MECHANISMS OF APOPTOTIC CELL DEATH OF LYMPHOCYTES IN AGING AND IN ALZHEIMER'S DISEASE

The thesis

for the degree of

Doctor of Natural Sciences

(Dr. phil. nat.)

submitted to the Faculty of

Chemical and Pharmaceutical Sciences

of the

Johann Wolfgang Goethe-University

Frankfurt am Main

by

Katharina Schindowski

from Frankfurt am Main

Frankfurt am Main

2001

(D F 1)

MECHANISMEN DES APOPTOTISCHEN ZELLTODES AN LYMPHOZYTEN IM ALTERUNGSPROZESS UND IN DER ALZHEIMER DEMENZ

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften (Dr. phil. nat.)

vorgelegt beim Fachbereich 14
(Chemische und pharmazeutische Wissenschaften)
der
Johann Wolfgang Goethe-Universität,
Frankfurt am Main

von

Katharina Schindowski

aus Frankfurt am Main

Frankfurt am Main

2001

(D F 1)[1]

(DF1: D = Dissertation / F1 = Bibliothekskennzeichen)

vom Fachbereich 14 (Chemische und pharmazeutische Wissenschaften) der Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekan: Prof. Dr. Walter E. Müller

Gutachter: Prof. Dr. Walter E. Müller, Prof. Dr. Theo Dingermann

Datum der Disputation: 06. Dezember 2001

meiner Familie gewidmet

Die vorliegende Arbeit wurde von September 1997 bis Mai 2001 am Pharmakologischen Institut für Naturwissenschaftler der Universität Frankfurt am Main unter der Leitung von Prof. Dr. Walter E. Müller angefertigt.

Mein aufrichtiger Dank gilt insebsondere:
Herrn Prof. Dr. Walter E. Müller für die Überlassung des Themas, das Vertrauen in meine Arbeit und die Möglichkeit, an internationalen Kongressen teilzunehmen.
Herrn Prof. Dr. Dingermann möchte ich für die Begutachtung der Arbeit danken.
Frau PD Dr. Anne Eckert danke ich für die konstruktive Kritik, die Hilfestellung zum selbstständigen Arbeiten und die Korrekturen der vielen Manuskripte und Abstracts.
Dem gesammten Team der Gedächtnisambulanz der Psychiatrischen Klinik, vor allem aber Dr. Lutz Frölich, Corinna Gorriz, Uta Schramm, Thomas Weinandi und Jürgen Peters sowie Prof. Dr. Konrad Maurer für die gute Zusammenarbeit. Ausserdem den zahlreichen, freiwilligen sowie nicht ganz so freiwilligen, alten und jungen Blutspendern und allen, die bei der Blutentnahme geholfen haben.
Fr. Bozyk für ihre hervorragende und hingebungsvolle Pflege der Versuchstiere. Herrn Dr. Moser für die exzellente Organisation des Arbeitskreises. Den drei entzückenden Damen vom Sekretariat für ihre immer offenen Ohren, ihre heiteren Wesen und die Organisation meiner zahlreichen 'Extrawünsche'. Hebbes für ihre Hilfe und unglaubliche Kreativität bei Problemen mit Hard- und Software. Dr. Barbara Steiner für die grosse Hilfestellung beim Verfassen und Korrigieren zahlreicher Manuskripte sowie ihre hervoragende Organisation des Zellabors. Claudia für die Hilfe bei der Genotypisierung der Mäuse und Ina für die vielen Tipps und Anregungen.

Der gesammten Arbeitsgruppe Schmalzing, vor allem aber Armaz Aschrafi und Cora Büttner, dass sie so geduldig mein Schnorren von molekularbiologischen Materialien und Tipps ertrugen („Ich mach dann auch das nächste Mal kompetente Zellen...").
Silke Leutner, von der ich mehr über Pharmakologie, Studiendesign, kritische Hinterfragung und Umgang mit Versuchstieren gelernt habe, als sie warscheinlich ahnt. Ihr, ebenso wie Sabine Kressmann, Stefanie Müller, Celio Marques und Katrin Schüssel vielen Dank für die vielen lustigen und schönen Stunden im Labor 120, an die ich mich immer gern erinnern werde.
Den Ex-Radioleuten Andrea Singer und Meinolf Wonnemann für die sehr aufschlussreiche und vergnügliche Zeit, die vielen kritischen und nicht so ernsten Diskussionen im und ausserhalb des Radiolabors. Ausserdem danke ich den 'Radios' und Michael Sych für die schönen manchmal vielleicht leicht chaotischen Reisen zum Neuroscience-Meeting.
Sabine Kressmann für die Hilfe bei der Ginkgostudie und die vielen Süssigkeiten, die sie schwesterlich mit mir teilte. Michael Sych für seine Hilfe und Diskussion zu den Presenilin Western Blots. Dr. Christian Luckhaus und Dr. Henrike Hartmann für die vielen sehr konstruktiven Disskusionen, exzellenten Anregungen und ihr offenes Ohr während ihrer leider viel zu kurzen Zeit am Institut.
Andrea K., Gisellé, Barbara Siebert, Bettina sowie den anderen Mitglidern des KO-Teams und allen hier aus Platzgründen nicht aufgezählten Mitgliedern des Instituts für die wundervolle Zeit, die wir hatten.
Dr. Dipl.Chem. 'mit Ausszeichnung' Duy Ngyuen für die hervoragende Korrektur des Manuskripts.
All meinen Freunden und ehemaligen Kommilitonen für die tolle und ablenkende Zeit ausserhalb der Doktorarbeit und für ihre offenen Arme während der Entstehung dieser Arbeit.
Meiner Oma, die den Abschluss dieser Arbeit leider nicht mehr erleben konnte. Meinem Bruder Alexander für die Hilfe beim Druck dieses Manuskript. Meiner gesamten Familie für ihre Unterstützung und Rückhalt nicht nur während dieser Arbeit.

Nach Wissen suchen,
heisst Tag für Tag dazu gewinnen
(Lao Zi)

In Gedenken an die zahllosen Mäuschen,
die ihr Leben für diese Arbeit lassen mussten.

Table of Contents

Introduction

As medical care improved outstandingly and is available to nearly everybody in the industrialized countries and rescued countless people with life threatening diseases in their middle age, life expectancy increased dramatically within the last century. Therefore, aging and age-related diseases are becoming more and more important for our society and our health care system. Not only the costs of retirement and care facilities explode with rising life span, moreover especially the costs of the medical care are an essential problem for the whole community.

Nevertheless, elderly people provide a very important impact and benefit to human social life, ethnic culture and family bands. 'Healthy aging', therefore, is one of the major aims for modern medicine.

Hallmarks of age-related health problems are degenerative and/or atrophic changes of several tissues. Degeneration most likely occurs due to increased damage of differentiated cells that are often substituted by connective tissue or inappropriate origin tissue only, thereby leading to atrophy. Many theories try to explain why in the aged organisms the number of damaged cells increases. The most important of those theories relates to the phenomenon that the detoxifying system against reactive oxygen species (ROS), that are generated during aerobic metabolism, decreases or becomes impaired with aging.

Aging

Aging is a condition associated with many social changes likely to induce psychological stress. Many behavioral changes that normally occur over 65 years of age are also related with changes in life-style. Retirement represents '*per se*' a stressing event. The income is less than that of the mean working population, leading to reduced expensive habits and increased economic troubles. In addition, progressive loneliness caused by the death of the spouse, death of friends and/or being forsaken by children and ancestors, generates a situation of isolation (Guidi *et al.,* 1998). Age-dependent neurological, articular, and muscular modifications and diseases can cause a progressive loss of motility and disability. In modern society, 'efficiency' is a fundamental quality of life, thus the old age period of 'unproductivity' can be perceived as a condition of uselessness (Blazer and Williams, 1980). The outcome of all these psychosocial changes can be a condition of psychological stress which, depending on personality features and previous experiences, can be perceived as

uncontrollable and lead in some cases, on a long term, to clinically relevant depression (Guidi *et al.,* 1998). However, acute and chronic stress have a strong impact on immune function (summarized in Guidi *et al.,* 1998).

Molecular aspects of aging

The lifespan or life-expectancy of a cell or an organism, respectively, is biologically terminated. At the end of each chromosome, there are telomeres, which regulate lifespan by modulating genomic silencing. Telomere shortening has been observed in somatic cells of aging humans and in primary cells as they divide in culture (Nicanor *et al.,* 1997). Loss of telomeric DNA and gradual shortening of telomeres have been proposed to result, after a certain number of cell divisions, the inability of cells to divide again. Loss of telomeric repeats is not an *in vitro* phenomenon as it is also observed in human cells *in vivo* (Pawelec *et al.,* 1999). Telomere shortening might therefore act as a mechanism counting the number of cell divisions that a cell population has experienced. Telomere length in human blood cells *in vivo* was shown to be related to donor age (Freedman *et al.,* 1994). Telomere attrition occurs more rapidly in premature aging syndromes, e.g. *Hutchinson-Gilford* progeria (Allsopp *et al.,* 1992) or trisomy-21 (Vaziri *et al.,* 1993).This shortening is due to the absence of telomerase in aging cells, which leads to a progressive loss of DNA sequences at the ends of replicated chromosomes. It has been suggested that telomere shortening serves as a mitotic clock, eventually triggering the senescence of cells and aging in people (Thoman and Weigle, 1989). Short telomeres could stop cell division by cell cycle arrest, by making damage to chromosomes in mitosis, or by deletion of telomere-proximal genes. In many tumor cell lines telomerase activity has been restored during immortalization (Tielen *et al.,* 1993).

Besides the biological termination of cell replication, there are other factors contributing to aging and senescence, which are specially relevant for post-mitotic cells like oxidative stress. Under normal physiological conditions, the oxygen consumption by cells with aerobic metabolism always generates potentially deleterious ROS, which are usually detoxified by several enzymes and physiological antioxidants. A mismatch between production and detoxification of ROS leads to oxidative stress and consequently to cellular damage. Increasing with age, oxidative stress is a major causal factor of cellular damage and enhanced programmed cell death in many aging tissues including brain and immune system. The mitochondrial chain failure has been implicated as a factor in the aging process (Miquel *et al.,*

1980). It has been proposed that accumulation of mitochondrial mutations may underlie this phenomenon (Wallace, 1992). Furthermore, elevated generation and dysfunctional elimination of ROS in cells from aged individuals seem to be involved in senescence and can trigger programmed cell death.

Damaged or presumably dysfunctional cells are eliminated to maintain the common wealth of the whole organism. In fact, these cells are not subject to an execution, they recognize that they are damaged and perform their own suicide, called apoptosis or programmed cell death (PCD).

Brain aging

The brain mainly consists of neurons, however, a smaller proportion of brain tissue is of non-neuronal origin, the so-called glia or glial cells. The glia (from Greek, meaning 'glue') was first recognized in the 1800s. Initially biologists mistakenly thought of the glia as a single unit that served only as the uninteresting putty between neurons and spinal cord. But the 1920 microscopists had identified three kinds of glial cells: astrocytes, oligodendrocytes and microglia. In the 1970s it was found that astrocytes can sop up excessive neurotransmitters in order to protect neuron from to much stimulation while oligodendrocytes produce the myelin sheath that insulates axons (Streit and Kincaid-Colton, 1995). All cell types provide important immunological activity.

Neurons are mostly post-mitotic structure and usually do not proliferate anymore. Vascular changes in aging occur in brain blood vessels as in the periphery, resulting in a lower supply of nutrients and oxygen to brain cells. Neurons are cells that get rather old, as their plasticity stores memories, that shall survive for a life-time. Malnutrition of neurons due to vascular insults is a major reason for vascular dementia.

