town et al., 2002) and passed in 0.5 mL aliquots into 24-well plates at $3 \times 10^6$ cells/mL. These cells were cultured for 48 h in the presence or absence of Con A (5 µg/mL), or Aβ1-42 (20 µg/mL). Supernatants were then collected and assayed by IFN-γ, IL-2, and IL-4 cytokine ELISA kits in strict accordance with the manufacturer's instruction (R&D Systems). The Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) was performed to measure total cellular protein from each well prior to quantification of cytokine release by ELISA, and cytokine secretion is expressed in pg/mg total cellular protein (mean ± SD). To verify whether stimulation of splenocytes produced any between-groups differences on cell death that might account for altered cytokine profiles, LDH release assay was carried out as described (Townsend et al., 2005) and LDH was not detected in any of the wells studied.

3.3.9 Aβ-specific lymphocyte neurotoxicity assay
Primary cultured neuronal cells were used as target cells in $^{51}$Cr release assay for Aβ-specific lymphocyte neurotoxicity (Tan et al., 1999). We co-cultured primary neuronal cells from PSAPP mice or their littermates with CD3$^+$ T-cells (including CD4$^+$ and CD8$^+$ T-cells) isolated from primary cultures of splenocytes derived from Aβ1-42/IgG- or Aβ1-42/CD40L antibody-treated PSAPP mice as described above. As in our previous studies (Tan et al., 1999; Town et al., 2002), primary neuronal cells were labeled with $^{51}$Cr as target cells and co-cultured with T-cells as effectors. Four hour-$^{51}$Cr release assay was then carried out. Total release represents the radioactivity released after lysis of target cells with 5% Triton X-100.

3.3.10 Statistical analysis

All data were normally distributed; therefore, in instances of single mean comparisons, Levene’s test for equality of variances followed by t-test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance (ANOVA) was used, followed by post-hoc comparison using Bonferroni’s method. Alpha levels were set at 0.05 for all analyses. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, Illinois) was used for all data analysis.

3.4 Results

3.4.1 CD40 deficiency modulates Aβ antibody production after Aβ vaccination
It is well-established that B-cells require CD40 engagement by T-cell-derived CD40L to produce IgG antibodies in response to vaccination (Kawabe et al., 1994; Bishop and Hostager, 2003). To determine the effects of Aβ1-42 vaccination on Aβ antibody production in the absence of CD40 expression, strain- and gender-matched CD40 deficient (CD40−/−) and wild type mice (n = 8 per group, 4♂/4♀) were immunized. We employed a four-month vaccination strategy according to modified previous methods (Schenk et al., 1999) utilizing synthetic aggregated Aβ1-42 peptide or PBS with MPL/TDM as adjuvant. Mouse plasma samples were collected monthly over this four-month vaccination period and subjected to ELISA for measurement of Aβ antibodies. Results indicated potent Aβ IgG production in wild-type mice, whereas CD40−/− mice produced no detectable Aβ IgG (Figure 6A, top panel). While CD40−/− mice did not produce detectable Aβ IgG, they did produce Aβ IgM that was not significantly different from wild-type mice [98.56 ± SD 7.45 vs. 86.98 ± SD 12.09 (µg/mL at 1 month after the first immunization).

To investigate the impact of CD40 deficiency on Aβ1-42 vaccination-induced humoral immune responses in a mouse model of AD, we vaccinated 8 month-old PSAPP mice with three CD40 genotypes (PSAPP/CD40+/+, PSAPP/CD40+/−, and PSAPP/CD40−/−) with either Aβ1-42 or vehicle (PBS). Blood samples from all mice were individually collected once monthly over the four month vaccination period. As expected, Aβ1-42 vaccinated PSAPP/CD40−/− mice demonstrated no detectable Aβ IgG, whereas PSAPP/CD40+/− and PSAPP/CD40+/+ mice produced similar increases in Aβ antibodies throughout the four-month Aβ1-42 vaccination program (Figure 6A, bottom panel).
3.4.2 Increased plasma Aβ1-40 and Aβ1-42 in heterozygous CD40 deficient PSAPP mice after Aβ vaccination

Activation of Aβ efflux from the CNS to the systemic circulatory system is a well-recognized clearance mechanism underlying Aβ vaccination in AD mouse models (Sigurdsson et al., 2002; Lemere et al., 2003). To determine the effect of partial or complete CD40 deficiency on activation of Aβ efflux after Aβ1-42 vaccination, we separately measured plasma Aβ1-40 and Aβ1-42 species by ELISA monthly over the four-month vaccination period. Importantly, PSAPP/CD40+/−/Aβ1-42 mice exhibited dramatically increased plasma Aβ1-40 and Aβ1-42 compared to PSAPP/CD40+/−/Aβ1-42 animals at the time points analyzed (Figure 6B, \( P < 0.001 \)), pointing to a shift in Aβ load from the CNS to the systemic circulation in this group. PSAPP/CD40+/−/Aβ1-42 mice displayed very low levels of plasma Aβ species, similar to unvaccinated mouse groups. The lack of elevated Aβ plasma levels in PSAPP/CD40+/−/Aβ1-42 mice can most likely be explained by the absence of Aβ IgG in this mouse group, as homozygous CD40 deficiency conferred absence of Aβ IgG production to either Aβ species (Figure 6A, bottom panel). Interestingly, PSAPP/CD40+/−/Aβ1-42 mice displayed similarly elevated plasma levels of Aβ1-40 and Aβ1-42 when compared with the PSAPP/CD40+/−/Aβ1-42 mouse group (\( P > 0.05 \)). These data further suggest that Aβ IgG production may be required for Aβ efflux from the CNS to the periphery in this vaccination paradigm.
Figure 6

A

Aβ1-42 immunization (n = 8, 4♂/4♀)

Wild-type mice  CD40-/- mice

Aβ IgG (μg/mL)

0  200  400  600  800

0 month  1 month  2 months  3 months  4 months

Time of blood sample collection

0  1st  2nd  3rd  4th (month)
**Figure 6.** Evaluation of the effects of CD40 deficiency on Aβ antibody generation and Aβ efflux in Aβ1-42-immunized mice. Peripheral blood samples were collected monthly throughout the four-month Aβ immunization course. (A) The graph shows antibody levels for wild-type vs. CD40−/− mice (top panel) and PSAPP mice deficient for CD40 vs. appropriate controls as indicated (bottom panel) following Aβ1-42 vaccination. PSAPP/CD40+/+ Aβ1-42 and PSAPP/CD40+/− Aβ1-42 mice produced similar elevations in Aβ IgG antibodies, in contrast to PSAPP/CD40−/− Aβ1-42, PSAPP/CD40+/+/PBS, PSAPP/CD40+/−/PBS, and PSAPP/CD40−+/PBS mice that produced undetectable levels of Aβ IgG antibodies. Data are presented as mean ± SD of plasma Aβ antibodies (µg/mL). (B) Plasma Aβ1-40 and Aβ1-42 peptides were measured separately by ELISA. Data are represented as mean ± SD of Aβ1-40 (top panel) or Aβ1-42 (bottom panel). PSAPP/CD40+/+ Aβ1-42 and PSAPP/CD40+/− Aβ1-42 mice produced similar elevations in plasma Aβ1-40 and Aβ1-42, in contrast to PSAPP/CD40−/− Aβ1-42, PSAPP/CD40+/+/PBS, PSAPP/CD40+/−/PBS, and PSAPP/CD40−−/PBS mice that produced minimal levels of plasma Aβ1-40 and Aβ1-42.
3.4.3 Reduced cerebral Aβ1-40 and Aβ1-42 in heterozygous CD40 deficient PSAPP mice vaccinated with Aβ1-42

As shown in Figure 7, results revealed that Aβ1-42 vaccination of mice completely (PSAPP/CD40−/−) and partially (PSAPP/CD40+/-) deficient for CD40 yielded decreased amounts of soluble (top panels) and insoluble (bottom panels) Aβ1-40 and Aβ1-42 in brain homogenates as measured by ELISA. Most importantly, a significantly greater reduction in both soluble and insoluble Aβ1-40 and Aβ1-42 levels was evident in the PSAPP/CD40+/-/Aβ1-42 group compared to either PSAPP/CD40+/+/Aβ1-42 or PSAPP/CD40−/-/Aβ1-42 groups (*P < 0.05; **P < 0.001). We further observed that β-amyloid histopathology was also markedly reduced in the PSAPP/CD40+/-/Aβ1-42 group as determined by Aβ antibody immunohistochemical analysis (Figure 8A), and congo red staining (Figure 8B) of mouse coronal brain sections from Aβ vaccinated mice. Quantitative analysis of results revealed significantly reduced Aβ antibody- and congo red-positive β-amyloid plaque burden in each brain region examined from PSAPP/CD40+/-/Aβ1-42 mice as compared to either PSAPP/CD40+/+/Aβ1-42 or PSAPP/CD40−/-/Aβ1-42 groups (Figure 8C, **P < 0.001). Together, these data indicate that CD40 heterozygosity confers the greatest reduction in Aβ load in PSAPP mice when compared to all other groups following Aβ1-42 vaccination, and suggest that partial disruption of CD40 signaling could maximize Aβ1-42 vaccination efficacy.
Figure 7
Figure 7. Cerebral Aβ levels are significantly reduced in Aβ1-42-immunized PSAPP mice heterozygous for CD40. Detergent-soluble Aβ1-40 and Aβ1-42 (A) and insoluble (5M guanidine-soluble) Aβ1-40 and Aβ1-42 peptides (B) were measured separately in brain homogenates by ELISA. Data are presented as mean ± SD of Aβ1-40 or Aβ1-42 (pg/mg protein).
Figure 8

Hippocampal subfield

PSAPP/CD40+/PBS

PSAPP/CD40+/Aβ1-42

PSAPP/CD40+/Aβ1-42

CA3

CA1

DG

10 X
C

**Aβ burden (%)**

<table>
<thead>
<tr>
<th>Group</th>
<th>CC/H</th>
<th>EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ1-42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSAPP/CD40+/+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSAPP/CD40+-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSAPP/CD40-/-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Congo red burden (%)**

<table>
<thead>
<tr>
<th>Group</th>
<th>CC/H</th>
<th>EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ1-42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSAPP/CD40+/+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSAPP/CD40+-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSAPP/CD40-/-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = 16 mice (8♂/8♀)
Figure 8. β-amyloid pathology is reduced in Aβ1-42-immunized PSAPP mice heterozygous for CD40. Mouse coronal brain sections were embedded in paraffin and stained with monoclonal human Aβ antibody (A), or were stained with congo red (B), and the hippocampus is shown. (C) Percentages [plaque area/total area; mean ± SD with n = 16 mice (♂/♀)] of Aβ antibody-immunoreactive Aβ plaques (top panel) and congo red-positive Aβ deposits (bottom panel) were calculated by quantitative image analysis for each brain region (CC/H: cingulate cortex and hippocampus; EC: entorhinal cortex) as indicated.
Interestingly, Aβ plaque reduction in PSAPP/CD40\(^{-/-}\)/Aβ\(_{1-42}\) mice was significantly reduced when compared to PSAPP/CD40\(^{+/+}\)/Aβ\(_{1-42}\), or PSAPP/CD40\(^{-/-}\)/PBS groups (Figure 8C, \(*P < 0.05\)). These data are additionally supported by ELISA analyses of both soluble and insoluble Aβ\(_{1-40}\) and Aβ\(_{1-42}\) in brain homogenates (Figure 7), and suggest other mechanisms besides Aβ IgG production, such as cellular immune responses, might be involved in the observed reductions of cerebral Aβ/β-amyloid in PSAPP mice after Aβ\(_{1-42}\) vaccination.

3.4.4 Aβ\(_{1-42}\) vaccination results in markedly increased anti-inflammatory cytokines and reduced plasma soluble CD40L in PSAPP/CD40\(^{-/-}\) mice

Due to the observed lack of Aβ IgG antibodies in PSAPP/CD40\(^{-/-}\) mice following Aβ\(_{1-42}\) vaccination, we wished to investigate whether cellular immune responses could be involved in the reductions of cerebral Aβ and β-amyloid deposits in these animals. To test this hypothesis, we performed ELISA to examine anti-inflammatory cytokine profiles in brain homogenates from PSAPP/CD40\(^{+/+}\), PSAPP/CD40\(^{+/+}\), and PSAPP/CD40\(^{-/-}\) mice vaccinated with either Aβ\(_{1-42}\) or vehicle (PBS). As shown in Figure 9A, analysis of data revealed significantly (\(**P < 0.001\)) elevated expression of brain IL-10 from either PSAPP/CD40\(^{-/-}\)/Aβ\(_{1-42}\) or PSAPP/CD40\(^{-/-}\)/Aβ\(_{1-42}\) mice compared to PSAPP/CD40\(^{+/+}\)/Aβ\(_{1-42}\) mice, but not for IL-1β (\(P > 0.05\)). Moreover, we found a significant decrease in plasma soluble CD40L (sCD40L) when comparing PSAPP/CD40\(^{-/-}\)/Aβ\(_{1-42}\) to either PSAPP/CD40\(^{+/+}\)/Aβ\(_{1-42}\) or PSAPP/CD40\(^{-/-}\)/PBS mice (\(**P < 0.001\), Figure 9B). Reduced sCD40L in PSAPP/CD40\(^{-/-}\)/Aβ\(_{1-42}\) compared to PSAPP/CD40\(^{+/+}\)/Aβ\(_{1-42}\) mice occurred in
the absence of significantly different levels of Aβ IgM antibodies (data not shown).

These data indicate that reductions in cerebral Aβ after Aβ1-42 vaccination of PSAPP/CD40−/−/PBS mice is associated with a rise in the anti-inflammatory cytokines IL-4, IL-10 and TGF-β1, and a decrease in plasma sCD40L levels.
**Figure 9.** PSAPP/CD40−/− mice have increased anti-inflammatory IL-10 cytokine and decreased plasma soluble CD40L (sCD40L) after Aβ1-42 vaccination. (A) ELISA analysis of cytokine levels in brain homogenates from the indicated mouse groups. Data are presented as mean ± SD of each cytokine (pg/mg total protein). (B) ELISA for plasma sCD40L levels in the indicated mouse groups. Data are presented as mean ± SD of plasma sCD40L protein (pg/mL).
3.4.5 Neutralizing CD40L antibody increases circulating Aβ₁₋₄₀ and Aβ₁₋₄₂ levels and reduces cerebral amyloidosis in Aβ₁₋₄₂ vaccinated PSAPP and Tg2576 mice

To determine whether pharmacologic inhibition of CD40-CD40L interaction might produce a similar effect as genetic disruption on enhancing Aβ₁₋₄₂ vaccination efficacy, we administered neutralizing CD40L antibody to PSAPP mice in combination with active Aβ₁₋₄₂ vaccination described in “Materials and Methods.” Blood samples were individually collected from all mice at a monthly time interval. Similar to the effects observed in Aβ₁₋₄₂ vaccinated PSAPP/CD40⁺/⁻ mice, Aβ₁₋₄₂ vaccinated PSAPP mice treated with CD40L antibody displayed elevations in plasma levels of Aβ₁₋₄₀ and Aβ₁₋₄₂ and Aβ IgG antibodies that did not significantly differ from Aβ₁₋₄₂ vaccinated PSAPP mice injected with an isotype-matched IgG control antibody (Figure 10A, top to bottom, respectively). The PSAPP/Aβ₁₋₄₂/CD40L antibody and PSAPP/Aβ₁₋₄₂/IgG groups produced greater plasma levels of Aβ₁₋₄₀ and Aβ₁₋₄₂ species and Aβ antibodies than either PSAPP mice receiving PBS or treatment with CD40L antibody alone, confirming that 1) immunization of this cohort of animals was successful and 2) neutralizing CD40L did not hamper Aβ antibody production (Figure 10A). Additionally, no significant differences were revealed for either plasma Aβ₁₋₄₀ and Aβ₁₋₄₂ levels or Aβ antibodies when comparing PSAPP/Aβ₁₋₄₂/IgG to PSAPP/Aβ₁₋₄₂ groups (P > 0.05; data not shown).
Figure 10

A

- PSAPP/AB1-42/igG
- PSAPP/AB1-42/CD40L antibody
- PSAPP/CD40L antibody
- PSAPP/PBS

Time of blood sample collection

0 2nd 3rd 4th (month)
**Figure 10.** Peripheral and cerebral Aβ levels are reduced in Aβ1-42-immunized PSAPP mice treated with CD40L neutralizing antibody. (A) ELISA analysis for plasma levels of Aβ1-40 and Aβ1-42 and Aβ antibodies. Plasma Aβ1-40 (top panel) and Aβ1-42 (middle panel) were measured separately by ELISA. PSAPP/Aβ1-42/CD40L antibody and PSAPP/Aβ1-42/IgG mice produced similar elevations in plasma Aβ1-40 and Aβ1-42, in contrast to PSAPP/CD40L antibody and PSAPP/PBS mice which produced minimal levels of plasma Aβ1-40 and Aβ1-42. Data are represented as mean ± SD of Aβ1-40 or Aβ1-42 (pg/mL) in plasma. Aβ antibody levels (bottom panel) were measured by ELISA. PSAPP/Aβ1-42/CD40L antibody and PSAPP/Aβ1-42/IgG mice produced similar elevations in plasma Aβ IgG antibodies in contrast to PSAPP/CD40L antibody and PSAPP/PBS mice which had undetectable levels of plasma Aβ IgG antibodies. Data are presented as mean ± SD of Aβ antibodies (µg/mL) in plasma. No significant difference in Aβ antibody levels between PSAPP/Aβ1-42/CD40L antibody and PSAPP/Aβ1-42/IgG mice (P > 0.05) was observed. (B) Soluble Aβ1-40 and Aβ1-42 peptides (top panel) and insoluble Aβ1-40 and Aβ1-42 (bottom panel) in brain homogenates were measured separately by ELISA. Data are presented as mean ± SD of Aβ1-40 or Aβ1-42 peptides normalized to total protein (pg/mg).
We next examined the effect of neutralizing CD40L antibody on cerebral Aβ levels in PSAPP mice vaccinated with Aβ1-42. ELISA analysis revealed that PSAPP/Aβ1-42/CD40L antibody mice display significantly reduced amounts of cerebral soluble and insoluble Aβ1-40 and Aβ1-42 peptides as compared with PSAPP/Aβ1-42/IgG, PSAPP/CD40L antibody, or PSAPP/PBS groups (**P < 0.001; Figure 10B). Furthermore, PSAPP/Aβ1-42/CD40L antibody mice showed a marked reduction in cerebral Aβ deposits compared to PSAPP/Aβ1-42/IgG mice or other control groups (**P < 0.001; Figs. 11A–C). These data show additional reduction in cerebral Aβ and β-amyloid in the context of Aβ1-42 vaccination provided by pharmacologic blockade of CD40-CD40L interaction.

Together, these data demonstrate that disruption of the CD40-CD40L interaction by 1) a genetic approach or 2) pharmacologic depletion of available CD40L by neutralizing antibody enhances reduction of cerebral amyloidosis after Aβ1-42 vaccination. Recent studies have shown CAA and cerebral microhemorrhage often occur in AD mice following intraperitoneal injection (i.p) active or passive Aβ vaccination (Wilcock et al., 2004; Wilcock et al., 2007). Thus, we next investigated if disruption of CD40L activity could reduce CAA following Aβ vaccination in the Tg2576 mouse model of AD. Since Tg2576 mice are known to produce CAA pathology at 15 to 20 months of age (Christie et al., 2001; Li et al., 2003; Friedlich et al., 2004; Kim et al., 2007), we initiated i.p. injection of these mice with CD40L antibody at 12 months of age (n = 16, 8♂/8♀) in conjunction with active Aβ1-42 immunization using an identical procedure as described above. Four months later, we sacrificed these mice and examined CAA. As
shown in Figs. 11D – E, disruption of CD40L activity by the depleting antibody not only promotes additional reduction in total congo red after Aβ1-42 vaccination, but also further reduces vascular congo red signal. This was confirmed by statistical analysis, which revealed significant differences between Tg2576/Aβ1-42/IgG and Tg2576/Aβ1-42/CD40L antibody groups (**P < 0.001) for both total and vascular congo red. In addition, we also analyzed cerebral Aβ levels/β-amyloid deposits in these two groups by Aβ ELISA and immunochemistry. Similar to the effects observed in Aβ1-42/CD40L antibody-vaccinated PSAPP mice, Tg2576 mice receiving both Aβ1-42 immunization and depleting CD40L antibody displayed a marked decrease in cerebral soluble and insoluble Aβ1-40 and Aβ1-42 levels and β-amyloid load compared to Aβ1-42-alone-immunized mice (P < 0.001; data not shown).
Figure 11
PSAPP mice (n = 16, 8♂/8♀)
Tg2576 mice (n = 16, 8♂/8♀)
**Figure 11.** Cerebral β-amyloid deposits and cerebral amyloid angiopathy are reduced in Aβ1-42-immunized PSAPP or Tg2576 mice treated with CD40L neutralizing antibody. Mouse paraffin-embedded coronal brain sections from were stained with rabbit Pan-β-amyloid antibody (A) or with congo red (B), and the hippocampus is shown. (C) Percentages (plaque area/total area; mean ± SD) of Aβ antibody-immunoreactive deposits (top panel) or of congo red-stained sections (bottom panel) were calculated by quantitative image analysis. (D) Tg2576 received Aβ1-42 vaccination plus neutralizing CD40L antibody or isotype-matched control IgG both Aβ1-42, and brain sections were stained with congo red (hippocampus is shown). Positions of the hippocampal subfields CA1, CA3, and DG (dentate gyrus) are indicated in the upper left panel. Arrows indicate Aβ deposit-affected vessels. (E) Percentages (% of area) of congo red-stained plaques were quantified by image analysis [mean ± SD with (n = 16, 8♂/8♀)].
3.4.6 CD40 pathway blockade decreases MHC II and CD45-positive microglia and increases anti-inflammatory cytokines in Aβ1-42 immunized PSAPP mice

To evaluate the effects of CD40/CD40L blockade on pro-inflammatory APC-like microglial activation in the Aβ1-42 vaccination paradigm, we first stained brain sections from PSAPP/Aβ1-42/IgG and PSAPP/Aβ1-42/CD40L antibody mice with Iba1 antibody (Figure 12A). We quantified Iba1 positive microglia/macrophages with/without the spindle-shaped morphology, as it has been previously reported that microglial cells become spindle-shaped when exposed to stimuli including LPS and Con A that promote the pro-inflammatory APC phenotype (Washington et al., 1996; Bernhardi and Nicholls, 1999). Interestingly, disruption of CD40L activity significantly reduced spindle-shaped microglia/macrophages by morphologic analysis (**P < 0.001), but did not alter non-spindle-shaped cells (Figure 12B). To further evaluate whether CD40L neutralization mitigated APC-like microglia, we fluorescently labeled brain sections with MHC II and CD45 antibodies. As shown in Figure 12C, Iba1 positive microglial cells were largely positive for CD45 and MHC II in Aβ1-42/IgG immunized PSAPP mice, but not in Aβ1-42 and CD40L antibody co-immunized PSAPP mice. These data indicate that depletion of functional CD40L results in decreased pro-inflammatory APC-like microglial activation in PSAPP/Aβ1-42/CD40L antibody mice. It should be noted that Iba1 immunostaining does not distinguish resident microglia from peripherally-derived monocytes that may migrate to the CNS and take up a microglial phenotype.
Figure 12

A

Iba1 positive microglial cells

PSAPP/Aβ_{1-42}/CD40L antibody

CA3

10 X

40 X
B

Iba1 positive microglia (%)

<table>
<thead>
<tr>
<th>Spindle shaped</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSAPP/Aβ1-42</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>PSAPP/Aβ1-42/CD40L antibody</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

119
**Figure 12.** CD40L blockade inhibits APC-like microglial activation in Aβ1-42 vaccinated PSAPP mice and promotes anti-inflammatory cellular immunity. (A) Representative hippocampal sections from PSAPP/Aβ1-42/IgG and PSAPP/Aβ1-42/CD40L antibody mouse brains were stained with Iba1 antibody to illustrate both microglial load and morphology. (B) Quantitative image analysis of microglial load (Iba1 positive) and percentage of spindle-shaped Iba1 positive microglia is shown. (C) Representative hippocampal sections from PSAPP/Aβ1-42/IgG and PSAPP/Aβ1-42/CD40L antibody mouse brains were stained with Iba1 together with MHC II or CD45 antibodies to illustrate microglial load and activation status (DAPI was used as a nuclear counterstain). (D) Th1 and Th2 cytokine analysis by ELISA was conducted on mouse brain homogenates from PSAPP, PSAPP/Aβ1-42/IgG and PSAPP/Aβ1-42/CD40L antibody mice. Data are represented as mean ± SD of each cytokine in brain homogenates (pg/mg total protein) from PSAPP, PSAPP/Aβ1-42/CD40L antibody or PSAPP/Aβ1-42/IgG mice.
To further determine the consequences of this phenomenon in terms of inflammatory responses, we analyzed brain homogenates from PSAPP/Aβ1-42/CD40L antibody, PSAPP/Aβ1-42/IgG, and other control groups by ELISA for expression of pro-inflammatory and anti-inflammatory cytokines. Analysis of results revealed a significantly (**P <0.001) greater expression of anti-inflammatory TGF-β1 and IL-10 cytokines from PSAPP/Aβ1-42/CD40L antibody mice compared to the PSAPP/Aβ1-42/IgG mice (Figure 12D). Moreover, PSAPP/Aβ1-42/CD40L antibody mice also produced significantly (P <0.001) greater expression of TGF-β1 and IL-10 when compared to other controls including PSAPP/IgG and PSAPP/Aβ1-42/PBS mice (data not shown). No significant between-groups differences were revealed when considering TNF-α or IL-1β. These data indicate that CD40L blockade correlates with a rise in the anti-inflammatory cytokines TGF-β1 and IL-10, without affecting the pro-inflammatory cytokines TNF-α and IL-1β.

3.4.7 Aβ1-42-immunized PSAPP mice treated with CD40L neutralizing antibody exhibit increases in anti-inflammatory cytokines and decreases in neurotoxic inflammatory responses in vitro

To investigate Aβ-specific T-cell immune responses after Aβ1-42 immunization plus neutralizing CD40L antibody treatment, we established primary cultures of splenocytes from: PSAPP/Aβ1-42/IgG, PSAPP/Aβ1-42/CD40L antibody, PSAPP/CD40L antibody, and PSAPP/PBS mice. We then quantified key cytokines produced by activated T-cells (IFN-γ, IL-2, and IL-4) in supernatants by ELISA. Both non-specific
(Con A) and specific recall stimulation of primary cultured splenocytes with Aβ1-42 resulted in increases in the pro-inflammatory T-cell cytokines, IFN-γ and IL-2, in both PSAPP/Aβ1-42/IgG and PSAPP/Aβ1-42/CD40L antibody groups compared to the PSAPP/CD40L antibody group (Figure 13A). However, these pro-inflammatory T-cell cytokines were reduced in the PSAPP/Aβ1-42/CD40L antibody group versus PSAPP/Aβ1-42/IgG mice. Moreover, PSAPP/Aβ1-42/CD40L antibody mice demonstrated increased anti-inflammatory T helper type 2 cytokine IL-4 when compared to PSAPP/Aβ1-42/IgG mice. These data indicate that disruption of CD40L activity in Aβ1-42 vaccinated mice reduces pro-inflammatory Aβ-specific T-cell immune responses in favor of an anti-inflammatory response. One-way ANOVA followed by post hoc comparison revealed significant differences when comparing PSAPP/Aβ1-42/CD40L antibody to PSAPP/CD40L antibody or PSAPP/Aβ1-42/IgG mouse groups for levels of each of the three cytokines (**P < 0.001) after in vitro Aβ1-42 challenge. Of note, there were significant differences within mouse groups between IL-4 and either IFN-γ or IL-2 after Aβ1-42 recall challenge (##P < 0.001). Following challenge, no significant difference in cytokine release was observed from splenocytes between PSAPP/PBS and PSAPP/Aβ1-42/IgG control mouse groups (P > 0.05; data not shown).