Glial activation occurs even in healthy aging. Lost neurons are substituted by glia in the aging brain, thus gliosis (enhanced activation and proliferation of glia) seems to be one consequence for loss of neurons.

Apoptosis in aging

The only type of cell death recognized in classical pathology was necrosis. This is an involution phenomenon produced by severe injury to the cells. Its occurrence is usually

accompanied by inflammation with redness and swelling of the surrounding tissue. Necrosis is, therefore, unlikely to account for deletion of cells in normal animals.

The vision of naturally occurring cell death changed completely in 1972 when John Kerr et al. (1972) described that natural cell death is not an involution or a degeneration of tissues, but an active, inherently programmed phenomenon essential for organ development. They termed this process 'apoptosis', a word used in classical Greek for the falling of leaves from trees.

During the development of an animal from a single cell, the fertilized egg divides, and than the daughter cells divide, over and over again, generating a large number of cells, as many as 10^{13} (ten million million) in humans. Then each single cell undergoes differentiation i.e. takes on specific characteristics, such as becoming a nerve cell or muscle cell or a immune cell. Furthermore, all of these cells must interact so as to form groups of cells with the proper structure, like an arm or a leg, and correct interconnections as in the highly complex brain. These processes of cell division, cell differentiation and morphogenesis constitute the basic events of development and define the basic problems of developmental biology. In addition to these processes, there is another event that appears to be universal among developing animals, the process of cell death. Quite remarkably, many of the cells, that are generated as animals develop do not survive to form part of the animal, but instead die, often before they have had a chance to do anything.

It is apoptosis that removes tissues not needed and sculpts the body during fetal development. The formation of fingers, for example, occurs in the embryo by removal of interdigital webbed regions of the hand that starts as a paddle shaped structure or, as another example, the loss of the tail of the tadpole when it turns into a frog. Moreover, apoptosis is of extreme importance in natural tumor defense. Immunity relies on apoptosis to eliminate self-reacting lymphocytes, to induce suicide in virus-infected cells, and to eliminate normal activated lymphocytes after they have done their job in order to terminate an immune response.

Apoptosis is perhaps the most revolutionary concept ever introduced in biological sciences, because it shows that death is an integral part of life and that active and well programmed death is necessarily required for life to progress (Rappuoli, 2000). The new vision of cell death, now seen as active, programmed morphological event involved in cell turn over in healthy adult tissues and responsible for focal cell elimination during development took almost a decade to enter into the molecular world. This happened when genes of the nematode *Caenorhabditis elegans* (*C. elegans*) dedicated to apoptosis were identified and showed that cell death is the result of a programmed intracellular cascade of genetically determined steps

(Ellis and Horvitz, 1986). The two genes named *ced3* and *ced4* (where *ced* stands for cell death) were shown to be essential for the death of the 131 cells, that usually get lost during the normal development of the 1000-cells-worm.

Today, the occurrence of apoptosis in various physiological and pathological situations is confirmed and the programmed cell death is a subject of countless research studies to elucidate the underlying mechanism of human patho-biochemistry.

Apoptosis is generally conceived as a strictly regulated ('programmed') device for the removal of superfluous, aged, or damaged cells. It is fundamental for development, throughout embryogenesis, organ metamorphosis, and organogenesis, including synaptic interaction of neurons, and repertoire selection of T lymphocytes (Penninger and Kroemer, 1998). Increased resistance to apoptosis induction may lead to the persistence of severe damaged or mutated cells, thereby preceding to cancer, or of self reactive immune cells, leading to autoimmune diseases. In contrast, an enhanced susceptibility to apoptosis can cause degenerative diseases or functional immunodeficiency. In post-mitotic tissues cell replacement is not possible in response to damage, so the feasible outcomes are either complete repair, continued survival of a dysfunctional cell, or apoptosis.

The major source of most cell damage is assumed to be oxidative stress due to mitochondrial production of ROS. ROS presumably affect DNA, but also proteins and lipids. Maintenance of mitochondrial membrane integrity is also compromised by oxidative stress. Oxidative stress is responsible for the mitochondrial permeability transition, mitochondrial depolarization, decrease in ATP levels, and finally apoptotic cell death (Warner 1999). Possibly related to limited synthesis of ATP, the mitochondrial membrane may become permeable to cytochrome c, thereby initiating apoptosis. The antiapoptotic protein Bcl-2 inhibits apoptosis by blocking the release of cytochrome c (Yang *et al.,* 1997). Apoptotic cell death is associated with major changes in the redox status including a loss of nonoxidized glutathione (Macho *et al.,* 1997) which may be oxidized during the process of apoptosis, increased production and/or reduced detoxification of reactive oxygen species (Zamzami *et al.,* 1995), and oxidation of cellular constituents including membrane lipids (Hockenberry *et al.,* 1993).

During apoptosis, DNA fragmentation occurs *via* the activation of nucleases that generate mono- and oligomers of 180 – 200 bp corresponding to the length of the nucleosome.

Apoptosis can play a role in aging: elimination of damaged and presumably dysfunctional cells (e.g., lymphocytes) which can then be replaced by cell proliferation, thereby maintaining homeostasis and elimination of essential post-mitotic cells (e.g., neurons) which cannot be replaced so easily, thereby leading to pathology. Evidences indicate the age-related decreased susceptibility for apoptosis, although the molecular basis for these decreases appears to be different. Fibroblasts (and neurons) lose the ability to downregulate Bcl-2 in response to an apoptotic signal; thus, apoptosis is blocked even though an initiating signal has been received. In contrast, lymphocytes are not able to initiate the signal due to downregulation of the cell surface receptor Fas. There is limited information available for other tissue types, and nothing is known about why and how these age-related changes occur. An interesting observation, but not necessarily a crucial one, is that the frequency of upregulation of the Bcl-2 gene due to chromosome translocation increases with age. The role of apoptosis in regulating cell number is also a promising area of research. The studies on liver damage and neoplastic lesions suggest an extremely important role for apoptosis in controlling cancer. Apoptosis appears to be either 'on' or 'off' in cells, while the basic cell-killing machinery may often be present, but in an inactive form (Warner, 1997).

However, apoptosis is strictly linked to normal development and most of the physiological aspects of life in humans, animals and plants. Hence any unbalance in this process may lead to diseases such as tumors, degenerative disorders, and infections. The precise molecular knowledge of the fundamental events involved in apoptosis are today subject of intensive studies aiming at the development of drugs that may prevent or induce apoptosis to treat many diseases.

Alzheimer's diseases: an age-related neurodegenerative disorder

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by global cognitive decline including a progressive loss of memory, orientation, and reasoning. Alois Alzheimer extensively described a dementive syndrome of his patient Auguste D. whom he treated as neurologist and psychiatrist in Frankfurt am Main at the beginning of the last century (Alzheimer, 1907). He recorded a rapidly progredient memory loss of the 52 year old women. After her death, he examined her brain and found histological changes, that are specifically for AD.

Since the time of the discovery of the disease up to now, life expectancy further increased. Consequently, every 10^{th} person over 65 years is affected by the dementia of Alzheimer's

type (DAT) and further 10% with every decade. An irreversible loss of cognitive and mental abilities is the prognosis of this disorder. In later stages, AD patients are helpless and require full-time nursing care. Besides the personal and familial tragedies that are one aspect of the dementia, AD is a financial problem of health service and thereby a burden for the whole social community. And this cost will raise as more and more persons are aging and becoming elderly.

AD is a progressive neurodegenerative disorder with disastrous consequences, clinically characterized by a slow onset, the progress is associated with a loss of cognitive function and a fatal mental dysfunction. Histologically, the neurodegeneration is distinguished by neuropathological changes, protein deposits, mainly consisting of β-amyloid (Aβ) in the hippocampus, parietotemporal regions and cerebral cortex. The brains of AD patients contain abundant amounts of neurofibrillary tangles (NFT) and Aβ in the form of senile plaques and deposits in cerebral blood vessels.

Pathological changes in AD brain

The major histopathological lesions in AD brain are neurofibrillary tangles and plaques.

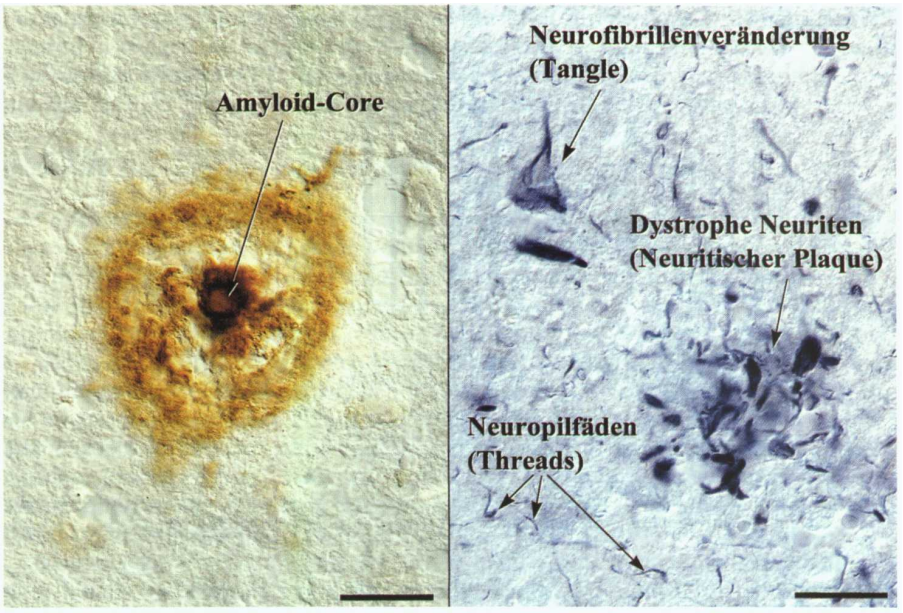


Figure 1. Pathological hallmarks in AD brain: a plaque, mainly consisting of Ab (left). The dense amyloid core is surrounded by an amyloid halo. Neurofibrillary tangles (right) are formed by dead neuron cell bodies due to cytoskeletal changes, for instance hyperphosphorylation of t-protein (picture taken from Arendt, 1999).

Neurofibrillary tangles

Tau (τ) is a microtubule-associated protein that, in a hyperphosphorylated form, comprises the main component of the paired helical filaments and neurofibrillary tangles found in AD brain. It is therefore important to understand the normal functioning and processing of τ protein, and the abnormal posttranslational processing of τ in AD pathology. Mutations in the τ gene have been found in several non-AD, autosomal dominant neurodegenerative disorders that exhibit extensive neurofibrillary pathology (Johnson and Harrtigon, 1998). In addition, there is increasing evidence that τ may be involved in signal transduction, organelle transport, and cell growth, independent of its microtubule-binding functions.

In Alzheimer's Disease brain τ is abnormally phosphorylated. This hyperphosphorylated form of τ is the major component of the paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) found in the AD brain. The mechanisms underlying the abnormal phosphorylation and accumulation of τ in AD are still under investigation (Johnson and Harrtigon, 1998).