To determine whether CD40L neutralization could mitigate potentially damaging effects of Aβ-specific T-cells, we co-cultured primary neuronal cells from PSAPP mice or their littermates with CD3+ T-cells (including CD4+ and CD8+ T-cells) isolated from the primary cultured splenocytes derived from PSAPP/Aβ1-42/IgG or PSAPP/Aβ1-42/CD40L antibody mice as described above. Per our previous reports (Tan et al., 2000;
Tan et al., 2002a; Town et al., 2002), we labeled primary neuronal cells with $^{51}$Cr as target cells and co-cultured them with T-cells as effectors, and carried out four-hour $^{51}$Cr release assay. ANOVA showed main effects of effector:target ratio for Aβ$_{1-42}$ vaccinated PSAPP mouse-derived T-cells (effectors) and PSAPP mouse-derived neuronal cells (target cells) (**$P < 0.001$; n = 8 mice for PSAPP/Aβ$_{1-42}$/IgG and PSAPP/Aβ$_{1-42}$/CD40L antibody groups; n = 5 mice for both control groups) (Figure 13B), but not when unvaccinated PSAPP mouse-derived T-cells were used (control 1) or when control littermate (non-transgenic mouse)-derived neuronal cells (control 2) were used ($P > 0.05$). ANOVA followed by post-hoc comparison revealed a significant difference across ratios between PSAPP/Aβ$_{1-42}$/IgG and PSAPP/Aβ$_{1-42}$/CD40L antibody T-cells (**$P < 0.001$), indicating an overall decrease in percentage of cell lysis as a result of disrupting CD40L activity.
Figure 13

A

![Graph showing cytokines (pg/mg, ± SD) for IFN-γ, IL-2, IL-4, IFN-γ, IL-2, and IL-4 in different conditions.

Challenge/Con A

- PSAPP
- PSAPP/Aβ1-42/IgG
- PSAPP/Aβ1-42/CD40L antibody
- PSAPP/CD40L antibody

Challenge/Aβ1-42

**

**

**
Figure 13. Aβ-specific neurotoxic inflammatory responses are reduced in Aβ1-42-immunized PSAPP mice deficient for CD40. (A) Splenocytes were individually isolated and cultured from mice as indicated after Aβ1-42 immunization and either CD40L antibody treatment or PBS injection (control). These cells were stimulated with Con A (5 µg/mL) or Aβ1-42 (20 µg/mL) for 48 hrs. Cultured supernatants were collected from these cells for IFN-γ, IL-2, and IL-4 cytokine analyses by ELISA. Data are represented as mean ± SD (n = 10) of each cytokine in supernatants (pg/mg total intracellular protein). (B) Aβ specific T cell-mediated neuronal cell injury was determined by ⁵¹Cr release assay. Data are reported as mean ⁵¹Cr release values ± SD, and n = 8 for each condition presented. PSAPP/Aβ1-42/IgG mouse group, effectors: Aβ1-42/IgG-immunized PSAPP mouse-derived T cells; target cells: PSAPP-mouse-derived primary neuronal cells. PSAPP/Aβ1-42/CD40L antibody mouse group, effectors: Aβ1-42/CD40L antibody-immunized PSAPP mouse-derived T cells; target cells: PSAPP-mouse-derived primary neuronal cells. Control 1, effectors: unvaccinated PSAPP mouse-derived T cells; target cells: PSAPP mouse-derived neuronal cells. Control 2, effectors: Aβ1-42-immunized PSAPP mouse-derived T cells; target cells: non-transgenic mouse-derived primary neuronal cells.
3.5 Discussion

We have previously shown the CD40-CD40L interaction enhances pro-inflammatory microglial activation triggered by cerebral Aβ deposits (Tan et al., 1999). This form of microglial activation is deleterious, as both genetic ablation of CD40L and CD40L neutralizing antibody reduce brain levels of several neurotoxic inflammatory cytokines and mitigate cerebral amyloidosis in AD mouse models (Tan et al., 2002a). To establish a possible mechanism to explain these results, we previously quantified microglial phagocytic activity in CD40 deficient versus CD40 sufficient AD mice. We observed inhibition of microglial Aβ phagocytosis upon CD40 ligation. This coincided with increased microglial co-localization of MHC class II with non-opsonized Aβ peptide. Moreover, this APC phenotype was accompanied by upregulation of pro-inflammatory Th1 cytokines such as TNF-α, IL-1β, IL-2, and IFN-γ (Townsend et al., 2005). These data suggest that CD40 pathway blockade induces “switching” of the microglial phenotype from a pro-inflammatory APC state to an anti-inflammatory, pro-phagocytic state (Townsend et al., 2005). Interestingly, brain Aβ clearance in the Aβ immunotherapy paradigm has previously been suggested to rely on microglial phagocytosis (Bard et al., 2000), and microglial Fc receptor (i.e., microglial phagocytosis of Aβ antibody-opsonized deposits) is not required for brain Aβ clearance (Das et al., 2003). Thus, we suggest that the combination of blocking the microglial CD40 pathway and Aβ immunotherapy further enhances microglial Abeta clearance.

Previous clinical investigation has revealed that active immunization with Aβ in humans confers the unacceptable risk of aseptic meningoencephalitis associated with T-
cell infiltration, gliosis, and associated rise in CNS pro-inflammatory mediators (Schenk et al., 1999; Schenk and Yednock, 2002; Nicoll et al., 2003). Based on these data and our work on the CD40-CD40L association with brain Aβ levels, we investigated whether CD40 blockade could reduce cerebral Aβ deposits without the undesirable inflammatory events in the CNS.

First, we tested whether active Aβ1-42 vaccination of CD40 deficient mice could produce significant levels of Aβ antibodies. Consistent with the requirement of CD40 signaling for IgM to IgG class switching (Kawabe et al., 1994), homozygous CD40 deficient mice vaccinated with Aβ1-42 did not produce detectable Aβ IgG antibodies, but had slightly increased levels of Aβ IgM antibodies vs. wild-type controls. However, active Aβ1-42 vaccination of PSAPP/CD40+/− mice produced elevated plasma anti-Aβ IgG antibodies comparable to CD40 sufficient PSAPP mice which are consistent with a gene dose-effect (Figure 6A). Interestingly, further reduction in cerebral amyloidosis in Aβ-vaccinated PSAPP/CD40−/− occurred essentially in the absence of Aβ IgG antibodies. It is well known that the CD40 pathway is essential for antibody isotype switching from IgM to IgG, and this result suggests that the additional therapeutic benefit from blocking the CD40 pathway is independent of IgG in our system. However, given 1) the requirement of CD40 signaling for a diverse set of immunological responses and 2) that we did not use IgG deficient mice, this conclusion should be taken with caveats.

Next, we quantified plasma levels of Aβ1-40 and Aβ1-42 species across the various groups of Aβ-immunized PSAPP mice in an attempt to determine the relationship between IgG, IgM, and efflux of Aβ from the brain to the periphery. Similar elevations
of plasma Aβ_{1-40, 42} species between CD40 heterozygous deficient or CD40 sufficient PSAPP mice were observed (Figure 6B). Thus, we suggest that Aβ IgG-mediated brain to blood efflux was operating similarly in CD40 heterozygous and CD40 sufficient PSAPP mice. By contrast, Aβ_{1-42} vaccinated PSAPP/CD40^{−/−} mice did not exhibit elevations in plasma Aβ_{1-40, 42} species (Figure 6B). This lack of elevated plasma Aβ_{1-40, 42} correlated positively with the lack of Aβ IgG antibody production in the homozygous CD40 deficient PSAPP mice. This is consistent with a lack of brain-to-blood efflux of Aβ via the peripheral sink hypothesis (DeMattos et al., 2001), and suggests that a distinct mechanism is operating in these PSAPP/CD40^{−/−} mice. While CD40 deficient mice do produce normal levels of Aβ IgM antibodies, no detectable elevation in peripheral Aβ was observed. This, too, is in accord with the notion that Aβ IgG is required for brain to blood efflux of Aβ (DeMattos et al., 2001). As pharmacotherapeutic “proof of principle”, we administered CD40L neutralizing antibody to Aβ_{1-42} vaccinated PSAPP mice, and confirmed greater reductions in cerebral amyloidosis compared to Aβ_{1-42} vaccinated PSAPP mice given control IgG. This experimental approach was performed to offset the possibility that genetic ablation, in and of itself, does not confer developmental changes that would result in modulation of Aβ loads.

We next went on to test T-cell specific immune responses against Aβ and found that recall stimulation of primary cultured splenocytes with Aβ_{1-42} peptide resulted in reduced IFN-γ and IL-2 production in the PSAPP/Aβ_{1-42}/CD40L antibody mouse group compared to PSAPP/Aβ_{1-42}/IgG mice (Figure 13A). Further, PSAPP/CD40^{+/-}/Aβ_{1-42} mice
exhibited an increase in the anti-inflammatory Th2 cytokine, IL-10, and produced less circulating soluble CD40L (sCD40L) compared to PSAPP/CD40+/+/Aβ1-42 mice (Figure 9). Taken together, these findings suggest partial CD40 pathway inhibition reduces pro-inflammatory but increases anti-inflammatory T-cell immune responses to Aβ challenge. This is particularly attractive as these results raise the possibility that 50% pharmacological inhibition of CD40 might be of benefit in the attenuation of the pro-inflammatory meningoencephalitis associated with active Aβ vaccination in humans, while still allowing for production of Aβ IgG antibodies.

In addition to developing parenchymal Aβ deposits and associated elevated inflammatory cytokines seen in the PSAPP model, the Tg2576 mouse model of AD also develops cerebrovascular Aβ deposits not unlike cerebral amyloid angiopathy (CAA), which is observed in the majority of AD patients (Nicoll et al., 2003; Wilcock et al., 2004; Wilcock et al., 2007). To determine if reducing available CD40L might also mitigate CAA, we administered neutralizing CD40L antibody to Aβ vaccinated Tg2576 mice. Indeed blockade of the CD40L with neutralizing antibody in combination with Aβ vaccination produced the highest therapeutic effect (reduction of parenchymal Aβ) and the most minimal CAA-like pathology as measured by parenchymal and congo red staining respectively (Figs. 11D – E). These results suggest CD40L antibody neutralization not only improves the cerebral amyloid reducing effect of Aβ vaccination, but also confers a reduction in undesirable CAA-like pathology in Tg2657 mice. Previously, we showed that CD40 ligation shifts microglial response to Aβ from an anti-inflammatory phagocytic phenotype to a pro-inflammatory APC response (Town et al.,
2002; Townsend et al., 2005). In accord, here we observed downregulation of microglial APC morphology in situ (Figs. 12A – B), and reduction in CD45 and MHC II-positive microglia from Aβ1-42 vaccinated PSAPP mice treated with CD40L neutralizing antibody (Figure 12C). Indeed, reduction in MHC II expression, a measure of microglial APC phenotype, correlated with elevated brain levels of anti-inflammatory Th2 cytokines TGF-β1 and IL-10 (Figure 12D). Although NK cell-mediated activation of microglia can bypass MHC and T cell receptors to produce a vaccine immune response, infiltration of NK cells into the CNS has not been reported in the Aβ vaccination paradigm, making this less likely.

Another possibility is that so-called “anti-ergotypic” responses, defined as an immune response to the vaccine-activated host immune cells, could play a role in enhanced efficacy of Aβ immunotherapy in conjunction with CD40 blockade. Short-term anti-ergotypic lines isolated from vaccinated Multiple Sclerosis (MS) patients demonstrate a mixed phenotype (both CD4+ and CD8+ cells). These cells secrete IFN-γ and TNF-α, but not TGF-β1 (Correale et al., 1997; Hellings et al., 2004). Although we can not fully rule out this mechanism in our studies of CD40 blockade enhancement of reduced cerebral amyloidosis after Aβ vaccination, it is interesting to note that our results in the CNS and in splenocytes show the converse: a reduction in IFN-γ and TNF-α, but an increase in TGF-β (Figure 12D and Figure 13A), suggesting the involvement of a different mechanism.

Given our previous observation CD40 ligation induces “switching” of the microglial phenotype from a pro-phagocytic state endorsing Aβ phagocytosis to a pro-
inflammatory APC state (Townsend et al., 2005), we propose that combined CD40 blockade and Aβ vaccination promotes microglial phagocytosis/clearance of Aβ from the CNS. Additionally, T-cells derived from neutralizing CD40L antibody-treated Aβ1-42 vaccinated PSAPP were dramatically less neurotoxic to Aβ producing neurons ex vivo (Figure 13B), suggesting further benefit afforded by combining these therapeutic approaches. However, it is worth noting that this latter result needs to be interpreted with the caveat that standard active Aβ vaccination of AD mouse models (unless modified with the addition of pertussis toxin) (Furlan et al., 2003) does not produce appreciable brain infiltrates of auto-aggressive T-cells as was observed in the active Aβ AN-1792 vaccine in AD patients (Nicoll et al., 2003; Town et al., 2005).

CD40-CD154 interaction is essential for initiating the adaptive immune response, as demonstrated by immune deficits observed in patients with mutations in the CD40 or CD40L genes. These patients develop type 1 hyper IgM immunodeficiency syndrome (HIGM1) that is characterized by recurrent bacterial and opportunistic infections (Durandy 2001; Fuleihan 2001; Levy et al., 1997a). Patients with homozygous mutations in the CD40 gene have a severe immunodeficiency termed HIGM3. Its clinical phenotype overlaps with that of HIGM1 and is characterized by defective generation of secondary antibodies and severe opportunistic infections, (Ferrari et al., 2001; Kutukculer et al., 2003) particularly Cryptosporidium enteritis and Pneumocystis carinii pneumonia (Levy et al., 1997b). Thus, complete systemic blockade of CD40-CD40L would likely have deleterious immunosuppressive side effects. For this reason, pharmacotherapy would need to be titrated such that the CD40-CD154 pathway was partially inhibited.
Interestingly, as we have shown in our proof-of-concept paradigm in transgenic mice, even a 50% blockade of CD40 is enough to increase the efficacy of the Abeta vaccine on reduction of cerebral amyloidosis.

Our observations suggest that partial blockade of CD40 signaling, either by genetic or by pharmacologic means, increases the effectiveness of Aβ1-42 vaccination by further reducing cerebral amyloidosis and simultaneously promoting anti-inflammatory cellular immune processes in the brain and in the periphery. If the benefit afforded by CD40 pathway blockade to Aβ1-42 vaccinated AD mouse models can translate to the clinical syndrome, then pharmacotherapy aimed at reducing CD40 signaling in conjunction with Aβ vaccination may represent an approach that is both safer and more effective in humans. Future studies will be required to isolate CD40-CD40L downstream signaling involved in reduced efficacy of Aβ vaccination, as this may uncover additional targets for pharmacologic intervention.
3.6 References


Jankowsky, J. L., Slunt, H. H., Ratovitski, T., Jenkins, N. A., Copeland, N. G., Borchelt,


CHAPTER FOUR

TRANSCUTANEOUS Aβ PEPTIDE IMMUNIZATION OF TRANSGENIC ALZHEIMER'S MICE RESULTS IN REDUCED CEREBRAL AB DEPOSITS IN THE ABSENCE OF T-CELL INFILTRATION AND MICROHEMORRHAGE

4.1 Abstract

Alzheimer’s disease (AD) immunotherapy accomplished by vaccination with β-amyloid peptide (Aβ) has proved efficacious in AD mouse models. However, “active” Aβ vaccination strategies for the treatment of cerebral amyloidosis without concurrent induction of detrimental side effects are lacking. We have developed a novel transcutaneous (t.c.) Aβ vaccination approach and have evaluated efficacy and monitored for deleterious side effects, including meningoencephalitis and microhemorrhage, in wild-type mice and in a transgenic mouse model of AD. We demonstrate that t.c. immunization of wild-type mice with aggregated Aβ1-42 plus the adjuvant cholera toxin (CT) results in high-titer Aβ antibodies (mainly of the immunoglobulin G1 class) and Aβ1-42-specific splenocyte immune responses. Confocal microscopy of the t.c. immunization site revealed Langerhans cells in areas of the skin containing the Aβ1-42 immunogen, suggesting that these unique innate immune cells participate in Aβ1-42 antigen processing. To evaluate the efficacy of t.c. immunization in
reducing cerebral amyloidosis, transgenic PSAPP (APPsw, PSEN1dE9) mice were immunized with aggregated Aβ₁₋₄₂ peptide plus CT. Similar to wild-type mice, PSAPP mice showed high Aβ antibody titers. Most importantly, t.c. immunization with Aβ₁₋₄₂ plus CT resulted in significant decreases in cerebral Aβ₁₋₄₀,₄₂ levels coincident with increased circulating levels of Aβ₁₋₄₀,₄₂, suggesting brain-to-blood efflux of Aβ.

Reduction in cerebral amyloidosis was not associated with deleterious side-effects of brain T-cell infiltration or cerebral microhemorrhage. Together, these data suggest that t.c. immunization constitutes a novel, effective and potentially safe treatment strategy for AD.

4.2 Introduction

Alzheimer’s disease (AD) is the most common dementing illness and is pathologically characterized by the presence of intracellular neurofibrillary “tangles” and extracellular senile plaques primarily composed of 40-42 amino acid Aβ peptides (Selkoe 2001). In a seminal report, Schenk and colleagues showed that intraperitoneal vaccination of the PDAPP transgenic mouse model of AD with Aβ₁₋₄₂ plus Freund’s adjuvant resulted in dramatic reduction of cerebral amyloidosis (Schenk et al. 1999). This therapeutic approach is clearly highly efficacious; however, the safety of this strategy has become an important concern. In a recent clinical trial, patients were administered a synthetic Aβ peptide (AN-1792) plus adjuvant and approximately 6% of these patients developed aseptic meningoencephalitis, most likely mediated by brain-infiltrating activated T-cells (Hock et al. 2003; Bayer et al. 2005). This serious side-
effect led to suspension of the clinical trial. Furthermore, passive transfer of Aβ antibodies to transgenic AD mice results in cerebral microhemorrhage, a potentially adverse side-effect (Wilcock et al. 2004; Racke et al. 2005). Uncovering of these adverse events has re-directed Aβ vaccination strategies towards the goal of developing an approach that is both safe and effective.

Studies examining the brains of Aβ vaccinated patients developing meningoencephalitis implicate Aβ reactive T-cell subsets as major components of this deleterious response to active Aβ vaccination (Nicoll et al. 2003; Ferrer et al. 2004). To subvert possible meningoencephalitis resulting from Aβ vaccination, various strategies have been attempted. Interestingly, recent works suggest that Aβ-derived peptides delivered intranasally to mucosal epithelial tissues (with adjuvant) results in effective clearance of Aβ plaques and improvement of cognitive function in animal models of AD. Moreover, T-cell reactivity appeared to be considerably reduced compared with other active immunization strategies (Maier et al. 2005; Maier et al. 2006). In other studies, differential T-cell responses were observed dependent upon the epitope/fragment of Aβ peptide utilized for vaccination. Specifically, portions of the Aβ peptide seemed to stimulate different T-cell responses, resulting in either pro-inflammatory T-helper cell type 1 (Th1) responses, or anti-inflammatory T-helper cell type 2 (Th2) responses. Such findings imply that Aβ vaccination is not only efficacious, but may also prove to be safe and therefore a feasible strategy for AD therapy depending upon a number of factors including route of delivery, adjuvant choice, and Aβ epitope administered.

The skin is a well-established effective route for vaccination, including delivery
of peptide-based vaccines (Beignon et al. 2005; Itoh; Celis 2005; Dell et al. 2006). Strong humoral and cellular immune responses have been elicited after transcutaneous (t.c.) vaccination (Giudice; Campbell 2006), largely owing to the diverse populations of resident antigen presenting cells (APCs) and other immune cells in the various dermal layers. Subsets of dermal-resident Langerhans cell (LC) precursors, known as migratory CD14+ LC precursors, are important immune regulators that demonstrate “professional” APC capability including reducing T-cell stimulatory function by producing anti-inflammatory cytokines (Larregina et al. 2001). Also, skin-resident keratinocytes release the anti-inflammatory cytokine interleukin (IL)-10 in response to certain stimuli. Keratinocyte-derived IL-10 serves to buffer harmful pro-inflammatory immune activation and thereby preserves skin barrier integrity (Niizeki; Streilein 1997).

Taken together, these lines of evidence led us to hypothesize that targeting Aβ immunotherapy to skin tissue may provide an immunotherapeutic approach that is both efficacious and safe. To evaluate this hypothesis, we tested a t.c. Aβ immunization strategy using both wild-type and the transgenic PSAPP (APPsw, PSEN1ΔE9) mouse model of AD (Jankowsky et al. 2001). We found that t.c. immunization of non-transgenic C57BL/6 mice with aggregated Aβ1-42 peptide plus the adjuvant cholera toxin (CT) resulted in high Aβ antibody titers [mainly immunoglobulin (Ig) G1], and Aβ1-42-specific splenocyte immune responses after re-challenge with the peptide. Confocal microscopy of the t.c. immunization site revealed CD207 and CD11c double-positive Langerhans cells in areas of the skin containing the Aβ1-42 immunogen, suggesting that these unique innate immune cells participate in Aβ1-42 antigen processing. Further,
transgenic PSAPP mice t.c. immunized with aggregated Aβ_{1-42} peptide plus CT manifested effective immune responses against Aβ in concert with reduced cerebral Aβ pathology, demonstrating the effectiveness of this approach. These mice showed high Aβ antibody titers and increased circulating Aβ levels, suggesting brain-to-blood efflux of Aβ. Importantly, brain T-cell infiltration and cerebral microhemorrhage were not observed after t.c. immunization, indicating that this immunization strategy is potentially safe.

4.3 Materials and methods

4.3.1 Reagents.

Lyophilized cholera toxin (CT), CT antibody, concanavalin (Con A) and mouse CD3 antibody were obtained from Sigma (St Louis, MO). Aβ_{1-42} peptide was purchased from U.S. peptides (Rancho Cucamonga, CA). As previously described (Schenk et al. 1999), Aβ_{1-42} peptide was added to 0.9% saline (4 mg/mL), vortexed, and incubated for 24 h at 37º C. This solution was aliquoted, frozen and stored at −80º C. Immediately prior to use, Aβ aliquots were thawed and then mixed with CT (reconstituted with distilled water to 0.5 mg/mL). Antibody 4G8 directed against Aβ (amino acids 17-26) was purchased from Chemicon/Millipore (Billerica, MA). Enzyme-linked immunoabsorbance assay (ELISA) kits for detection of IFN-γ, IL-2, and IL-4 were obtained from R&D Systems (Minneapolis, MN). Aβ_{1-40} and Aβ_{1-42} ELISA kits were purchased from IBL-American (Minneapolis, MN). Murine IgG and HRP-conjugated
goat anti-mouse IgG were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Biotinylated anti-mouse IgG1, IgG2a and IgG2b were purchased from Zymed® Laboratories (South San Francisco, CA). Alexa-Fluor™-conjugated secondary antibodies (including Alexa-Fluor™, 488, 594, and 647) were purchased from Invitrogen (Carlsbad, CA). Lactate dehydrogenase (LDH) kit was obtained from Promega (Madison, WI).

4.3.2 Animals

Wild-type C57BL/6 and PSAPP (APPsw, PSEN1dE9) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were housed and maintained in the College of Medicine Animal Facility at the University of South Florida, and all experiments were in compliance with protocols approved by the University of South Florida Institutional Animal Care and Use Committee. Brain sections from mice induced with experimental autoimmune encephalomyelitis were provided by Dr. Terrence Town and used as a positive control for CD3 staining. Brain sections for positive microhemorrhage staining from mice intraperitoneally passively given Aβ antibodies were provided by Dr. Dave Morgan (Wilcock et al. 2006).

4.3.3 Transcutaneous (t.c.) immunization of mice

To test whether t.c. delivery of Aβ peptide could stimulate immune responses, we first t.c. immunized wild-type C57BL/6 mice. These mice (n = 10, 5 male/5 female) were t.c. immunized with human Aβ1-42 peptide (200 μg/mouse) and CT (10 μg/mouse) or CT alone (10 μg/mouse) in 100 μL of 0.9% saline on a weekly basis for the first
Thereafter, these mice were continually t.c. immunized with Aβ1-42 (100 µg/mouse) and CT (5 µg/mL) or CT alone (5 µg/mouse) in 100 µL of 0.9% saline bi-weekly for the following 12 weeks. Transcutaneous immunization was performed according to a method described by Skelding and colleagues (Skelding et al. 2006) with minor changes. To ensure mice immobility for the duration of administration for each immunization, mice were anaesthetized. A small lower back section (1-2 cm²) was shaved with an extra precaution not to damage the skin. The skin was then swabbed with acetone to remove surface oils and enhance penetration, allowed to air dry, and then re-hydrated by swabbing with 0.9% saline. The shaved edge was coated with a thin petroleum jelly layer to prevent unnecessary leakage of the immunization solution. Lastly, 100 µL of Aβ1-42 in combination with CT or CT alone in 0.9% saline was placed on the shaved region and allowed to be absorbed for 2 h. At the end, the skin was washed with 0.9% saline and dried, so as to remove any remaining immunization solution. Mice were cleaned thereafter and returned to their cages. Blood was withdrawn on weeks 0 (immediately prior to the first immunization), 4, 8 and 16 (immediately prior to the sacrifice of these mice). We then t.c. immunized PSAPP mice (n = 9, 4 male/5 female) at 4 months of age using the same procedure described above. No mortality was observed over the course of the t.c. immunization and none of the animals exhibited symptoms causing their removal from the study.

4.3.4 Splenocyte cultures.

Cell suspensions of splenocytes from individual mice were prepared as
previously described (Town et al. 2002) and passed in 0.5 mL aliquots into 24-well plates at \(3 \times 10^6/\text{mL}\). These cells were treated for 48 h with ConA (5 \(\mu\text{g/mL}\)), anti-CD3 (1 \(\mu\text{g/mL}\)) or \(\text{A}\beta_1-42\) (20 \(\mu\text{g/mL}\)). Supernatants were then collected and assayed by cytokine ELISA kits in strict accordance with the manufacturer's instruction (R&D Systems). The Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) was performed to measure total cellular protein from each of the cell groups under consideration just prior to quantification of cytokine release by ELISA, and cytokine secretion is expressed in pg/mg total cellular protein (mean ± SD). To verify whether stimulation of splenocytes produced any between-groups differences on cell death that might account for altered cytokine profiles, LDH release assay was carried out as described (Tan et al. 2000) and LDH was not detected in any of the wells studied.