Although phosphorylation of specific sites may facilitate the aggregation of τ into filaments in AD brain, it is likely that other factors contribute to this process. For example, incubation of non-phosphorylated τ with sulfated glycosaminoglycans, such as heparin or heparan sulfate, resulted in the formation of PHF-like structures (Arrasate *et al.,* 1997). In addition, the repeat region of τ was essential for the induction of PHF formation (Goedert *et al.,* 1996). Heparin also prevented τ from binding to microtubules and promoted microtubule disassembly. Additionally, there is some evidence to suggest that heparan sulfate and τ may colocalize in NFT-containing neurons in AD brain (Goedert *et al.,* 1996). Considering this, it was hypothesized that an increase in sulphated glycosaminoglycans within the cytoplasm of nerve cells may trigger the hyperphosphorylation of τ, destabilize microtubules and induce assembly of PHFs.

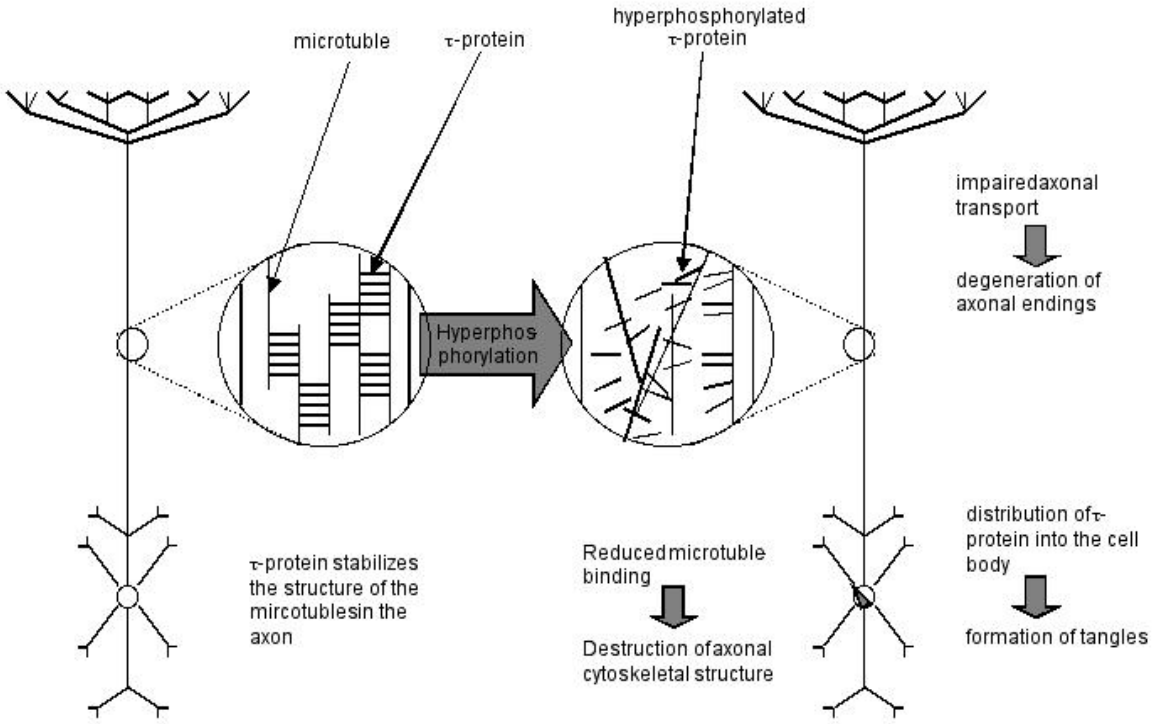


Figure 2. Molecular mechanisms of tangle formation. t-proteins stabilizes microtubles in axonal endings. Higher glutamate concentrations in AD brains induce hyperphosphorylation of t-protein, thereby destroying cytoskeletal structure. As a consequence, the axonal endings degenerates, t-protein is distributed into the cell body and initiate the evolution of tangles (adapted from Haass, 1999).

Amyloid plaques

A major feature of Alzheimer's disease (AD) is the region-specific formation of senile plaques in the brain. An amyloid deposit composed mainly of β–amyloid occupying the plaque center. Aβ is a peptide of 40 to 43 residues derived from a larger amyloid precursor protein (APP) located on chromosome 21 (Selkoe, 1994). Individuals with Down's syndrome (DS; trisomy 21) develop quite early an AD-like pathology, which might be due to a gene-dose effect of APP (Pallister *et al.,* 1997).

Senile plaques formation occurs age-dependent in physiological aging. However, when senile plaques transform into neuritic plaques, sites of inflammation form around the plaque involving mostly parts of the innate immunity (see Chapter

Neuroinflammation in AD).

Moreover, AD is mainly characterized by a dramatic loss of neurons and synaptic contacts in the affected brain areas. Loss of cholinergic neurons seem to be specially associated typical clinical symptoms, like memory deficits, impaired attention, cognitive decline, and reduced learning abilities. Aβ–toxicity and neuroinflammation elevate glutamate levels in neural tissue that further damage the insulted neurons. Mainly in later stages of the disorder, degeneration of dopaminergic neurons are involved as well, and Parkinson's-like symptoms with extrapyramidal impairment may occur (Kornhuber *et al.,* 1999).

Molecular mechanisms and genetical risks of AD pathology

While the etiological events that lead to AD remain unresolved, a small percentage of AD has been shown to be of genetic origin (Tanzi *et al.,* 1994; Wasco and Tanzi, 1995) and is termed familial Alzheimer's disease (FAD). FAD is a genetically heterogeneous disorder that can be categorized according to age-of-onset using 60 years as the cut-off for "early-onset" versus "late-onset" FAD. The gene defects responsible for early-onset (under 60 years) FAD are located on chromosomes 1, 14, and 21 and include the genes for presenilin 2 (PS2), presenilin 1 (PS1), and amyloid ß protein precursor (APP), respectively. In addition, a genetic "risk factor" for late-onset FAD (over 60 years old), the apolipoprotein E (ApoE) on chromosome 19 has been identified (Saunders *et al.,* 1993).

Amyloid-Precursor protein (APP)

APP is a transmembrane protein with various isoforms that result from alternative splicing (Selkoe, 1994). The Aβ peptide is generated by processing through β– and γ–secretases. By cleavage within the Aβ region of APP by α–secretase, the long N-terminal fragment (secreted APP, sAPP) is secreted into the extracellular space. It is found in plasma and cerebrospinal fluid (Ghiso *et al.,* 1989; Podlisny *et al.,* 1990). Considering the abundance of both membrane-bound APP and sAPP, they are likely to have significant biological functions. Conversely, proposed functions for sAPP include the regulation of blood coagulation (Van Nostrand *et al.,* 1990), wound-healing (Cunningham *et al.,* 1991), extracellular protease activity (Van Nostrand *et al.,* 1989), neurite extension (Robakis *et al.,* 1990), cell adhesiveness (Schubert *et al.,* 1989), cell growth, (Bhasin *et al.,* 1991), and differentiation

(Yamamoto *et al.,* 1994). Many different types of cells synthesize APP. In neurons it is abundantly located in the synaptic zone (Schubert, 1991). Different cell types produce different isoforms of APP. Differentiated neurons produce mainly the APP695 form whereas non-neuronal cells produce the *Kunitz*-type protease inhibitor (KPI)-containing forms of APP (Selkoe, 1994). Activated leukocytes display elevated levels of APP (Monning *et al.,* 1992; 1990).

In vitro studies demonstrated that Aβ may cause neuronal death, supporting the hypothetical involvement of Aβ in neurodegeneration in AD. To explain massive neuronal loss in the brain of AD patients, several models have been proposed placing Aβ as a principal culprit (Citron *et al.,* 1992; Mattson *et al.,* 1992; Pike *et al.,* 1993; Yankner *et al.,* 1989). Possible mechanisms of Aβ toxicity include kinase activation (Zhang *et al.,* 1994), generation of free radicals (Behl and Schubert, 1993), and enhancement of glutamate toxicity (Mattson *et al.,* 1992). Because APP plays a role in cell growth and neuronal maintenance and differentiation, understanding the relationship between sAPP's neurotrophic function and Aβ neurotoxicity is important. sAPP treatment could reduce the effect of Aβ on neurons (Goodman and Mattson, 1994) which suggests that the neurotrophic effect of sAPP is sufficient to overcome Aβ toxicity under normal conditions.

There have also been numerous studies examining the possible effects of Aβ on τ phosphorylation. Treatment of fetal rat hippocampal and human cortical neurons with fibrillar Aβ resulted in an increase of τ phosphorylation. The phosphorylation of τ in response to Aβ treatment resulted in a loss of microtubule binding capacity and accumulation of τ in the somatodendritic compartment (Busciglio *et al.,* 1995). One possible mechanism by which Aβ may induce increases in τ phosphorylation is by perturbing calcium homeostasis which would result in inappropriate increases in the activities of certain protein kinases contributing to τ hyperphosphorylation. Further, it has been hypothesized that Aβ-induced perturbation of calcium homeostasis could make the cells more vulnerable to other potentially ‘toxic‘ stimuli, including glutamate. Treatment of neuronal cells with Aβ resulted in elevated intracellular calcium levels over those of resting cells (Hartmann *et al.,* 1994a, 1994b, 1994c; Müller *et al.,* 1996). Addition of Aβ also made the cells more vulnerable to neurotoxicity induced by glutamate, kainate or N-methyl-D-aspartate (NMDA; Mattson *et al.,* 1992; Mattson *et al.,* 1993).

The first gene defects causing early-onset FAD were found in the APP gene (Goate *et al.,* 1991; Mullen *et al.,* 1992) and all six are missense mutations lying within or close to the domain encoding the Aß peptide, the major component of amyloid in AD. The APP mutations account for a very small proportion (2-3 %) of all published cases of FAD (Tanzi *et al.,* 1992) and 5-7 % of reported cases of early-onset FAD. The pathogenicity of these mutations has been strongly supported by the fact that they are virtually 100 % penetrant in FAD kindred where they occur in affected or at-risk individuals, but are absent in age-matched controls. Moreover, transgenic mice expressing the APPV717F 'London' mutation produce numerous Aß deposits in the form of classical senile plaques and the brains of these animals exhibit other neuropathological features of AD including neuronal and synaptic loss and gliosis (Games *et al.,* 1995). FAD mutations in APP have been shown to affect the release of Aß in transfected cells and carrier fibroblasts (Cai *et al.,* 1993; Suzuki *et al.,* 1994; Citron *et al.,* 1992). While the APP codon 717 mutations are associated with overproduction of Aß42 (increased ratio of Aß42:Aß40), the 'Swedish' FAD double missense mutant leads to an increase in total Aß secretion. The Swedish mutant involves the substitution of the two N-terminal amino acids of the Aß domain presumably rendering APP more susceptible to ß-secretase activity. Additionally, studies of polarized cells have revealed that while up to 90 % of α-secretase-cleaved APP is normally released from the basolateral surface (see Figure 4; Haass *et al.,* 1994), APP carrying the Swedish mutation is released primarily from the apical surface. (Selkoe *et al.,* 1995). Therefore, FAD mutations in the APP gene appear to affect both Aß release and the trafficking of APP, intracellularly.

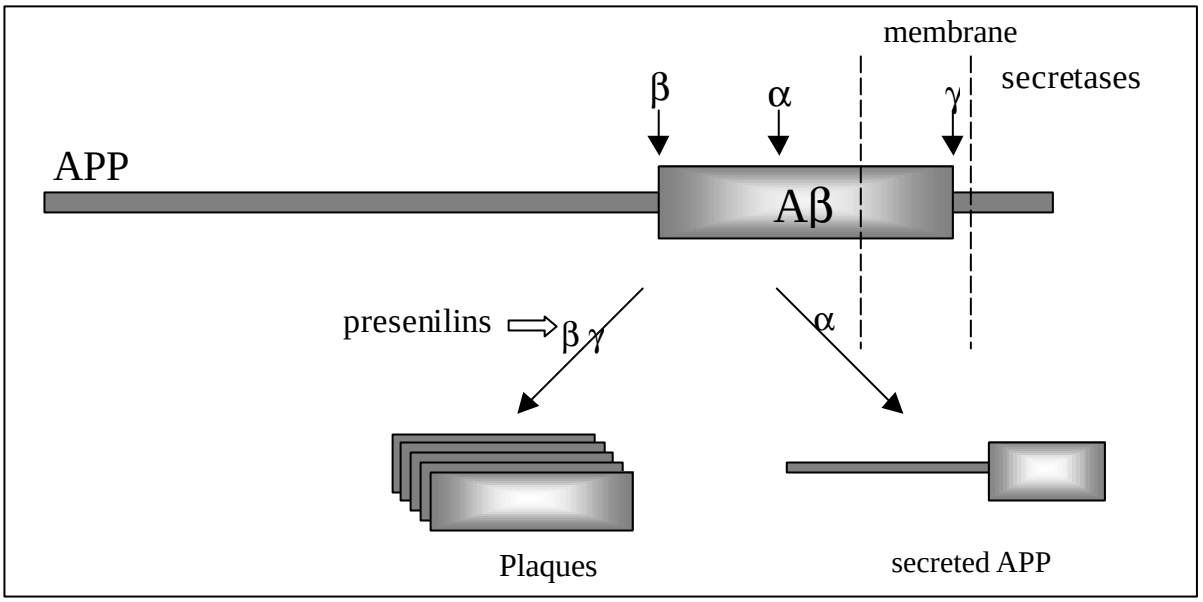


Figure 3. Molecular mechanisms of plaque evolution in AD brains (for details see text).

Presenilins (PS)

The largest portion of early-onset FAD cases have been associated with mutations in the presenilin genes, PS1 and PS2 (Sherrington *et al.,* 1995; Levy-Lehad *et al.,* 1995; Li *et al.,* 1995; Rogaev *et al.,* 1995). PS1 encodes a 467 amino acid polypeptide predicted to contain between 7-9 transmembrane spanning domains (Sherrington *et al.,* 1995; Slunt *et al.,* 1995) and includes a hydrophilic 'loop' region (amino acids 267-403). Plasma and fibroblasts from patients and pre-symptomatic carriers of mutations in PS1 and PS2 contain increased amounts of the more amyloidogenic version of the Aß peptide, $Aß_{1-42}$ or $Aß_{x-42}$ (Scheuner *et al.,* 1996). The mean age of onset of AD in PS1-linked FAD families is approximately 45 years with a range of 32 to 56 years. In contrast, the mean age of onset in the Volga German families with the N141I PS2 mutation is 52 years and individual ages of onset in these kindred range from 40-85 years (Tanzi, 1996). Whether considered as a group or individually, the PS1 FAD kindred exhibit a more narrow range in age of onset of AD than do the families with PS2 mutations. The known (>50) FAD mutations are distributed throughout the PS1 gene, although almost half of all reported mutations in PS1 are in predicted TM domains. Additionally, all FAD missense mutations occur in amino acids that are conserved between PS1 and PS2 (Tanzi, 1996).

The cellular distributions of the PS1 and PS2 are similar with both localized to the nuclear envelope, endoplasmic reticulum (ER) and the Golgi (Kovacs *et al.,* 1996). Overall, the cytoplasmic staining pattern observed for the presenilins is reticular and punctuate, suggestive of vesicular staining. Plasma membrane staining is not found for either of the presenilins (Kovacs *et al.,* 1996; Cook *et al.,* 1996). In comparison with the wild-type PS1 and PS2, no gross abnormalities in the subcellular localization of mutant presenilins have been reported. PS1 was also found in dendrites (but not axons) of human neuronal cell lines expressing PS1. Haass and colleagues have reported the phosphorylation of the N-terminus of PS2 but not of PS1 (Haass *et al.,* 1996), further supporting the related, but differential roles for these proteins.

While the biological functions and physiological roles of the presenilins are not yet known, both have been proposed to possibly act as receptors, ion channels, or molecules involved in protein processing or trafficking (Sherrington *et al.,* 1995; Levy-Lahad *et al.,* 1995; Kovacs *et al.,* 1996). The most valuable clues regarding possible functions of the presenilins have emerged from the recent findings that PS1 and PS2 are homologues of two *C.elegans* genes

known as *sel*-12 (50% identity; Levitan and Greenwald, 1995) and *spe*-4 (25% identity, L'Hernault and Arduengo, 1992). It is worth noting that greater than 80% of the FAD mutations identified in the PS1 and PS2 gene occur in amino acids that are perfectly conserved in *sel*-12, which encodes a member of the Notch family of receptors involved in intercellular signaling and the determination of cell fate (Levitan and Greenwald, 1995). The latter role is indirectly supported by our previous findings that PS1 and PS2 are localized to the ER and Golgi where they could conceivably participate in the processing and/or cellular trafficking of plasma membrane-bound and secretory proteins. PS1 has been reported to be processed into two fragments: a 27 kDa N-terminal 18 kDa C-terminal fragments (Thinakaran *et al.*, 1996).

These data suggest a common pathogenic pathway for the early-onset FAD gene defects in APP and the presenilin genes revolving around the increased production of $A\beta_{42}$.

ApoE polymorphism

In humans, *apo*ε (*apo*ε = gene; ApoE = protein) is a single gene located on chromosome 19q13.2 with three major allelic variants (ε2, ε3, ε4) encoding three protein isoforms ApoE2, ApoE3, and ApoE4 (Bales *et al.*, 2000). ApoE has been widely documented to play a role in sporadic late onset AD. ApoE first came to light in AD as a susceptibility gene (Strittmatter *et al.*, 1993). Whereas the *apo*ε2 and *apo*ε3 alleles have no effect or a even beneficial effect, possession of the *apo*ε4 allele, especially homozygosity for *apo*ε4, appears to shorten the date of AD onset by some 5-10 years (see Figure 4). In a recent assessment of the impact of ApoE4 on AD as a function of age of onset in over 300 families and affected sib-pairs with FAD (Blacker *et al.*, 1997), ApoE4 was found to be strongly associated with AD, however, the risk for AD due to inheritance of ApoE4 was most significant in FAD families with mean age of onset between 61 and 65 years. In addition, two copies of ApoE4 was associated with a lower age of onset in this study (Blacker *et al.*, 1997). AD and Down's syndrome patients who are *apo*ε4-positive exhibit increased amyloid burden in the brain presumably due to increased aggregation or decreased clearance of Aß (Hyman *et al.*, 1995). AD patients who are heterozygous, and especially those who are homozygous for apoε4 tend to have more congophilic angiopathy (Lue *et al.*, 1999), perhaps suggesting a connection with current vascular/ cholesterol hypotheses of AD (Sparks, 1997). Still other alternatives may follow from the finding that ApoE can influence microglial expression of several inflammatory indicators (Laskowitz *et al.*, 1998), and this effect seems to be isoform-dependent (Barger and

Harmon, 1997). In addition, ApoE promotes axonal growth and synaptogenesis, probably because it regulates the transcellular transport of cholesterol and phopholipids (Mesulam *et al.,* 2000). The ApoE/lipoprotein complex seems to act as scavenger of normally secreted extracellular lipophilic/non-aggregated Aβ *in vivo* (Poirier *et al.,* 2000). In brief, the ApoE initially binds to the lipophilic region (amino acids 18-42) of soluble Aβ, *via* the LDL receptor the lipoprotein with the bound Aβ is internalized into the cell and directed *via* chlathrin-coated pits to endosomal compartments, where it is processed through the usual endosomal/lysosomal pathway.

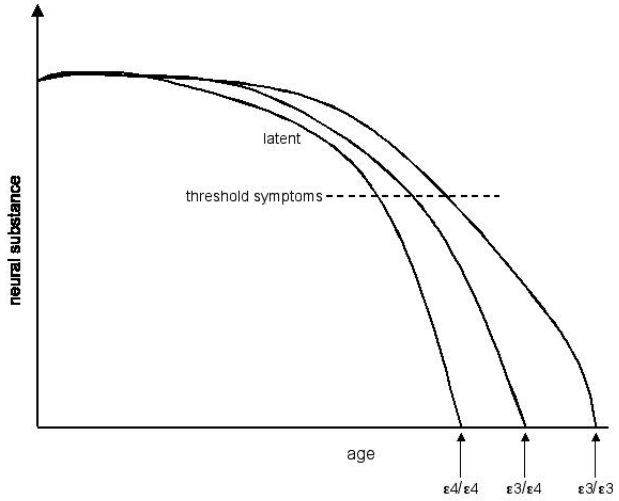


Figure 4. Onset of AD pathology and loss of neural substance in relation to the ApoE genotype.

Apoptosis in AD

Recent data in cell culture have shown that brain neurons are particularly vulnerable towards degeneration by apoptosis. Further the inducers that activate the program (e.g. Aβ, oxidative damage, low energy metabolism) correspond to conditions present in the AD (Cotman and Anderson, 1995) and DS brain (Seidl *et al.*, 2001). Furthermore, some neurons in vulnerable regions of the AD brain show evidence of DNA damage, nuclear apoptotic bodies, chromatin condensation (Dragunow *et al.,* 1995), and the induction of selected genes characteristic of apoptosis (Giannakopoulos et al., 1999; Sawa, 1999; Tortosa et al., 1998; de la Monte et al., 1997; Nishimura et al., 1995) in cell culture and animal models. This suggests the possibility that apoptosis may be one of the mechanisms contributing to neuronal loss in this disease. On the other hand, DNA damage is present in the majority of neurons in vulnerable regions in early and mild cases. In most tissues, cells in fully activated apoptosis degenerate and are

removed within hours to days and thus it seems all DNA damage is unlikely to signify terminal apoptosis. The presence of extensive DNA damage suggests an acceleration of damage, faulty repair process, loss of protective mechanisms, or an activation and arrest of parts of the apoptotic program. DNA damage is unlikely to be an artifact of *post mortem* delay or agonal state. The existence of protective mechanisms for neurons may exist as these cells are nondividing and essential. In this context it is interesting that Bcl-2 is upregulated in most neurons with DNA damage (Satou *et al.*, 1995; Su *et al.*, 1996). Further, apoptosis-related gene expression is altered (Su *et al.*, 1997). Recently, a close involvement of caspases, executor molecules of apoptosis, in Aβ toxicity and in AD brain was found (Eckert *et al.*, 2001a; Lu *et al.*, 2000; Gervais *et al.*, 1999; Weidemann *et al.*, 1999; Pellegrini *et al.*, 1999). Thus it appears as if neurons are in a struggle between degeneration and repair. As research advances it is critical to reduce the stimuli that cause the neuronal damage and discover the key intervention points to assist neurons in the repair processes (Cotman and Su, 1996).