### 4.3.5 Immunofluorescence staining.

Non-transgenic C57BL/6 mice were transcutaneously treated with \(\text{A}\beta/\text{CT}\), CT alone, or PBS for 18 h, as described above. The dorsal skin was removed by careful razor slicing around pre-labeled regions (1.5 cm in diameter) where the vaccine was applied. Skin or brain samples were routinely prepared for immunofluorescence staining. The staining was carried out using the following primary antibodies: anti-mouse CD207 (Langerin; 1:250; eBioscience San Diego, CA), anti-mouse CD11c (1:50; Pierce Biotechnology, Rockford, IL), and/or anti-human \(\text{A}\beta\) antibody (clone 4G8; 1:500; Chemicon/Millipore, Billerica, MA) or rat anti-mouse CD3 antibodies (1:200; eBioscience) overnight at 4°C, followed by appropriate secondary antibodies conjugated
with AlexaFluor®488, 594, and/or 647 (1:500; Invitrogen) for 45 min. Sections were then washed 3 times in PBS, and mounted with fluorescence mounting media containing DAPI (Vector Laboratories, Inc., CA) to counter-stain cell nuclei, and then viewed under an Olympus BX-51 microscope or visualized in independent channels using a Zeiss LSM510 META confocal microscope equipped with a 2-photon laser that was used for exciting DAPI.

4.3.6 Aβ antibody ELISA.

Aβ antibodies in mouse plasma and brain homogenates were measured according to previously described methods (Maier et al. 2005). Briefly, human Aβ1-40 peptide was coated at 2 µg/mL in 50 mM carbonate buffer, pH 9.6 (coating buffer) in 96-well immunoassay plates overnight at 4°C. The plates were washed with 0.05% Tween 20 in PBS (washing buffer) five times and blocked with blocking buffer (PBS with 1% BSA and 5% horse serum) for 2 h at room temperature. Murine IgG was serially diluted in coating buffer (1,000 - 0 ng/mL) to generate a standard curve. Mouse plasma and brain homogenate samples were diluted in blocking buffer at concentrations ranging from 1:400 to 1:102,400, added to the plates, and incubated for 2 h at room temperature. After 5 washes with washing buffer, a detection antibody (HRP-conjugated goat anti-mouse IgG, 1 mg/mL) was diluted (1:4,000), added to the plates and incubated for 1 h at 37°C. Following 8 washes, tetramethylbenzidine (TMB) substrate was added to the plates and incubated for 15 min at room temperature. Fifty µL of stop solution (2 N NaN₂SO₄) was added to each well to terminate the reaction. The optical density of each well was
immediately determined by a microplate reader at 450 nm. Aβ antibodies were represented as ng per mL of plasma (mean ± SD).

ELISAs for Aβ antibody isotypes were carried out as previously described (Town et al. 2001; Lemere et al. 2002). Ninety-six well plates were coated with Aβ1-40 peptide (2 µg/mL) in coating buffer and incubated for 2 h at 37° C. Following three washes in washing buffer, wells were blocked with blocking buffer for 2 h at room temperature. Mouse plasma was serially diluted in blocking buffer, added to each well, and incubated for 1 h at 37° C. After five washes, the plates were incubated in secondary antibodies (biotinylated anti-mouse IgG1, IgG2a or IgG2b) diluted at 1:1,000 in blocking buffer for 1 h, followed by 30 min in streptavidin-HRP (1:200 in blocking buffer) at room temperature. TMB substrate was added to the plates and incubated for 15 min at room temperature. Fifty µL of stop solution was added to each well to terminate the reaction. The optical density of each well was immediately determined by a microplate reader at 450 nm. The ratios of IgG1 to IgG2a or IgG1 to IgG2b were calculated for each time point from each mouse individually using optical density values and then average ratio for each group (mean ± SD).

4.3.7 Aβ ELISA.

Mouse brains were isolated under sterile conditions on ice and placed in ice-cold lysis buffer as previously described (Rezai-Zadeh et al. 2005). Brains were then sonicated on ice for approximately 3 min, allowed to stand for 15 min at 4°C, and centrifuged at 15,000 rpm for 15 min. This fraction represented the detergent-soluble
fraction. Aβ_{1-40,42} species were further subjected to acid extraction of brain homogenates in 5 M guanidine buffer (Johnson-Wood et al. 1997), followed by a 1:10 dilution in lysis buffer. Soluble Aβ_{1-40,42} were directly detected in plasma and brain homogenates prepared with lysis buffer described above at a 1:4 or 1:10 dilution, respectively. Aβ_{1-40,42} was quantified in these samples using the Aβ_{1-40,42} ELISA kits (IBL-America, Minneapolis, Minnesota) in accordance with the manufacturer’s instructions, except that standards included 0.5 M guanidine buffer in some cases. Aβ_{1-40,42} were represented as pg per mL of plasma and pg per mg of total protein (mean ± SD).

### 4.3.8 Immunohistochemistry and image analysis.

As previously described (Tan et al. 2002), five coronal sections from each brain (5 μm thickness) were cut with a 150 μm interval. Sections were routinely deparaffinized and hydrated in a graded series of ethanol before preblocking for 30 min at ambient temperature with serum-free protein block (Dako Cytomation, Carpinteria, CA). Aβ immunohistochemical staining was performed using anti-human amyloid-β antibody (clone 4G8; 1:100) in conjunction with the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, CA) coupled with diaminobenzidine substrate. 4G8-positive Aβ deposits were examined under bright field using an Olympus (Tokyo, Japan) BX-51 microscope. For congo red histochemistry, sections were routinely deparaffinized and rinsed in 70% (v/v) ethanol before staining with fresh-filtered 1% (w/v) congo red diluted in 70% ethanol for 5 min. These sections were rinsed three times for 5 min each in 70% ethanol, hydrated for 5 min in 0.9% saline, and mounted in Vectashield fluorescence
mounting media (Vector Laboratories). Congo red-positive β-amyloid plaques were visualized using an Olympus BX-51 microscope. Quantitative image analysis was performed for both immunohistochemistry and Congo red-stained tissue sections with an Oncor V150 image analysis system. The software uses hue, saturation, and intensity (HSI) to segment objects in the image field. Operationally, thresholds for object segmentation are established by using a series of standard slides that have extremes of intensity for the stain being measured. Thresholds in HSI space are established that accurately identify objects on all standard slides, and these segmentation thresholds remain constant throughout the analysis session. We do not adjust thresholds for each section to avoid the introduction of experimenter bias into the estimation. Section-to-section variability in immunostaining is minor, owing to rigid fixation and staining protocols. In most experiments using the same reaction product (e.g., DAB-peroxidase), the same thresholds settings can be used for different antibodies. After establishing the threshold parameters, the image field is digitized with a frame grabber. The computer software then corrects for heterogeneity in background illumination (blank field correction) and calculates the measurement parameters for the entire field.

For quantitative image analysis of immunohistochemistry, we combined unbiased sampling of sections with the videodensitometric procedures we have used extensively in the past (Wilcock et al., 2004). All procedures were performed by an individual blind to the experimental condition of each specimen. Sample numbers were randomized before the start of the tissue processing, and code was broken only after completion of the analysis. We sampled every fifth section from the mouse brain, starting with a section between 1 and 5, identified by a random number generator. Frontal cortical
measurements were performed on laminae II-VI of the most anterior portion of the cortex. Hippocampal measurements were performed by circumscribing the entire hippocampus or selected hippocampal subfields. Using the standard nomenclature, "Aβ load" is defined as percent of area in the measurement field occupied by reaction product. Similarly, "Congo red staining" (or "amyloid load") refers to the percent of the area that is stained with Congo red. All values from a single mouse were averaged to represent the single value for that animal in statistical analyses. Statistical analyses were performed by using ANOVA followed by Fischer's LSD post hoc means comparison test (Statview software from SAS). The numbers of animals, not numbers of sections, have been used for statistical comparisons.

4.3.9 Perl’s Prussian blue reaction for ferric ion-hemosiderin.

Sections were deparaffinized, hydrated through descending grades of ethanol, washed in distilled water, and incubated for 20 min in a solution containing 20% hydrochloric acid and 10% potassium ferrocyanide (VWR). These sections were washed 3 X for 5 min with H₂O and counterstained with hematoxylin solution (Sigma) for 15 seconds and then mounted (Wilcock et al. 2006).

4.3.10 Statistical analysis.

Means and standard deviations were calculated according to standard practice. In instances of multiple comparisons of the means, one-way analysis of variance (ANOVA) was carried out with post-hoc comparison by Bonferroni’s method. In instances of single mean comparisons, a t-test for independent samples was used to assess significance. P-
values less than 0.05 were considered to be statistically significant. Data were analysed using the Statistical Package for the Social Sciences (SPSS), release 13.0 (SPSS Inc., Chicago, IL).

4.4 Results

4.4.1 Transcutaneous immunization of mice with Aβ1-42 peptide plus cholera toxin results in high Aβ antibody titers.

We first sought to determine whether t.c. administration of Aβ1-42 to mice could result in Aβ antibody production. Twenty non-transgenic C57BL/6 mice at eight weeks of age were used for this experiment, and ten mice received aggregated Aβ1-42 peptide with CT (Aβ/CT), while the remaining ten received CT alone. Mice were t.c. immunized over a 16-week time course; weekly for the first 4 weeks and bi-weekly for the following 12 weeks. To characterize the kinetics of the humoral immune response following immunization, blood samples were taken at week 0 (baseline, immediately prior to the first immunization), 4, 8 and 16 (immediately prior to the sacrifice of these mice). Plasma Aβ antibody titers were measured by ELISA. Aβ antibody isotypes were determined by an IgG isotyping assay using an isotype-specific secondary antibody (Town et al. 2001). Aβ antibodies were first detected at week 4 in all immunized mice and dramatically increased thereafter [(Figure 14.4) P <0.001]. Consistent with our previous studies that utilized an intraperitoneal route of administration of Aβ plus Fruend’s adjuvant (Town et al. 2002), Aβ antibodies of the IgG1 isotype were produced.
at the highest level, while IgG2a antibodies directed against Aβ were present in significantly lesser quantity ($P < 0.001$). Aβ IgG2b antibody was least detectable (Figure 14B, C). Transcutaneous immunization was further evaluated by assaying CT antibody titers in plasma from these mice at weeks 0, 4, 8 and 16 following immunization. A similar pattern of results was observed, albeit CT antibody titers were higher in plasma from mice t.c. immunized with either Aβ/CT compared with CT alone (data not shown). As an additional control group, we injected non-transgenic C57BL/6 mice with PBS alone ($n = 10$) in parallel, and we were unable to detect Aβ antibody titers in these animals, confirming the specificity of our titer assay (data not shown).
Figure 14. Generation of immune responses in wild-type C57BL/6 mice t.c. immunized with aggregated Aβ1-42 peptide plus CT. (A) Aβ antibody titers were measured by ELISA. Data are presented as mean ± SD (n = 10) of Aβ antibodies (ng/mL plasma). One-way ANOVA followed by post hoc comparison revealed significant differences in anti-Aβ titers when comparing week 4 to weeks 8, 12, or 16 (**P < 0.001). IgG isotypes were determined by an Ig isotyping assay and represented as ratios (mean ± SD; n = 10) of IgG1 to IgG2a (B) or IgG1 to IgG2b (C). One-way ANOVA followed by post hoc comparison revealed significant differences between the ratio of IgG1 and IgG2a versus IgG1 and IgG2b at each week shown (**P < 0.001). (D) Splenocytes were individually isolated and cultured from wild-type mice t.c. immunized with Aβ1-42/CT, CT alone, or PBS (control). These cells were stimulated with Con A (5 µg/mL) or Aβ1-42 (20 µg/mL) for 48 h. Cultured supernatants were collected from these cells for IFN-γ, IL-2 and IL-4 cytokine analyses by ELISA. Data are presented as relative fold mean ± SD (n = 10) of each cytokine over PBS control. One-way ANOVA followed by post hoc comparison revealed significant differences between groups for levels of each of three cytokines [IFN-γ, IL-2 and IL-4 (**P < 0.001)] following in vitro Aβ1-42 challenge. As noted, there was also a significant difference in cytokine levels between IL-4 and either IFN-γ or IL-2 following Aβ1-42 challenge (##P < 0.001). (E) To characterize the dermal immune responses to Aβ/CT t.c. immunization, skin tissues were prepared from non-transgenic C57BL/6 mice t.c. immunized for 18 h with PBS (control, top panels), CT alone (middle panels) or with Aβ/CT (bottom panels) as indicated and then analyzed by laser scanning confocal microscopy with the indicated antibodies (antibody 4G8 was used to reveal Aβ).
Note the presence of CD207+CD11c+ LCs in Aβ-positive regions in the Aβ/CT t.c. immunized group.
Figure 15. Aβ/CT t.c. immunization resulted in LC recruitment into dermal layers. To characterize the dermal immune responses to Aβ/CT t.c. immunization, skin tissues were prepared from nontransgenic C57BL/6 mice treated with Aβ/CT (Left), CT alone (Center), or PBS (Right) as indicated for 18 h and then analyzed. Skin frozen sections were stained with anti-mouse CD207 antibody/FITC-labeled anti-rat IgG2a, rabbit anti-human Aβ antibody/Alexa Fluor-555-labeled anti-rabbit IgG, and DAPI. As noted, blue staining indicates all nucleated cells, and green indicates CD207 positive cells (presumed LC, ×40).
4.4.2 Aβ-specific immune responses in splenocytes from mice transcutaneously immunized with Aβ plus cholera toxin.