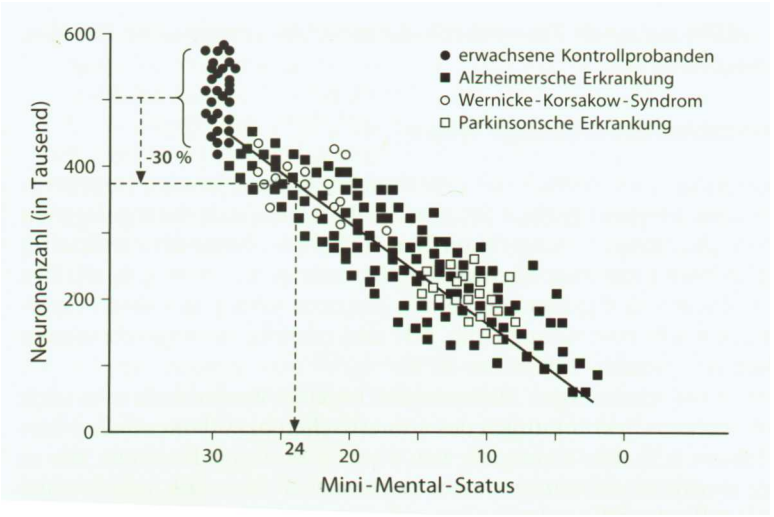


Figure 5. Loss of cholinergic neurons in neurodegenerative disorders (y-axsis: number of neurons in *Nucleus basalis Meynert*) correlates with the cognitive decline measured as Mini Mental State Score (figure taken from Arendt, 1999; x-axsis; ●, adult controls; ■, AD patients).

The cognitive decline of patients with neurodegerative dementias correlates significantly with neuronal cell loss (see Figure 5). One current hypothesis is that at least some human degenerative disorders are caused by ecotopic programmed cell death, that are mechanistically similar to those that occur in normal development but that are for some reason, however, being expressed by the wrong cells or at the wrong time (Arendt, 1999)

It has long been recognized that there is a loss of neurotransmitters in the AD brain. New findings have extended these observations to evaluate localized fiber loss. In particular, axon length in the area of Aβ deposits has been reported to be reduced in comparison with the neighboring neurophil, a result that has been hypothesized to reflect local axotomy and to explain the mechanism for synaptic loss (Cotman and Anderson, 2000).

Oxidative stress in AD

Dysfunction of mitochondrial chain activity may predipose for neurodegenerative disorders, since most of these diseases are associated with elevated levels and byproducts of oxidative stress (Ojaimi et al, 1999). Immunohistochemical data have demonstrated many of the hallmark modifications of oxidative damage in AD brain, including the presence of proteins modified with advanced glycation end products (AGE; Takeda *et al*., 1998), malondialdehyde, 8-hydroxy-deoxyguonosine, 4-hydroxynonenal (Markesberry and Carney, 1999) and nitrotyrosine (Good *et al*., 1996), along with increased amounts of lipid peroxidation (Markesberry and Carney, 1999). Oxygen free radical stress not only leads to direct cellular injury, it may also influence neuronal integrity by triggering redox-sensitive, NF-κB-mediated transcription of various pro-inflammatory and apoptosis-related genes in surrounding cells (Kaltschmidt et al., 1997). Although the majority of research on AD oxidative stress had focused on the possibility of free radical generation by affected neurons (Behl, 1997; Mattson and Pederson, 1998) as a result of impaired electron transport chain function during mitochondrial oxidative metabolism, the concept of free radical toxicity actually has its roots in inflammation processes, where the secretion of ROS and nitrogen radicals by inflammatory cells is a major mechanism for attacking opsonized target. Alternative sources of free radicals in the AD brain are therefore likely to induce activated microglia (see: Cellular mediators of inflammation in the AD brain), which have the potential to produce large amounts of ROS as a result of activation of the NADPH complex (Della-Bianca *et al*., 1999).

Neuroinflammation in AD

A virtual textbook of inflammatory mediators has been observed in the Alzheimer's disease brain over the last 15 years. These mediators are typically undetectable or background levels in samples from non-demented elderly persons, and have been investigated at immunohistochemical, biochemical, and molecular levels. As with all new developments in science, perception of these findings has evolved over time. Initially, resistance was high. Many of the early results were dismissed as artifact, an impossibility given in the ''immunologic privilege'' of the brain (Hickey and Kamura, 1988; Weckerle *et al*., 1986, 1987; Aisen and Davis, 1994; McGeer and McGeer, 1995). It is now clear that the brain may have many unique immunologic properties, but it is by no means an immunologicalyy isolated organ.

With this understanding, new challenges have arisen. Are inflammatory mechanisms actually causing damage in AD or they present merely to remove the detritus from other, more primarily pathologic processes? Are anti-inflammatory drugs a viable therapeutic option for AD?

Cellular mediators of inflammation in the AD brain

Microglia

Activated microglia clusters at sites of aggregated Aβ deposits and deeply interdigitate neuritic plaques. Since microglial cells are related to peripheral macrophages (Ling *et al*., 1993), they are one of the most obvious targets for research in AD inflammation. Although in the normal brain microglia also play neurotrophic roles (Streit *et al*., 1999), their potential neurotoxic action have been emphasized in AD research. Once activated *in vitro*, microglia are capable producing a variety of pro-inlammatory molecules (McGeer *et al*., 1989) and potentially neurotoxic substances (Banati *et al*., 1993) that could contribute to localized or more widespread CNS injury. These include complement, cytokines, ROS, secreted proteases, glutamate and NO (Banati *et al*., 1993). Moreover, Aβ stimulates microglial production of IL-1, IL-6, TNF-α, MIP-1α, MCP-1, and ROS (Cotman and Tenner, 1996; Giulian *et al*., 1996). In addition, activated microglia release the excitotoxins glutamate (Piani *et al*., 1992) and quinolinic acid (Espey *et al*., 1997). Exposure to those substances may not necessarily kill the entire cell, however, could result in a disastrous degree of dentritic pruning (Mattson and Barger, 1993), thereby leading to cognitive impairment that is not unavoidably related to

neuronal loss. Moreover, AD patients show elevated levels of glutamate in CSF (Jiménez-Jiménez *et al.*, 1998).

AD microglia reportedly upregulate their expression of the macrophage scavenger receptor (MSR; El Khoury *et al*., 1998) and RAGE (receptor for AGE; Yan *et al*., 1996), both of which may have Aβ as ligands. It has also been shown that adhesion of microglia to Aβ fibrills *via* scavenger receptors lead to immobilization of the cells and induces them to produce ROS (El Khoury *et al*., 1998). Moreover, there are findings that support a role for microglia in transforming diffuse plaques into neuritic plaques (Cotman and Tenner, 1996; Mackenzie *et al*., 1995), probably a key process in AD pathology.

Astrocytes

Like microglia, astrocytes are found at sites of Aβ deposition, but in contrast the positioning of astrocytes in plaques differs from that of microglia. Astrocyte somas form a corona at the perimeter of the neuritic halo that, in turn surround a dense core Aβ deposit (Mrak *et al*., 1996). Astrocytes may impair the natural ability of microglia to clear plaques (Snow *et al*., 1988). In addition, astroglial cells are capable to produce complement factors (Gasque *et al*., 1998), an ApoJ/ApoE complex (LaDu *et al*., 1998), IL-1 (Del Bo *et al*., 1995), IL-6 (Van Wagoner *et al*., 1999), S100-β (Van Eldik *et al*., 1994), TGF-βs (Kriegelstein *et al*., 1995), α_1-ACT (Das *et al*., 1995), ICAM-1 (Akiyama *et al*., 1993), prostaglandins and COX-2 (Bauer *et al*., 1997), and iNOS (Lee *et al*., 1993) upon activation.

Neurons

Perhaps surprisingly, neurons themselves have been consistently identified as a cell type capable of producing inflammatory molecules mediators, including complement (Shen *et al*., 1997), COX (Oka and Takashima, 1997), the cytokines IL-1, IL-6 and TNF-α as well as their receptors (Yan *et al*., 1995) and M-CSF (Yan *et al*., 1997). It is therefore possible that neurons themselves may exacerbate inflammatory reactions in their vicinity and thus contribute to their own destruction in AD.

<u>Inflammatory pathways in the AD brain</u>

Just as damaged tissue and the chronic presence of highly inert abnormal materials are classical stimulants of inflammation in the periphery, thus Aβ, tangles, and neurodegeneration

are the most likely sources for inflammation in the AD brain. From there, as in the periphery, a nearly bewildering number of inflammatory subsystems are involved, each characterized by an abundance of amplifying and dampening loops, as well as multiple interactions with the other subsystems.

Complement

The classical component pathway is made up of some 20 or more components, many of them serine proteases that can be sequentially activated as an amplifying cascade.

Some complement factors (C5b, C6, C7, C8 together with multiple C9 components) may form the membrane attack complex (MAC). When assembled on a cell membrane, this macromolecular terminal reaction complex forms a ring-like structure. The transmembrane channel caused by MAC assembly at the cell surface permits the free diffusion of ions and small molecules into and out of the cell, disrupting cellular homeostasis, particularly Ca^{2+} homeostasis, and ultimately resulting in cell lysis in case a sufficient number of MAC complexes have assembeled on the cell. Notably, the MAC can also induce bystander lysis of healthy adjacent tissue (Neuroinflammation Working Group, 2000).

β-pleated, fibrillar Aβ (Webster *et al*., 1997) and, more recently, τ-containing neurofibrillary tangles (Rogers *et al*., 1998) have been shown to directly activate the classical complement pathway fully *in vitro*, and to do so in the absence of antibody. For Aβ the details of binding and activation are relatively clear. A 13-15 amino acid sequence on the human C1q A-chain collagen-like tail contains five cationic side chains that bind in a charge-based fashion to anionic side chains along the N-terminus of human Aβ (Jiang *et al*., 1994). Notably, rodent Aβ differs from human Aβ by three amino acids in the N-terminal region (Velazquez *et al*., 1997) and mouse C1q lacks two of the positively charged residues in the A-chain site critical for Aβ/C1q binding (Jiang *et al*., 1994). The latter finding may have implications for transgenic mouse models of AD that express human Aβ but do not express human C1q. Human Aβ activates human C1 more efficiently than mouse C1 (Webster *et al*., 1999).

By binding multiple Aβ molecules or by stabilizing already-formed oligomeres of Aβ, C1q appears to nucleate the formation of Aβ fibrills (Webster *et al*., 1999). In addition to Aβ aggregates and neurofibrillary tangles, other potential sources for classical pathway activation exist in the AD brain. Neurodegeneration, for example, may ultimately expose DNA and

neurofilaments to the extracellular environment, thereby activating complement cascades (Linder *et al*., 1979).

Initiation of the complement induces chemotactic and activating signals to inflammatory cells bearing appropriate receptors, and so do microglia and astrocytes (Gasque *et al*., 1998). Therefore, reactive astrocytes and activated microglia are all highly co-localized with plaque containing aggregated Aβ (McGeer *et al*., 1989). Neurons also reportedly express receptors for C3a and C5a (Davoust *et al*., 1999), but the functional significance of the finding has not been conclusively determined.

Upregulation of complement defense proteins, including C4 binding protein (C4bp), vitronectin, and clusterin (apolipoprotein J; ApoJ), also occurs in the AD brain. ApoJ has been shown to bind Aβ, potentially facilitaing Aβ transport across the cerebrovasculature (Zlokovic *et al*., 1996). Although the receptors for ApoJ transport are not well understood at this time, they may include an endocytosing member of the low density lipoprotein (LDL) receptor family (Chun *et al*., 1999).