To further investigate splenocyte responses to Aβ, primary cultures of splenocytes were established from individual mice (non-transgenic C57BL/6) t.c. immunized with Aβ/CT, CT alone, or PBS only (control) and immune responses were compared. We quantified key cytokines produced by activated T-cells [interferon-γ (IFN-γ), interleukin (IL)-2, IL-4] in splenocyte supernatants by ELISA as an indicator of immune responsiveness. Non-specific mitogenic stimulation of cultured splenocytes with concanavalin A (Con A) resulted in over two-fold increases in IFN-γ, IL-2, and IL-4 production in cells from mice immunized with Aβ/CT or CT versus PBS-immunized controls (Figure 14D). No statistically significant difference was noted between Aβ/CT and CT alone groups for each cytokine ($P > 0.05$). Similar results were observed in cultured splenocytes stimulated with anti-CD3 (data not shown). On the other hand, specific recall stimulation with Aβ$_{1-42}$ peptide of primary cultured splenocytes from Aβ/CT-t.c. immunized mice resulted in significantly increased production of IFN-γ, IL-2 and IL-4 compared to splenocytes cultured from mice immunized with CT alone ($P < 0.001$, Figure 14D). Importantly, regarding the anti-inflammatory cytokine IL-4, an 8-fold increase in its secretion by splenocytes from Aβ/CT-immunized mice following Aβ$_{1-42}$ recall stimulation was observed ($P < 0.001$) (Figure 14D). Taken together with the predominantly IgG1 Aβ-specific humoral response in Aβ/CT-t.c. immunized wild-type mice, this IL-4 result suggests an anti-inflammatory Th2-type immune response.
4.4.3 Transcutaneous immunization with Aβ plus CT promotes recruitment of dermal Langerhans cells.

Given that LCs often play a key role in skin immune responses, we asked whether LCs might be recruited to the site of t.c. Aβ/CT immunization. Skin tissues and frozen sections were prepared from non-transgenic C57BL/6 mice 18 h after t.c. vaccination with Aβ/CT or CT alone, and they were co-stained with antibodies against mouse CD207 antibody (Langerin, a pan-LC marker), mouse CD11c (a marker of an LC subset (Douillard et al. 2005) and/or rabbit anti-human Aβ. Aβ/CT t.c. immunization resulted in LC recruitment into dermal layers compared with CT alone or PBS-immunized controls (Figure 14E and Figure 15), where dermal LCs were much less frequently observed. Furthermore, these LCs tended to be found in regions of the skin that stained positive for Aβ peptide by 4G8 Aβ antibody (Figure 14E). These data show the migratory action of LCs in response to Aβ/CT t.c. stimulation and suggest that this effect is important in mediating the initial immune response to Aβ/CT t.c. immunization.

4.4.4 Transcutaneous immunization of PSAPP mice with Aβ plus CT results in Aβ-specific immune response and increased circulating Aβ.

Having shown high Aβ antibody titers and cultured splenocyte immune responses to Aβ in Aβ/CT t.c. immunized non-transgenic C57BL/6 mice, we then asked whether this immunization strategy would reduce cerebral amyloidosis in a mouse model of AD. Eighteen double-transgenic (APPswe/PSEN1dE9) PSAPP mice, which overproduce human Aβ and develop significant amyloid deposits by 8 months of age (Jankowsky et
al. 2001), were immunized at four months of age in this study. Half of them (n = 9) received aggregated Aβ1-42 peptide with CT, while the remaining half received CT alone. The 16-week procedure that we employed was identical to that used above for non-transgenic C57BL/6 mice. Blood samples were taken at weeks 0, 4, 8 and 16 following immunization. Plasma Aβ antibody titers from these mice were measured by ELISA. Significant increases in Aβ antibody titers were observed in PSAPP mice t.c. immunized with Aβ/CT (P < 0.001) (Figure 16A). Similar to non-transgenic C57BL/6 mice, Aβ antibodies were first detected at week four in plasma from Aβ/CT-immunized PSAPP mice, and dramatically increased thereafter. By contrast, these Aβ antibodies were not detected in plasma from CT-vaccinated control mice (Figure 16A). Two weeks after the final immunization, primary splenocytes were isolated and cultured from individual mice. Recall stimulation of splenocytes from Aβ/CT-t.c. immunized PSAPP mice with Aβ1-42 peptide resulted in significantly increased production of IFN-γ and IL-2, and particularly IL-4 (data not shown), similar to results from Aβ/CT-t.c. immunized non-transgenic C57BL/6 mice.
**Figure 16.** Increased systemic Aβ after Aβ$_{1-42}$/CT t.c. immunization of PSAPP mice.

For Aβ analysis, blood samples were individually collected from Aβ/CT or CT alone t.c.-immunized PSAPP mice at the time points indicated. (A) Plasma Aβ antibody titers were measured by ELISA. Data are presented as mean ± SD (n = 9) of Aβ antibodies (pg/mL plasma). ANOVA followed by *post hoc* comparison revealed significant differences between Aβ/CT and CT t.c.-immunized PSAPP mice for plasma Aβ antibody levels at each time point as indicated (***P <0.001). Moreover, this analysis revealed significant differences between time points within the Aβ/CT t.c. immunized group as indicated (##P <0.001). (B and C) Plasma Aβ$_{1-40,42}$ peptides were measured separately by Aβ ELISA. Data are presented as mean ± SD (n = 9) of Aβ$_{1-40}$ or Aβ$_{1-42}$ (pg/mL plasma). One-way ANOVA followed by *post hoc* comparison revealed significant differences between Aβ/CT and CT alone t.c.-immunized PSAPP mice for plasma Aβ$_{1-40,42}$ levels at each time point as indicated (*P <0.05; **P <0.001). Arrows below the panels show each t.c. immunization with respect to time of blood sample collection.
It has been hypothesized that passively-administered Aβ antibodies create a “peripheral sink”; in essence, shifting the equilibrium of Aβ levels from the brain to the blood (DeMattos et al. 2001; Matsuoka et al. 2003). To determine whether this phenomenon was occurring in the Aβ/CT-t.c. immunization paradigm, we quantified Aβ levels in the blood by ELISA. In support of this hypothesis, we found significantly increased circulating Aβ1–40, 42 in PSAPP mice t.c. immunized with Aβ/CT as early as 4 weeks after immunization (Figure 16B and C). Importantly, plasma Aβ1–40, 42 levels increased rapidly to the highest values of 781 ± 118 pg/mL and 129 ± 46 pg/mL, respectively, by week 8 (two weeks after the third booster t.c. immunization). Thereafter, plasma Aβ levels remained relatively constant through to the time of sacrifice following 16 weeks of immunization.

4.4.5 PSAPP mice transcutaneously immunized with Aβ plus cholera toxin show reduced cerebral amyloidosis in the absence of T-cell infiltrates or cerebral microhemorrhage.

Having shown significantly increased circulating Aβ levels in PSAPP mice t.c. immunized with Aβ/CT, we sought to evaluate Aβ/β-amyloid pathology in these mice. Using a sandwich ELISA-based method, detergent-soluble Aβ1–40, 42 levels were reduced by approximately 53 and 48%, respectively ($P < 0.001$) (Figure 17A). Insoluble Aβ1–40, 42 (prepared by acid extraction of detergent-insoluble material in 5 M guanidine and subsequent ELISA) levels were reduced by 50 and 54%, respectively, in Aβ/CT t.c.-immunized PSAPP mice ($P < 0.001$) (Figure 17B). We further analyzed β-amyloid plaques in brains of mice that received t.c. immunization with Aβ/CT or CT alone by
4G8 immunohistochemistry and congo red histochemistry (Figure 17D and F, respectively). At 10 months of age, Aβ/CT-t.c. immunized PSAPP mice showed 42-58% (Figure 17E) and 61-65% (Figure 17G) reductions in 4G8 immunoreactive and congo red-positive Aβ deposits, respectively, across hippocampal and cortical brain regions examined. Together, these results demonstrate that t.c. immunization with Aβ/CT is effective in reducing cerebral amyloidosis in PSAPP mice.

As shown above, we found that peripheral levels of human Aβ1-40, 42 were increased in mice t.c. immunized with Aβ/CT. To determine this systemic increase in human Aβ1-40, 42 was associated with a reduction in cerebral Aβ levels, we performed correlation analysis and noted an inverse correlation between plasma and brain soluble Aβ (Figure 17C and Figure 18), suggesting that circulating Aβ antibodies play an important role in clearance of Aβ from brain to blood via the “peripheral sink” hypothesis (DeMattos et al. 2001; Matsuoka et al. 2003). This effect is likely not solely responsible for reduced cerebral amyloidosis, as, interestingly, we detect Aβ antibodies in brain homogenates from Aβ/CT-t.c. immunized PSAPP mice [18.87 ± 6.25 (mean ng/mg total protein ± SD)], while Aβ antibodies were undetectable in PSAPP mice t.c. immunized with CT-alone (data not shown). Given the presence of these Aβ antibodies in the brain, it is possible that additional Aβ clearance mechanisms (i.e., mediated by the Fc receptor on phagocytic microglia) are operating.
PSAPP mice following t.c. Aβ1-CT immunization

Increased Plasma Aβ levels

Aβ deposits (4G8 positive)

Insoluble Aβ

Soluble Aβ

Insoluble Aβ

Insoluble Aβ

Soluble Aβ

Soluble Aβ

Decreased brain Aβ levels

Aβ levels (% over control) mean ± SD

100  75  50  25  0  -25  -50  -75  -100
Figure 17. Reduction of cerebral Aβ/β-amyloid pathology in PSAPP mice t.c. immunized with Aβ_{1-42}/CT. Detergent-soluble Aβ_{1-40, 42} peptides (A) and insoluble Aβ_{1-40, 42} prepared from 5 M guanidine extraction (B) in brain homogenates were measured separately by ELISA. Data are presented as mean ± SD (n = 9) of Aβ_{1-40} or Aβ_{1-42} (pg/mg protein), and reductions for each group are indicated. (A and B), A t test revealed a significant between-group difference for either soluble or insoluble Aβ_{1-40, 42} (P < 0.001). (C) A significant inverse correlation (P < 0.001) between plasma and brain soluble Aβ levels was revealed. Plasma Aβ levels are presented as percentage mean ± SD (n = 9) of soluble circulating Aβ at 16 weeks following t.c. immunization of PSAPP mice with Aβ/CT over CT control mice. (D) Mouse brain coronal paraffin sections were stained with monoclonal anti-human Aβ antibody 4G8. Left, Aβ_{1-42}/CT t.c. immunized PSAPP mice; right, CT t.c. immunized PSAPP mice. The top panels are from the cingulate cortex (CC), the middle panels are from the hippocampus (H), and the bottom panels are from the entorhinal cortex (EC). (E) Percentages (plaque “burden”, area plaque/total area) of Aβ antibody-immunoreactive Aβ plaques (mean ± SD; n = 9) were calculated by quantitative image analysis, and reductions for each mouse brain area analyzed are indicated. (F) Mouse brain sections from the indicated regions were stained with congo red. Left, Aβ_{1-42}/CT t.c. immunized PSAPP mice; right, CT t.c. immunized PSAPP mice. (G) Percentages of congo red-stained plaques (mean ± SD; n = 9) were quantified by image analysis, and reductions for each brain region are indicated. (E and G) a t test for independent samples revealed significant differences (P < 0.001) between-groups for each brain region examined.
Figure 18
Figure 18. Simultaneous analysis of plasma and brain soluble Aβ levels on a mouse-by-mouse basis. Data are presented as percentage of circulating or brain soluble Aβ at 16 weeks after Aβ/CT t.c. immunization of individual PSAPP mice (n = 9) relative to average Aβ levels of control mice t.c. immunized with CT alone. Each color bar represents plasma Aβ levels and brain-soluble Aβ levels from each individual mouse as indicated.
In a recent clinical trial, patients were administered a synthetic Aβ peptide (AN-1792) plus adjuvant and approximately 6% of these patients developed aseptic meningoencephalitis, most likely mediated by brain-infiltrating activated T-cells (Hock et al. 2003; Bayer et al. 2005). This serious complication led to suspension of the clinical trial. We sought to investigate whether Aβ/CT t.c. immunization might induce T-cell infiltration into the brain. We immunostained brain sections from mice immunized with Aβ/CT, CT alone, or, as a positive control, non-transgenic C57BL/6 mice subcutaneously injected with myelin oligodendrocyte protein emulsified in complete Freund’s adjuvant (brains were isolated 20 days after immunization, when copious amounts of T-cells have infiltrated the brain). As shown in Figure 19A-C, we did not detect CD3-positive T-cells in brains from mice t.c. immunized with CT or Aβ/CT; however, this was not due to a technical issue as T-cells were detected in the positive control tissue (Figure 19A). Further, we also immunostained brain sections from the above mice with CD4 or CD8 antibodies (which stain different subsets of T-cells) and did not detect T-cell infiltration in brains of mice t.c. immunized with CT or Aβ/CT, while such cells were detected in our positive control tissue (data not shown).

It has been reported that mice receiving passive transfer of Aβ antibodies manifest cerebral microhemorrhage (Wilcock et al. 2004; Racke et al. 2005). To investigate this potentially adverse side-effect of Aβ/CT t.c. immunization, we carried out microhemorrhage analysis via Perl’s Prussian blue stain (which can detect even minute amounts of deposited iron derived from blood (Wilcock et al. 2004; Racke et al. 2005). We did not detect positive staining using this method in either t.c. immunized group, but
did observe staining in our positive control tissue [in this case, from mice intraperitoneally passively given Aβ antibodies (Wilcock et al. 2006)] (Figure 19D-F). As an additional indicator of possible blood-brain-barrier breakdown, we analyzed apolipoprotein B (present in blood but not normally in brain) levels in these brain tissues by Western blot and it was undetectable in the t.c. immunized groups (Figure 20). It is noteworthy that both the Prussian blue stain and apolipoprotein B analyses were negative in t.c. immunized PSAPP mice, suggesting that detection of Aβ antibodies in the brains of these mice (as mentioned above) was not due to poor perfusion efficiency or detectable breakdown of the blood-brain-barrier, but rather was likely due to “physiological” entry of Aβ antibodies into the brain parenchyma.
Figure 19
**Figure 19.** Absence of T-cell infiltration or brain microhemorrhage in Aβ/CT t.c. immunized mice. To characterize the safety of the vaccine, brain sections were stained for CD3 as an indicator of T-cell infiltration (top panels). Staining for hemosiderin was also performed to identify microhemorrhage (bottom panels) in mice immunized with Aβ/CT (B and E), or CT alone (C and F). These stained sections were compared to positive controls. For CD3 staining, the positive control consisted of CD3-positive brain sections from EAE mice (A). For microhemorrhage, experimental sections were compared to sections from mouse brains suffering microhemorrhage (D). Each panel is representative of staining repeated in triplicate for each brain section for either CD3 or hemosiderin. The brain region shown for each panel is the neocortex. Magnification for CD3 staining is 10 X while it is 20 X for microhemorrhage.
<table>
<thead>
<tr>
<th>kDa</th>
<th>-220</th>
<th>-129</th>
<th>-32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain tissue homogenates (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ/CT t.c. immunized PSAPP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution series of mouse plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B 100-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 20
Figure 20. Apolipoprotein B protein was undetectable in brain tissue homogenates derived from both Aβ/CT and CT t.c.-immunized mice. Brain homogenates were prepared from PSAPP mice t.c.-immunized with Aβ/CT or CT, and an aliquot corresponding to 100 µg of total protein was electrophoretically separated by using 4% SDS-polyacrylamide gels. In addition, a series of mouse plasma dilutions (100, 50, and 25 µg per lane) was loaded as positive controls. Western blot analysis by apolipoprotein B antibody (ab20737, Abcam, Inc, Cambridge, MA) shows apolipoprotein B100 in plasma but not in brain homogenates as indicated.
4.5 Discussion

To translate animal Aβ immunization approaches into successful clinical AD therapies, such strategies should not only be efficacious, but also be safe, including avoiding meningoencephalitic reactions to Aβ immunization previously observed in humans (Janus 2003; Monsonego et al. 2003). Experimental and postmortem evidence suggests that such aseptic meningoencephalitis observed in AD patients after Aβ vaccination resulted from CNS invasion by Aβ reactive T-cells (Monsonego et al. 2003; Maier et al. 2006). The requirement of Aβ-reactive T-cells for cerebral amyloid plaque clearance mediated by “active” Aβ vaccination strategies still remains unclear (Monsonego et al. 2003; Orgogozo et al. 2003; Ferrer et al. 2004). A previous study utilizing intranasal delivery of short Aβ-derived peptides lacking T-cell reactive epitopes with a specific immune-modulating adjuvant (mutant Escherichia coli heat-labile enterotoxin; LT R192G), demonstrated the possibility of potentiating an effective humoral anti-Aβ response while minimizing Aβ reactive T-cells (Maier et al. 2006), suggesting that Aβ-reactive T-cells are not necessary for an effective Aβ antibody response. If this proves to be true in humans, such an approach may represent reasonable therapeutic potential; however, it should be noted that anosmia/hyposmia may limit the usefulness of intranasal Aβ immunization. Further evidence that Aβ-reactive T-cells are likely not required for Aβ immunotherapy efficacy comes from “passive” immunization studies, which have shown that humoral responses alone may be sufficient to effectively reduce cerebral amyloid burden, and thereby mitigate neurodegeneration (Dickstein et al. 2006; Ma et al. 2006).
Here, we investigated the potential of t.c. Aβ immunization for the treatment of AD-like cerebral amyloidosis in transgenic mice. Transcutaneous immunization is an attractive route of delivery, as it is convenient, relatively painless, and minimally invasive. This strategy is also appealing because the epidermal and dermal immune systems provide a unique environment for immune stimulation due to LC antigen presentation (Rozis et al. 2005; Renn et al. 2006; Schiller et al. 2006; Strid et al. 2006). Indeed, following Aβ/CT t.c. immunization, we observed cells double-positive for CD207 and CD11c in dermal regions that stained positive for Aβ, showing that these LCs migrate to the t.c. immunization site and likely participate in antigen processing. The skin immune environment has likely evolved over millennia, owing to constant bombardment of the skin with various antigenic stimuli, resulting in a delicate balance between immunogenic and tolerogenic responses. It is noteworthy that transcutaneous/epicutaneous immunization has been successful in mitigating neurodegenerative disease in both induced and spontaneous forms of experimental autoimmune encephalomyelitis (EAE), mouse models of the demyelinating disease multiple sclerosis (Bynoe et al. 2003; Bynoe et al. 2005).

To determine the ability of Aβ t.c. immunization to effectively produce Aβ antibodies, we began our investigation in non-transgenic C57BL/6 mice, where we measured the kinetics of Aβ-specific antibody titers. Remarkably, the Aβ antibody response was observed as early as week four in all immunized mice and dramatically increased thereafter, remaining elevated through 16 weeks post-initial vaccination. Antibody isotype characterization demonstrated a predominantly IgG1
(IgG1>IgG2a>IgG2b) response in line with our previous report utilizing an intraperitoneal route of Aβ vaccination plus Freund’s adjuvant (Town et al. 2002). Production of IgG1 and IgG2b are typically due to anti-inflammatory Th2 cytokine signaling, whereas IgG2a typically results from pro-inflammatory Th1 signaling (Abbas et al. 1996). The Th2 cytokine profile is likely favorable for inducing antibody production and thus Aβ clearance without the overt pro-inflammatory (i.e., possibly contributing to auto-immune responses) Th1-type activation that typifies cellular immune responses (Romagnani 2000; Schwarz et al. 2001; Town et al. 2002). Accordingly, to circumvent meningoencephalitic reactions, many studies investigating vaccination methods for reducing cerebral amyloidosis in AD have attempted to bias Th cell responses towards Th2 profiles utilizing various strategies (Kim et al. 2005; Dasilva et al. 2006; Ghochikyan et al. 2006). The effectiveness and potential safety of these strategies seems promising, but further investigation is needed to confirm whether the link between Th cell responses and meningoencephalitis in AD patients is causitive.

Notwithstanding the need for these critical studies, Aβ immunization appears to modulate immune responses based on three major criteria: 1) tissue route of delivery, 2) antigen epitope utilized for immunization, and 3) properties of the co-administered adjuvant. Whether Th2 polarization in this study occurred due to route of delivery, CT adjuvant choice, or the genetic background of the C57BL/6 strain (Rosas et al. 2005; Fukushima et al. 2006) remains to be fully determined in future studies. It has been reported that CT promotes an anti-inflammatory Th2 immune response (Eriksson et al. 2003), and our data demonstrating IgG1 subtype antibodies produced in the greatest
proportion (compared to IgG2a or IgG2b antibodies) supports this notion. Of note, CT antibody titers were observed (data not shown), indicating an immunogenic response to this adjuvant. To confirm specific systemic versus local immune cell activation, we analyzed primary cultures of isolated splenocytes from t.c. immunized non-transgenic C57BL/6 mice and found that Aβ/CT t.c. immunization conferred Aβ-specific T-cell response as measured by secretion of cytokines IFN-γ, IL-2 and IL-4 upon aggregated Aβ1-42 peptide recall challenge. Importantly, there was a marked increase in IL-4 secretion compared to IFN-γ or IL-2, further suggesting Th2 immune responses after Aβ/CT t.c. immunization. This is in agreement with our previous study, where we found Th2-type cytokine responses both in vivo and ex vivo after intraperitoneal Aβ vaccination with Freund’s adjuvant (Town et al. 2002). Further, the Th2-type response that we observed following Aβ/CT t.c. immunization is important as anti-inflammatory Th2-type immune responses are likely preferred to pro-inflammatory Th1-responses in the Aβ vaccination paradigm, given that pro-inflammatory Th1 cells likely contributed to the aseptic meningoencephalitis in the human clinical trial of AN-1792 (Nicoll et al. 2003; Town et al. 2005). When taken together, these findings show that Aβ/CT t.c. immunization of non-transgenic C57BL/6 mice produces both Aβ-specific local LC immune response and systemic immune response characterized by high Aβ antibody titers that are sustained throughout the immunization protocol.

To determine the potential therapeutic efficacy of Aβ t.c. immunization, 6 month-old double-transgenic (APPsw, PSEN1dE9) PSAPP mice [which develop robust amyloid pathology at 8 months of age (Jankowsky et al. 2001)], were immunized against Aβ/CT
or CT alone for 16 weeks. Results showed consistently high and sustained Aβ antibody titers throughout the 16 week immunization period only in the Aβ/CT immunized group. Interestingly, the magnitude of Aβ antibody response in Aβ/CT t.c. immunized was only about half of that in wild-type mice (compare Figure 14A with Figure 15A), supporting the notion that transgenic mouse models of AD are hypo-responsive to Aβ vaccination, probably owing to overexpression of the human APP transgene throughout their lives (Monsonego et al. 2001). This humoral response correlated with high plasma levels of Aβ1-40, 42 peptides which peaked around eight weeks and remained relatively constant through to 16 weeks. Immunohistological and histochemical analyses of Aβ immunoreactive plaques and congophilic plaques, respectively, in cortical and hippocampal brain sections showed reductions by approximately 50% compared to CT t.c. immunized PSAPP mice, and a negative correlation existed between brain Aβ and blood Aβ levels following Aβ/CT t.c. immunization. Taken together, these results show that Aβ/CT t.c. immunization is effective at mitigating cerebral amyloidosis and suggest activation of Aβ brain-to-blood clearance. This “peripheral sink” mechanism has been reported by others following passive transfer of Aβ antibodies to AD transgenic mice (DeMattos et al. 2001; Matsuoka et al. 2003).

In order for AD immunotherapy approaches to be useful, they must not only be efficacious, but such approaches must also be safe and well-tolerated. Importantly, while we did observe peripheral Aβ-specific T-cell responses consistent with an anti-inflammatory Th2 response (characterized \textit{in vivo} by IgG1 Aβ antibody production and \textit{ex vivo} by IL-4 secretion after Aβ recall stimulation of splenocytes) after Aβ/CT t.c.
immunization, no signs of aseptic meningoencephalitis and/or cell-mediated immunity were observed in brains as evidenced by lack of CD3 positive T-cell infiltrates. However, we did observe evidence of humoral immunity in brain as demonstrated by Aβ antibody titers in brain homogenates, similar to data from previous reports using other modes of Aβ immunotherapy (Bard et al. 2000; Bacskai et al. 2001), suggesting transport of Aβ antibodies across the blood-brain-barrier. This observation was not due to poor PBS perfusion at the time of sacrifice, as Perl’s stain (which normally detects even trace amounts of iron which could be present due poor perfusion) results were consistently negative. Finally, other investigators have reported that passive transfer of Aβ antibodies to transgenic AD mice results in cerebral microhemorrhage (Wilcock et al. 2004; Racke et al. 2005). Importantly, Perl’s stain did not show this potentially adverse side-effect in mice t.c. immunized with Aβ/CT. Thus, when taken together, t.c. immunization holds potential as a novel, effective, and safe potential treatment strategy for AD.
4.6 References


Rozis, G., de Silva, S., Benlahrech, A., Papagatsias, T., Harris, J., Gotch, F., Dickson, G., Patterson, S., 2005. Langerhans cells are more efficiently transduced than dermal dendritic cells by adenovirus vectors expressing either group C or group B fibre protein: implications for mucosal vaccines. Eur J Immunol. 35, 2617-2626.


Strid, J., Callard, R., Strobel, S., 2006. Epicutaneous immunization converts subsequent and established antigen-specific T helper type 1 (Th1) to Th2-type responses. Immunology.


increased vascular amyloid and microhemorrhage. J Neuroinflammation. 1, 24.

Alzheimer’s disease is the most common progressive neurodegenerative disorder. Based on genetic, biochemical, and post-mortem evidence, Aβ peptides are key etiological contributors to AD pathogenesis (Selkoe, 2001). In addition to parenchymal Aβ deposits, deposition of Aβ in the cerebral vasculature (known as CAA) is another pathological feature of AD, and occurs with 83% frequency in AD patients (Ellis et al., 1996; Selkoe, 2001; Jellinger, 2002; Green et al., 2005). It is well known that there is a chronic activation of inflammatory pathways in AD brain, including the production of proinflammatory cytokines and acute-phase reactants in and around Aβ plaques. In addition, a number of other molecules important in the proinflammatory acute-phase response are also found at high levels in AD brain, including the activated glial product S100 beta, the protease α-1-antichymotripsin and α-2-macroglobulin, the prostaglandin – generating enzymes cyclooxygenases 1 and 2, and components of the classical protein complement cascade such as C1q and C3 (Akiyama et al., 2000; McGeer and McGeer, 2001). Thus, the neuroinflammatory response can be regarded as a central feature of AD brain.
5.1 Microglia and central nervous system

Microglia are innate immune cells of myeloid origin that take up residence in the central nervous system (CNS) during embryogenesis. While classically regarded as macrophage-like cells, it is becoming increasingly clear that reactive microglia play more diverse roles in the CNS. Microglial “activation” is often used to refer to a single phenotype; however, a continuum of microglial activation exists, with phagocytic response (innate activation) at one end and antigen presenting cell function (adaptive activation) at the other. Where activated microglia fall in this spectrum seems to be highly dependent on the type of stimulation provided.