Cytokine and chemokine pathways

IL-1 is an immunoregulatory cytokine that is overexpressed within affected cerebral cortical regions of the AD brain (Griffin *et al*., 1998). IL-1 overexpression seems to occur early in plaque evolution, suggesting that it plays a orchestrating role in plaque formation. It is already evident in diffuse, non-neuritic Aβ deposits, and can be observed in autopsied brain samples from fetuses and young children with Down's syndrome (Griffin *et al*., 1995). In particular, IL-1 promotes the synthesis (Mackenzie, 2000) and the processing (Buxbaum *et al*., 1992) of APP and may therefore promote further amyloid production and deposition in plaques. A reciprocal relationship also seems to exits wherein the secreted form of APP (sAPP) activates microglia and induces excessive production of IL-1 (Barger and Harmon, 1997). Moreover, IL-1 activates astrocytes to express several acute phase and/or Aβ-binding proteins, including α_1-antichymotrypsin (α_1-ACT; Das *et al*., 1995), ApoE (Das *et al*., 1995) and complement factors (Barnum *et al*., 1994). One of the most important trophic actions of IL-1, however, is induction of S100β overexpression, a neurite growth promoting cytokine, by reactive astrocytes (Sheng *et al*., 1996). A significant correlation between cross-sectional area of dystrophic neurites in Aβ plaques and the number of plaque associated S100β immunoreactive astrocytes, suggesting that its neurite growth-promoting action is directly reponsible for dystrophic neurite growth near Aβ deposits (Mrak *et al*., 1996). Finally, IL-1

may also influence AD patho-pharmacology by its ability to induce expression and activity of acetylcholineesterase (AChE). Presumably such an effect would exacerbate cholinergic decline and dysfunction in AD.

IL-6 is a pleitropic cytokine that mediates immune response and inflammatory reactions affecting CNS cell growth and differentiation (Hirano *et al*., 1997). Normally at barely detectable levels in the adult CNS, it is strongly induced under pathological conditions (Vallieres and Rivest, 1997) and induces acute phase reactants, act as a major pyrogen, increases vascular permeability, lymphocyte activation and antibody synthesis (Strauss *et al*., 1992). IL-6 seems to modulate APP synthesis and may also act on neurons, inducing the expression of acute phase proteins α_2-macroglobulin (α2-MAC) and metallothionine (Bauer *et al*., 1993). It has been suggested that IL-6 may appear before neuritic changes rather than neuritic degeneration. In the CSF, AD patients exhibit significantly decreased concentrations of soluble IL-6 receptors compared to healthy, age-matched controls, whereas IL-6 protein levels are unaltered (Hampel *et al*., 1998). Interestingly, patients with multiple sclerosis, like AD patients, show decreased CSF soluble receptor levels (Padberg *et al*., 1999).

Elevated TNF-α levels in AD serum (Fillit *et al*., 1991), CSF, cortex, and glial cell cultures after exposure to Aβ (Tarkowski *et al*., 1999) were found. The pathophysiologic action of TNF-α in AD are controversial given its role as a potent pro-inflammatory, cytotoxic polypeptide in the periphery and in such other CNS disorders as brain trauma (Mullberg *et al*., 1995), multiple sclerosis (De Vos *et al*., 1998), and Parkinson's disease (Hsu *et al*., 1996). Low levels of TNF-α can act neuro-protective (Bruce *et al*., 1996), however, excess of TNF-α is able to kill human cortical neurons (Good *et al*., 1996; McKee *et al*., 1998). Activation of neurons by TNF-α induces the expression of protective molecules, including manganese superoxide dismutase (SOD). In contrast, TNF-α is a potent stimulator of NF-κB, that elevates the expression of pro-inflammatory factors such as complement and cyclooxygenase (COX; Cardinaux *et al*., 2000), as well as survival factors such as Bcl-2 (Tamatani *et al*., 1999).

TGF-βs are multifunctional peptide growth factors that play pivotal roles in tissue development, homeostasis and repair (Kriegelstein *et al*., 1995). TGF-βs have been shown to modulate a wide range process that are implicated in AD, including brain injury, brain

inflammatory response and microglial activation, extracellular matrix production, accumulation and regional distribution of amyloid, regulation of known and potential AD risk factors (e.g., APP (Monning *et al*., 1994), α_2-MAC (Webb *et al*., 1994), and COX-2 (Luo *et al*., 1998)), and inhibition of cell death (Böttner et al., 1996; Unsicker *et al*, 1991). TGF-β1 has been detected in plaques, and higher TGF-β1 levels were found in cerebrospinal fluid (Chao *et al*., 1994a) and serum (Chao *et al*., 1994b) of AD cases than in nondemented controls.

Although well known as an anti-inflammatory cytokine, TGF-β also exerts pro-inflammatory effects in certain pathological conditions. In addition, TGF-β1 is a potent chemo-attractant for microglia, stimulates prostaglandin-E_2 (PGE_2) synthesis, increases COX-1 expression in astrocytes and COX-2 expression in both astrocytes and neurons (Luo *et al*., 1998). Altered levels of TGF-β could be responsible for the elevated levels of COX observed in AD (Ho *et al*., 1999). Experiments with transgenic mice identified TGF-β1 as an inducer of vascular amyloid deposition and modulator of Aβ accumulation in the neuropil (Wyss-Corey *et al*., 1997). TGF-β protected against Aβ and glutamate neurotoxicity in cell culture possibly by upregulating antiapoptotic or calcium-stabilizing factors such as Bcl-2, Bcl-$_{XL}$, and calbindin. Alternatively, TGF-β protection against Aβ toxicity may be limited to paradigms involving only short term Aβ exposure, as long term exposure has the opposite effect (Prehn *et al*., 1996).

Blood coagulation and fibrinolysis systems

Originally discovered as mechanisms that regulate the flow and coagulation of blood in the vasculature and sites of vascular injury, the blood coagulation and fibrinolysis system have recently been recognized as playing important roles in inflammatory and tissue repair processes in extravascular tissues. Like tissue factor and thrombin, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), and plasminogen activator inhibitor type I (PAI-I) are present in senile plaques (Rebeck *et al*., 1995). Interestingly, a secreted form of APP with a *Kunitz*-type inhibitor domain is identical to protease nexin-2, a potent inhibitor of factor IX and XI. Factor XIIIa found in reactive microglia in AD brains may function as a transglutaminase to cross-link cytoplasmic proteins upon apoptotic cell death (Greenberg *et al*., 1991).

Acute phase proteins

The acute phase proteins are a diverse set of molecules that arise early in inflammation as the acute phase response. Like many other inflammatory mediators, a wide range of acute phase reactants has been found in association with senile plaques and extracellular neurofibrillary tangles. α_1-ACT is consistently co-localized with Aβ deposits in the AD brain, and has been suggested to play a role in their formation by enhancing the conversion of nonfibrillar forms of the molecule to Aβ fibrills (Eriksson *et al*., 1995). Polymorphisms in α_1-ACT, the most common of which is located in the signal peptide, have been investigated as risk factors for AD in several studies; most of them found a weak association (Kamboh *et al*., 1995).

α_2-MAC is a potent, broad spectrum protease inhibitor, probably evolved as primitive host defense systems. The formed protease/α_2-MAC complex is removed by endocytosis following binding of this domain to the α_2-MAC receptor/LDL receptor-related protein (α_2-MACR/LRP). This systems was shown to serve as a clearance system for inflammatory proteins, including α_2-MAC itself, ApoE, APP, IL-1β, TGF-β and several others (Borth, 1992). α_2-MAC and its receptor have been implicated in several AD pathophysiological processes and was detected in neuritic plaques and neurofibrillary tangles (Strauss *et al*., 1992). Aβ also reportedly forms a complex with α_2-MAC that is subsequently removed by α_2-MACR/LRP endocytotic clearance mechanisms (Narita *et al*., 1997). Recently, a new locus for familial Alzheimer's disease (FAD) had been proposed on chromosome 12, where both α_2-MAC and α_2-MAC/LRP genes are located (Pericak-Vance *et al*., 1997).

sAPP bears a number of properties in common with acute phase proteins. It is elevated at sites of tissue damage, and its synthesis and release are at least partly mediated by pro-inflammatory stimuli as IL-1β (Buxbaum *et al*., 1998). When applied to microglia at subnanomolar concentrations, sAPP stimulates NF-κB activity, IL-1 and inducible nitric oxide synthase (iNOS) expression, and neurotoxicity (Barger and Harmon, 1997). This pro-inflammatory activity of sAPP is inhibited by binding to ApoE, with ApoE3 being more effective than ApoE4.

A short graphical summary of the main neuroinflammatory events taking place in AD pathology shows Figure 82 at the end of thesis.

Anti-inflammatory drug studies in AD

Anti-inflammatory therapy should be beneficial in delaying the onset or slowing the progression of AD, assuming the neuroinflammation in the neurodegenerative disorder. Epidemologic retrospective analysis showed a clear association between the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and relative risk for AD. However, all recent intervention trials have only been able to slower the progression of AD by about one year and failed to indicate a relevant therapeutic benefit after the clinical onset of AD (Neuroinflammation Working Group, 2000). AD inflammation is chronic but weak, accumulating significant damage only over many years duration. Interestingly, anti-inflammatory drugs like NSAIDs, including COX-2 inhibitors, cyclosporin and FK-506 reduce not only glial activation but do also reduce the levels of Aβ (Lee and Wurtmann, 2000).

The major epidemiological studies concerning anti-inflammatory treatment are summarized in the following table.

study	criteria	relative risk/ results/ outcome
Inferred anti-inflammatory treatment		
Case control studies. No differentiation between osteoarthritis and rheumatoid arthritis		
Heyman *et al*., 1984	40 AD patients	1.19 for arthritis
French *et al*., 1985	78 AD patients in Minneapolis/ USA	0.62 for arthritis
Broe *et al*., 1990	178 AD patients in Australia	0.56 for arthritis
Li *et al*., 1992	70 AD patients and 140 control from the same neighborhood in China	0.16 for arthritis
The Canadian Study of Health and Aging, 1994	201 AD patients matched with 468 controls in Canada	0.54 for arthritis
Breitner *et al*., 1994	26 elderly twin pairs in whom one twin developed AD 3 or more years before the other	0.64 for arthritis
Breitner *et al*., 1995	sibships with differential onset of AD	0.45 for arthritis
Case control studies focused on rheumatoid arthrits		
Graves *et al*., 1990	130 AD patients and 130 matched controls from Seatlle/ USA	1.18 for arthritis
Jenkinson *et al*., 1989	92 AD patients and 92 non-AD patients	0.17 for arthritis
population based reports		
Beard *et al*., 1991	rheumatoid arthritis patients, no age specified	4.4% higher prevalence for dementia
McGeer *et al*., 1996	Only low potent salicylates were used for the treatment	only four cases of dementia
McGeer *et al*., 1990	973 established rheumatoid arthritis patients age over 65 in the US	0.39% with co-diagnosis of dementia
McGeer *et al*., 1990	7490 patients over 65 with diagnosis of rheumatoid arthritis in Canada/ USA	

Myllykangas-Luosujarvi and Isomaki, 1994	government statistics from Finland on causes of death rheumatoid arthritis general population	 0.12% 0.54%
NSAIDs		
<u>case control epidemiological surveys</u>		
McGeer *et al*., 1996	Four case control studies (The Canadian Study of Health and Aging, 1994; Breitner *et al*., 1994; 1995; Stewart *et al*., 1997). Risk for AD under NSAID users.	0.475 for AD
In't Veld *et al*., 1998 and Stewart *et al*., 1997	Risk for AD under NSAID users	prolonged use of NSAIDs further reduces th risk for AD
<u>population based studies</u>		
Lucca *et al*., 1994	Review of the data from two clinical AD trials in Italy. Percentage of NSAID user among AD patients general elderly patients	 0.8%; 0.0% 22.8%, 20.3%,18.5%
Andersen *et al*., 1995	NSAID users age 55 or over suffering from AD in a total cohort of 5893 in Rotterdam: NSAID users non-NSAID users	 1.4% 2.5%
Rich *et al*., 1995	Comparsion of 32 AD patients who used NSAIDs with 177 non-NSAID users with AD in Baltimore/ USA over one year	NSAID user exhibited significantly slower disease progression

post mortem evaluations			
Mackenzie and Munoz, 1998	brain pathology of NSAID users was compared with age matched controls	Similar degree of senile plaque and neurofibrillary tangle pathology, NSAID use was associated with a significant reduction in the number of senile plaques-associated, activated microglia.	
Steroid anti-inflammatory agents			
epidemiological studies			
The Canadian Study of Health and Aging 1994, Breitner *et al*., 1995, Graves *et al*., 1990	risk of steroid users was compared with controls	failed to obtain a significant odds ratio indicative of a preventive action against AD	
Breitner *et al*., 1994	risk of steroid users was compared with controls		odds ratio 0.25 for AD
post mortem evaluation			
Mackenzie and Munoz, 1998	evaluation of activated, plaque-associated microglia in non-demented elderly humans		no effect of steroids

Table 1. Summary of epidemiological studies about the involvemnt of NSAIDs use and deceased risk for AD.