Here, we suggest a model wherein microglial cells exist in at least two functionally discernable states once “activated”, namely a phagocytic phenotype (innate activation) or an antigen presenting phenotype (adaptive activation), as governed by their stimulatory environment. When challenged with certain PAMPs (particularly CpG-DNA), murine microglia seem to activate a “mixed” response characterized by enhanced phagocytosis and pro-inflammatory cytokine production as well as adaptive activation of T cells. In the EAE model, murine microglia seem to largely support adaptive activation of encephalitogenic T cells in the presence of the CD40-CD40 ligand interaction. In the context of Aβ challenge, CD40 ligation is able to shift activated microglia from innate to adaptive activation. Further, it seems that the cytokine milieu that microglia are exposed to biases these cells to innate activation (i.e., anti-inflammatory Th2-associated cytokines such as IL-4, IL-10, TGF-β1) or an adaptive form of activation (i.e., pro-inflammatory Th1-associated cytokines such as IFN-γ, IL-6, and TNF-α; summarized in Fig. 21).
Figure 21
Figure 21. Model for innate versus adaptive microglial activation responses. In the context of β-amyloid challenge, microglia activate a phagocytic response. If co-stimulated with CD40 ligand, a shift from innate activation to adaptive antigen-presenting cell response ensues. Additionally, certain anti-inflammatory Th2-type cytokines shift this balance back towards innate phagocytic response, while some pro-inflammatory Th1-associated cytokines tip the balance further towards adaptive activation of microglia. See the text and Table for references. Abbreviations used: APC, antigen presenting cell; CD40L, CD40 ligand; Th1, CD4+ T helper cell type I response; Th2, Th type II response; TGF, transforming growth factor; IL, interleukin; IFN, interferon, TNF, tumor necrosis factor.
Not all forms of microglial activation are deleterious, as activated microglia may serve a protective role as was shown in Aβ1-42-immunized mouse models of AD. It seems that enhanced microglial phagocytosis of β-amyloid plaques is at least partly responsible for the therapeutic benefit in these animals, so perhaps stimulation of innate microglial activation contributes to these reported benefits. In conclusion for microglial activation, if we can learn how to better harness microglia in order to produce specific forms of microglial activation, this could be key to turn a pathogenic cell into a therapeutic modality.

5.2 Immunotherapy for Alzheimer disease

In a seminal report, Schenk and colleagues showed that peripheral immunization of the PDAPP mouse model of AD with Aβ1-42 peptide resulted in high antibody titers, a small fraction of which (0.1%, (Bard et al., 2000) crossed the blood-brain-barrier and entered the brain parenchyma. Most importantly, these authors found that Aβ1-42 vaccination markedly diminished β-amyloid plaque burden (Schenk et al., 1999). These authors also found evidence of cells in the brains of the Aβ1-42 immunization animals that contained Aβ. Many of these cells stained for the activated microglia marker MHC II and phenotypically resembled activated microglia, suggesting that these cells were able to phagocytose Aβ deposits. In a follow-up report, Bard and colleagues supported this hypothesis by showing ex vivo that certain antibodies against Aβ peptides could trigger microglial phagocytosis and subsequent clearance of Aβ through the Fc receptor (Bard et al., 2000) (Bard et al., 2003). Clearance of brain β-amyloid deposits was beneficial, as
Aβ1-42-vaccinated mice had markedly reduced cognitive impairment as assayed by behavioral testing in AD mice (Janus et al., 2000) (Morgan et al., 2000). Thus, in mouse models of AD, innate (phagocytic) microglial activation mediated by the Fc receptor in the presence of antibody-opsonized Aβ appears beneficial rather than deleterious.

Based on the above-mentioned data, a human clinical trial was begun to peripherally administer a synthetic Aβ1-42 peptide (AN-1792) with an adjuvant to AD patients. Unfortunately, the trial was halted when a small percentage of patients developed aseptic T cell meningoencephalitis. This response most likely occurred because of an immune reaction to Aβ mediated by infiltrating T cells (Pfeifer et al., 2002). In the post-mortem brain of one patient who died as a consequence of this side-effect of treatment, there was significant clearance of Aβ plaques in parts of the neocortex and, in other areas where plaques remained, Aβ-immunoreactivity was associated with microglia (Nicoll et al., 2003). It is not yet clear whether this fulminate infiltration of T cells in AD patients who developed aseptic T cell meningoencephalitis was due to adaptive activation of microglia, but this is a distinct possibility given that microglia did seem to recognize antibody-opsonized Aβ (Nicoll et al., 2003) (Monsonego and Weiner, 2003). These results indicate the potentially damaging and overwhelming effects of a full-blown T cell autoimmune response, which does not normally occur in AD, and which may have been mediated by adaptively activated microglia. Thus, to translate animal Aβ immunization approaches into successful clinical AD therapies, such strategies should not only be efficacious, but also be safe, including avoiding meningoencephalitic reactions to Aβ immunization previously observed in humans.
et al., 2006). Taken together, these lines of evidence led us to hypothesize that targeting Aβ immunotherapy to skin tissue may provide an immunotherapeutic approach that is both efficacious and safe. Here, we first found that t.c. immunization of non-transgenic C57BL/6 mice with aggregated Aβ₁₋₄₂ peptide plus the adjuvant cholera toxin (CT) resulted in high Aβ antibody titers [mainly immunoglobulin (Ig) G1], and Aβ₁₋₄₂-specific splenocyte immune responses after re-challenge with the peptide. Similar response was observed in transgenic PSAPP mice and resulted in effective immune responses against Aβ in concert with reduced cerebral Aβ pathology, demonstrating the effectiveness of this approach. These mice showed high Aβ antibody titers and increased circulating Aβ levels, suggesting brain-to-blood efflux of Aβ. Importantly, brain T-cell infiltration and cerebral microhemorrhage were not observed after t.c. immunization, indicating that this immunization strategy is potentially safe. Aβ immunization appears to modulate immune responses based on three major criteria: 1) tissue route of delivery, 2) antigen epitope utilized for immunization, and 3) properties of the co-administered
adjuvant. Whether Th2 polarization in our study occurred due to route of delivery, CT adjuvant choice, or the genetic background of the C57BL/6 strain (Rosas et al., 2005; Fukushima et al., 2006) remains to be fully determined in future studies. It has been reported that CT promotes an anti-inflammatory Th2 immune response (Eriksson et al., 2003), and our data demonstrating IgG1 subtype antibodies produced in the greatest proportion (compared to IgG2a or IgG2b antibodies) supports this notion. Further we showed that splenocytes isolated from t.c. immunized mice and found Aβ-specific T-cell responses as measured by secretion of cytokines IFN-γ, IL-2 and IL-4 upon aggregated Aβ1-42 peptide recall challenge. Importantly, there was a marked increase in IL-4 secretion compared to IFN-γ or IL-2, further suggesting Th2 immune responses after Aβ/CT t.c. immunization. This is in agreement with our previous study, where we found Th2-type cytokine responses both in vivo and ex vivo after intraperitoneal Aβ vaccination with Freund’s adjuvant (Town et al., 2002). Further, the Th2-type response that we observed following Aβ/CT t.c. immunization is important as anti-inflammatory Th2-type immune responses are likely preferred to pro-inflammatory Th1-responses in the Aβ vaccination paradigm, given that pro-inflammatory Th1 cells likely contributed to the aseptic meningoencephalitis in the human clinical trial of AN-1792 (Nicoll et al., 2003; Town et al., 2005). Finally, other investigators have reported that passive transfer of Aβ antibodies to transgenic AD mice results in cerebral microhemorrhage (Arendash et al., 2001; Racke et al., 2005). Importantly, Perl’s stain did not show this potentially adverse side-effect in mice t.c. immunized with Aβ/CT. Thus, when taken together, t.c. immunization holds potential as a novel, effective, and safe prospective treatment
strategy for AD.

We have previously shown the CD40-CD40L interaction enhances pro-inflammatory microglial activation triggered by cerebral Aβ deposits (Tan et al., 1999a). This form of microglial activation is deleterious, as both genetic ablation of CD40L and CD40L neutralizing antibody reduce brain levels of several neurotoxic inflammatory cytokines and mitigate cerebral amyloidosis in AD mouse models (Tan et al., 2002a). Blocking the CD40-CD40L interaction mitigates Aβ-induced inflammatory responses and enhances Aβ clearance (Tan et al., 2002b; Townsend et al., 2005). Here, we utilized genetic and pharmacologic approaches to test whether CD40-CD40L blockade could enhance the efficacy of Aβ_{1-42} immunization, while limiting potentially damaging inflammatory responses. We show that genetic or pharmacologic interruption of CD40-CD40L interaction enhanced Aβ_{1-42} immunization efficacy to reduce cerebral amyloidosis in the PSAPP and Tg2576 mouse models of AD. Potentially deleterious pro-inflammatory immune responses, cerebral amyloid angiopathy (CAA) and cerebral microhemorrhage were reduced or absent in these combined approaches. Pharmacologic blockade of CD40L decreased T-cell neurotoxicity to Aβ-producing neurons. Further reduction of cerebral amyloidosis in Aβ-immunized PSAPP mice completely deficient for CD40 occurred in the absence of Aβ immunoglobulin G (IgG) antibodies or efflux of Aβ from brain to blood, but was rather correlated with anti-inflammatory cytokine profiles and reduced plasma soluble CD40L. Recently soluble CD40L was reported to be significantly increased in AD patients vs. healthy elderly controls, further supporting a role for this receptor/ligand dyad in the pathogenesis of AD (Desideri et al., 2006). These
results suggest CD40-CD40L blockade promotes anti-inflammatory cellular immune responses, likely resulting in promotion of microglial phagocytic activity and Aβ clearance while precluding generation of neurotoxic Aβ-reactive T-cells. If the benefit afforded by CD40 pathway blockade to Aβ1-42 vaccinated AD mouse models can translate to the clinical syndrome, then pharmacotherapy aimed at reducing CD40 signaling in conjunction with Aβ vaccination may represent an approach that is both safer and more effective in humans. Future studies will be required to isolate CD40-CD40L downstream signaling involved in reduced efficacy of Aβ vaccination, as this may uncover additional targets for pharmacologic intervention.

5.2.2. Immunotherapy: human umbilical cord blood cells

Considering that inflammation plays a crucial role in propagation of Alzheimer’s disease, a second or parallel therapy would be desired. This alternative therapy approach would mainly immunomodulate peripheral immunity such that desired anti-inflammatory Th2 responses would predominate, in other words an increased Th2/Th1 ration should be observed. A possible strategy would involve a partial blockade of CD40-40L. However, it is rather challenging to partially block CD40-40L interaction with the use of CD40 agonists or CD40L antagonists since it could lead to immunodeficiencies and hyper IgM syndrome. Human cord blood cells (HUCBC) have unique immunomodulatory potential. HUCBC have been shown to oppose the pro-inflammatory Th1 response, as demonstrated in an animal model of stroke where HUCBC infusion promoted a strong anti-inflammatory Th2 response (Vendrame et al., 2004). Importantly, this effect was
associated with reduced infarct volume and rescue of behavioral deficit (Vendrame et al., 2004). HUCBC infusion has also shown therapeutic benefit in other neuroinflammatory conditions including multiple sclerosis, amyotrophic lateral sclerosis, age-related neuromacular degeneration, and Parkinson’s disease (El-Badri et al., 2006; Garbuzova-Davis et al., 2006; Henning et al., 2006). In AD preclinical models, administration of these cells to the PSAPP mouse model of AD was associated with extension of lifespan, although high doses were administered in this paradigm (Ghorpade et al., 2001). Here, we showed a marked reduction of Aβ levels/β-amyloid plaques and associated astrocytosis following multiple low dose infusions of HUCBC. HUCBC infusions also reduced cerebral vascular Aβ deposits in the Tg2576 AD mouse model. Interestingly, these effects were associated with suppression of the CD40-CD40L interaction as evidenced by decreased circulating and brain soluble CD40L (sCD40L) and elevated systemic IgM levels, attenuated CD40L-induced inflammatory responses, and reduced surface expression of CD40 on microglia. Importantly, deficiency of CD40 abolishes the effect of HUCBC on elevated plasma Aβ levels. Moreover, microglia isolated from HUCBC-infused PSAPP mice demonstrated increased phagocytosis of Aβ. Further, sera from HUCBC-infused PSAPP mice significantly increased microglial phagocytosis of Aβ1-42 peptide while inhibiting IFN-γ-induced microglial CD40 expression. Increased microglial phagocytic activity in this scenario was inhibited by addition of recombinant CD40L protein. Thus, the data suggests that HUCBC infusion confers mitigation of AD-like pathology by disrupting CD40L activity. When taken together, our results provide the basis for a novel immunomodulatory strategy for AD using HUCBC.
In conclusion, there is no current treatment for Alzheimer’s disease. Immunotherapy is emerging as a potential therapy not just for AD but other diseases as well. Previous vaccines were very effective yet came with undesired side effects for a small population of patients. Since then, the accent have been given to safety as much as to efficacy of any treatment. Alternative routes, adjuvants, and antigens could be used to bypass this problem. Here, we showed that transcutaneous immunization of a whole Aβ peptide with a cholera toxin adjuvant could be the answer. This vaccine could further be enhanced with inhibitors of yet to be identified specific CD40-40L signaling molecules involved in inflammation. Lastly, human cord blood cells could be used in parallel with the vaccine as they provide the host with required tools for needed immunomodulation. In addition, these cells do not represent any ethical concerns and they are readily available. Based on these finding and current trends in research it is highly likely that future AD treatments will incorporate both the vaccine- and the cell-based therapies. Future studies are needed to further examine the safety of these approaches since both have shown very effective.
REFERENCE


Human umbilical cord blood progenitor cells are attracted to infarcted myocardium and significantly reduce myocardial infarction size. Cell Transplant. 15, 647-58.


ABOUT THE AUTHOR

William Veljko Nikolic received his Bachelor’s degree in Biology from Florida Atlantic University in 2002 and his Master’s degree in Biology microbiology/immunology track in 2004. During his Master’s program he worked on systemic lupus erythematosus in the laboratory of Dr. James X. Hartmann. In the fall of 2004 he joined the Medical Science Ph.D. program at the University of South Florida in the laboratory of Dr. Jun Tan where he worked on immunotherapy for Alzheimer’s disease. His formal appointment was in the department of Molecular Medicine within college of Medicine. He successfully defended his doctoral dissertation on June 13, 2008 at the University of South Florida.