A summary of the major neuroinflammatory events taking part in AD pathology is found in Figure 82. After all, it seems that AD is not associated to only one of the above mentioned conditions, but a multi-factoral disorder where all of thses risk factors and/ or familail predispositions are involved. However, the major risk for getting AD is increasing age.

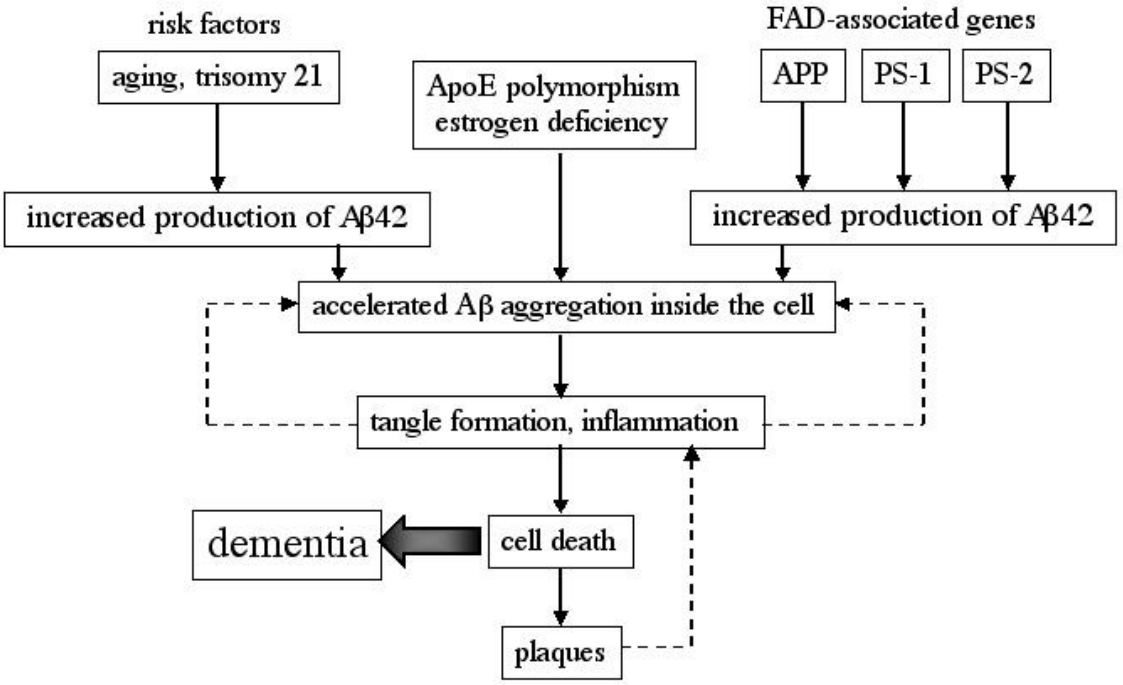


Figure 6. Scheme of AD pathology. Several factors like age or associated genes induces neurodegeneration mainly via apoptosis contributing to dementia (according to Haass, 1997).

Peripheral immuno-competent cells

The immune system can be divided into the innate and the adaptive immunity. Cells of the innate immunity are granulocytes, natural-killer (NK) lymphocytes and monocytes. Monocytes can differentiate into macrophages upon stimulation and infiltrate various tissues. Monocytes and microglia origin from the same progenitor cells. Moreover, the complement belongs to innate immune system. The adaptive immunity involves the T- and B-lymphocytes, whereas the T cells trigger mostly of the cellular immunity while the humoral immunity is mainly carried out by B cells.

Density centrifugation of whole blood enriches a fraction of the leukocytes, the so-called peripheral blood mononuclear cells (PBMC) that consists of T lymphocytes, mainly, and to a lesser content of NK- and B-lymphocytes and some monocytes.

T lymphocytes ($CD3^+$) gained their name as they developed in the microenviroment of the thymus, an organ located in the mediastinum anterior to the heart. T cells can be divided into two subsets: the T helper/ inducer or according to their antigen $CD4^+$ cells and T cytotoxic/ suppressor or $CD8^+$ cells. $CD4^+$ T lymphocytes are stimulated by MHC-II positive e.g. professional antigen presenting cells for example macrophages that phagocytize non-self particles and presents them via MHC class II. In addition, $CD4^+$ T cells induce humoral immunity by binding to appropiate B cells ($CD19^+$). In contrast, $CD8^+$ T lymphocytes recognize antigens which are presented on MHC-I, therefore playing an important role in natural anti-viral and tumor defense. Subsequently to binding to a non-self 'foreign' antigen presenting cell, the cell is killed or apoptosis is induced by the cytotoxic T lymphocyte (Janeway and Travers, 1997). Under certain condition, $CD8^+$ lymphocytes are capable of inhibiting immune response in an antigen-specific manner (Niijkamp and Parnham, 1999).

Binding of naive T cells to their specific MHC-I or MHC-II presented antigen *via* the T cell receptor (TCR) and in combination with some costimulatory receptors forms the immunological synapse. Here, the two cells are getting in very close and tight contact. Simillar to a neurological synapse, information is transmitted via secreted molecules (cytokine). Subsequently, the TCR autophosphorylates its intracellular domain, and the signal is further mediated by the Ras/Raf-kinase cascade, protein kinase C and phospholipase activation (see Figure 54). The naive T lymphocyte differentiate into an effector cell, that execute its immunological function. Most T cells are eleminated by apoptosis after the

infection in order to terminate the immune response, however, some T cells survive as memory T cells (Janeway and Travers, 1997).

B cells are capable to produce different classes of immunoglobins and are MHC-II positive. B lymphocyte activation is initiated by specific-recognition of antigen receptor, i.e. membrane-bound immunoglobins or via interaction with $CD4^+$ T cells. Similar to memory T cells, some B cells resist as plasma cells (Janeway and Travers, 1997).

NK cells are lymphocytes with cytotoxic potential against virus-infected and certain tumor-transformed cells. NK cells differ from cytotoxic T lymphocytes in their human lymphocytte antigen (HLA)-independent manner of target-cell recognition. However, both NK and cytotoxic T lymphocytes carry the CD8 antigen (Janeway and Travers, 1997).

Apoptosis of peripheral immune-competent cells

The immune system is an example of a system in which programmed cell death (PCD) is a major mechanism of regulation. In the lymph nodes, PCD is a basic mechanism that allows the termination of the normal immune response and the prevention of the formation of activated autoimmune B and T cells. The mechanism whereby apoptotic cells are efficiently identified, removed, and degraded by phagocytes in mammalian cell are not well understood, scavenger receptors, CD14 or the receptor for oxidized LDL are supposed to be involved (Mevorach, 1999). Phospahtidyserine appears on the outer membrane upon the initiation of apoptosis, and seem to be a part of recognition of apoptotic cells and their fragments.

Although cell death by apoptosis is measured in hours, the removal of apoptotic fragments is normally so rapid that apoptotic cells are rarely seen, even in tissues such as thymus where up to 95% of cells undergo apoptosis (Surh and Sprent, 1994). It is thought that uptake of these cells by specific receptors in phagocytes results in disposal of cellular contents without induction of inflammation (Savill *et al*., 1993).

Immunogerontology

Gerontology, especially immunogerontology, is becoming a more and more important discipline in medicine, since more people are getting older in the industrialized countries. A decline of some immune function, termed immunosenescence, is known since decades (Guidi *et al*., 1998), mainly involving specific immunity. The content of autoreactive antibodies raises with increasing age and thereby enhances the risk for immuneregulatory dysfunction

(Hijmans *et al*., 1984). Other immune functions (e.g. $CD25^+$ T cells, $CD50^+$ T cells, density of CD45RA or CD45RO on T cells, CD4/CD8-ratio) remain unmodified with aging (Ginaldi *et al*., 2000; Yen *et al*., 2000). However, the causes for the immunosenescence remain unclear. Membrane changes taking place in the aging process are involved in altered binding of hormones, antigens, and cytokines (Woda *et al*., 1979) as well as in defective transmission through ion channels (Griffith *et al*., 2000). Moreover, other theories explain declined cellular activity within aging by increased oxidative stress (Guyton *et al*., 1998). Previous studies in lymphocytes revealed elevated byproducts caused by ROS (e.g. lipidperoxidation (Hendricks and Heidrick, 1998), oxidized proteins (Berlett and Stadtman, 1997; Stadtman, 1992), and DNA-damage (Wei *et al*., 1998)). Altered and impaired signal transduction in general seems to be a major cause for impaired cellular function in all aged tissues (Müller *et al*., 1996; Eckert *et a*l., 1994). The performance of the immune system is closely related to the health situation of the individual. Longevity is associated with appropriate immune function (Sansoni *et al*., 1997; 1992; Franceschi *et al*., 1995). Moreover, recent research indicates that the immune system is not autonomously. It interacts with the whole organism in bi-directional ways (Guidi *et al*., 1998). Close interaction of the central nervous system (CNS) and immune cells become more evident in the recent years. Immune cell function is modulated by neuroendocrines and as well by neurotransmitters and vice a versa (Carson and Sutcliffe, 1999; Hanisch *et al*., 1996). In addition, neurodegenerative diseases like dementia of Alzheimer type (DAT (Eckert *et al*., 2001; 1998)), Down's Syndrome (DS (Park *et al*., 2000)) and the dymyelating disease multiple sclerosis (MS (Crucian *et al*., 1995a)) are accompanied by altered lymphocyte function and apoptosis. Chronic psychosocial and emotional stress is associated with declined and/ or impaired immune function (Berkenbosch *et al*., 1991).

The clinical relevance of immunosenescence is quite high, since impaired function of the immune network contribute to pathologies like cancer, increased susceptibility to infectious diseases and autoimmune disorders, cardiovascular diseases and the not clearly defined general multimorbidity (Bruunsgaard *et al*., 2000a). Moreover, a prospective study emphasized the close relation between decreased T cell proliferation after mitogenic activation and increased mortality (Murasko *et al*., 1987).

The relevance of physiological immune aging is, in addition, of great interest with respect to determine disorders with pathologic immune function in aging individuals. However, there are growing evidences that neurological disorders are often associated with changed immune

properties. Immune cells provide a good accessibility and are therefore a useful tool to explore the mechanism taking place within this disorders

AD and peripheral immune cells

Peripheral blood cells provide a good accesibility and are therefore used as cell model in several studies determing molecular mechanism of AD pathology (Monning *et al*., 1990, 1992; Bauer *et al*., 1991; Eckert et al, 1998). Moreover, as lymphocytes are detected in AD brain and are undetectable in healthy aging brains, they seem to be an interesting target for further research (Itagaki *et al*., 1988; Rogers *et al*., 1988).

There are some studies that show altered muscarinic receptor signal transduction in AD lymphocytes: Density (B_{max}), affinity (K_d), and norepinephrine-stimulated cyclic adenosine monophosphate (cAMP) generation of the β-adrenergic receptor on lymphocytes in patients with dementia of the Alzheimer type and in normal aged controls showed no differences (Gietzen *et al*., 1989). However, the B_{max} of ^{3}H-N-methyl-scopolamine (^{3}H-NMS) was significantly decreased both in early and late onset AD groups as compared with age-matched controls, whereas the K_d was the same in AD and control group. In addition, the average B_{max} in early AD was significantly lower than in late AD. The density of the binding of ^{3}H-NMS was also significantly lower in a subgroup of old subjects with Down's syndrome, whereas no changes were found in younger individuals with DS or in patients with multi-infarct dementia. In the AD group, the difference in binding sites was unrelated either to the severity of dementia or disease duration. Treatment of the patients with cholinergic agents did not alter the binding values in any of the examined group (Ferrero *et al*., 1991).

Moreover, Aβ is detectable on peripheral circulating monocytes, is upregulated upon activation with pyrogen (lipopolysaccharide, LPS) and its expression is increased in AD (Jung *et. al*., 1999).

Levels of APP in PBMC are increased in aging, AD and in Down's syndrome (Pallister *et al*, 1997). Lymphocyte APP was non significantly higher in AD vs. aged controls. In addition, the ratio of lymphocyte APP751:APP770 mRNA levels was significantly lower in AD subjects compared to a healthy old cohort. This decreased ratio is most likely due to an average 31% increase in the lymphocyte APP770 isoform in AD patients compared to 12% in the old cognitively intact group (Ebstein *et al*, 1996).

Soluble Aβ induces IL-2 receptor expression and proliferation in peripheral T cells from healthy individuals, but not from patients with AD (Trieb *et al*, 1996). Moreover, human dendritic cells (DC), propagated from the peripheral blood of healthy individuals, were incubated with $A\beta_{25-35}$ and no apoptosis or necrosis, production of TNF-α was induced. DC pulsed with Aβ aggregates were unable to stimulate T cells in an autologous coculture system. The results demonstrate that amyloid may escape immune recognition by its failure to activate antigen-presenting cells and by inhibiting MHC class II surface expression (Schmitt *et al*, 1997).

Consistent with observations of reduced depolarization-induced Ca^{2+} rises in dissociated neurons of aged mice, corresponding age-related changes of reduced mitogen-induced Ca^{2+} responses were observed both in mouse lymphocytes and, more importantly, in circulating human lymphocytes (Eckert *et al*., 1994). Beta-amyloid or its fragment, 25-35, interacts with neuronal calcium regulation. Moreover, exposure of Aβ to peripheral lymphocytes elevated the phytohemagglutinin (PHA)-induced Ca^{2+} mobilization (Eckert *et al*., 1993). This amplifying effect of Aβ on the mitogen-induced rise of free intracellular calcium in circulating lymphocytes was strongly reduced in AD patients. Moreover, low Aβ responses were significantly correlated with the presence of the ApoE4 (Eckert *et al*., 1995).

Neither the basal $[Ca^{2+}]_i$, nor the activation-induced Ca^{2+} responses differed among neutrophils or lymphocytes from aged controls and AD patients. However, a delayed Ca^{2+} response was found in AD lymphocytes after PHA-stimulation (Eckert *et al*., 1997).

Apoptosis contributes to neuronal death in AD. Particularly in lymphocytes, apoptosis plays an important physiological role, as explained above. The susceptibility to apoptosis seems to altered in PBMC from AD patients compared to non-demented controls (Eckert *et al*., 1998a; Eckert *et al*., 1998b; Eckert et al, 2001a). Interestingly, lymphocytes of AD patients are more vulnerable to oxidative-stress induced apoptosis.

Aim of the thesis

The research concerning Alzheimer's disease is one of the most dynamically changing and controversy fields within medical science in the last decades. Still little final data are known about the molecular mechanisms of this disorder. Moreover, AD occurs only in aging humans, so that no animal model is available. For long time, the only possible material for research was *post-mortem* brain tissue of AD patients. However, no functional studies are possible in dead tissue, only the status-quo of just prior to death situation could be assessed. Brain cells of alive AD patients are inaccessible for biochemical studies. Thus newer developments of transgenic animals bearing AD-associated mutations seem to be promising. In addition, another focus in AD investigations was cell culture with neuronal cell lines or primary cell transfected with AD risk genes. Most neural cell lines derive from neoplastic origin, while most primary neuronal cells are made from embryonic/ fetal tissue. Both models have intrinsic problems for AD research. It is not associated with a neoplastic situation and is definitely a disorder restricted to the aging process. Embryonic brain cells may not harbor the gene constellation that is required for AD. Thus the AD patient appears to be the best model for investigating this disorder. Therefore, our laboratory used peripheral blood cells of AD patients as model to investigate membrane changes, impaired Ca^{2+} homeostasis (Eckert *et al.*, 1993), Aβ toxicity in AD and apoptosis in peripheral cells from AD patients.

The aim of this thesis is based on previous findings indicating an enhanced apoptosis of peripheral blood cells in patients suffering from sporadic AD (Eckert et al., 1998). These changes should be investigated in more detail and characterized more firmly with new assays, and should again be compared with aged lymphocytes from non-demented control persons. A major focus is to find the cellular target and the mechanisms for altered apoptotic regulation in AD.

Material

Apparatus:

Agitator, type REAX 2000, Heidolph, Merck Eurolab GmbH, D-60487 Frankfurt

Biomax™ TranScreen-LE, Eastman Kodak, Rochester, New York, USA

CO_2-incubators Heraeus, Typ BB 6220, Heraeus Instruments GmbH, D-63450 Hanau

Coverslips for haemacytometer (Neubauer chamber), Superior, Merck Eurolab GmbH, 60487 Frankfurt

Culture dishes and flasks, Corning® 25 cm^2, Polystyren (tissue culture treated), Dunn Labortechnik GmbH, D-53567 Asbach

Developer, Replenisher, GBX reagents, Eastman Kodak, Rochester, New York, USA

Distillery for $aqua_{dd}$, type Destamat Bi 18E, Heraeus Quarzglas, D-63801 Kleinostheim

FACSCalibur™ (E1034), Becton Dickinson, D-69126 Heidelberg

Horizontal slab gel electrophoresis apparatus 10 x 15 cm, PeqLab Biotechnologies GmbH, D-91058 Erlangen

Incubator for bacteria, Kelvitron kl. Heraeus Instruments GmbH, D-63450 Hanau

Inverse microscope, Modell TMS, type I04, Nikon, Japan

Laminar flow hood, Heraeus InstrumentsGmbH, D-63450 Hanau

Latex examination gloves, Ansell GmbH, D-81829 München

Liquid scintilattion counter, Wallac 1409 Berthold, Turku, Finland

Magnetic agitator, type Poly 15, H + P Labortechnik, Merck Eurolab GmbH, 60487 Frankfurt

Microtiter plates IWAKI, 96 well with flat bottom, Dunn Labortechnik GmbH, D-53567 Asbach

Microtiter plate, U-shaped botttom, sterile, Greiner Labortechnik, D-72636 Frickenhausen

Microcentrifuge, type GS-6R Centrifuge, Beckman, D-47807 Krefeld

Monovette® (blood collection systems), NH_4-Heparin 9 ml, Sarstedt, D-51588 Nümbrecht

Multifly® cannula-Set 21G, 0,8 mm, 30 cm tube, Sarstedt, D-51588 Nümbrecht

Multipette® plus 4981, Eppendorf, Merck Eurolab GmbH, D-60487 Frankfurt

Neubauer chamber Superior, Merck Eurolab GmbH, D-60487 Frankfurt

Parafilm® M, Merck Eurolab GmbH, D-60487 Frankfurt

pH-meter, type CG 825, Schott, D-65719 Hofheim

Pipetus®-akku, Hirschmann, Merck Eurolab GmbH, D-60487 Frankfurt

Plastibrand® Tip-Rack, 5 – 300 µl, Brand, Merck Eurolab GmbH, D-60487 Frankfurt

Pipettes model Pipetman 10, 20, 200, 1000 und 5000 µl, Abimed, D-40764 Langenfeld

Pipettes sterile packed 5, 10 and 25 ml, Greiner Labortechnik, D-72636 Frickenhausen

Power supplies for gel electrophoresis, PowerPac 300; 1000, respectively, Bio-Rad,D-80939 München

PP-tubes 15 ml and 50 ml, Cellstar®, Greiner Labortechnik, D-72636 Frickenhausen

Transferpette®-8, 10 - 100 µl, Brand, Merck Eurolab GmbH, D-60487 Frankfurt

Transferpette®-8, 30 - 300 µl, Brand, Merck Eurolab GmbH, D-60487 Frankfurt

UV/VIS-photometer, type U-2000 Spectrophotometer, Hitachi, D-40547 Düsseldorf

Vertical slab gel electrophoresis apparatus, Bio-Rad, D-80939 München

Scale, type Mettler AT 261 DeltaRange®, Mettler-Toledo, Greifensee, Schweiz

Water bath, type Thermomix 1441, B.Braun-Melsungen, D-40595 Düsseldorf

Water bath, type 1003, GFL, Merck Eurolab GmbH, D-60487 Frankfurt

X-ray films, Biomax™ MR films for gels with ^{33}P or ^{35}S samples, Biomax™ MS films for gels with ^{35}S samples in combination with screen, Eastman Kodak, Rochester, New York, USA

Chemicals:

7-Aminoactinomycin D (7-AAD), Moleculare Probes, Leiden, The Netherlands

Ammonium persulfate (APS), Merck Eurolab GmbH, D-60487 Frankfurt

Aqua bidest

CellWash®, Becton Dickinson, D-69126 Heidelberg

Citric acid-monohydrat, pro analysi , Merck Eurolab GmbH, D-60487 Frankfurt

Dimethylsulfoxide (DMSO), pro analysi, Merck Eurolab GmbH, D-60487 Frankfurt

Ficoll separating solution Seromed®, Biochrom KG, D-12247 Berlin

Dithiothreitol (DTT), Merck Eurolab GmbH, D-60487 Frankfurt

Fetal calf serum (FCS), GibcoBRL, Life Technologies GmbH, D-76131 Karlsruhe

Hydrochloric acid, HCl 37%, Merck Eurolab GmbH, D-60487 Frankfurt

Isopropanol, LiChrosolv® Gradient Grade, Merck Eurolab GmbH, D-60487 Frankfurt

$KHCO_3$, pro analysi, Merck Eurolab GmbH, D-60487 Frankfurt

methanol, Rotisolv® HPLC gradient grade, Roth, D-76185 Karlsruhe

L-(^{35}S)-methionine, Amersham Buchler; D-39528 Braunschweig

Ortho-phosphoric acid 85%, pro analysi, Merck Eurolab GmbH, D-60487 Frankfurt

PBS, Dulbeccos´s Phosphate Buffered Saline, GibcoBRL, Life Technologies GmbH, D-76131 Karlsruhe

Propidium iodide, Sigma Aldrich Chemie GmbH, 89552 Steinheim

RPMI 1640 medium with Glutamax-1, with 25MM HEPES, GibcoBRL, Life Technologies GmbH, D-76131 Karlsruhe

Sodium-monohydrogenphosphate-dihydrat, pro analysi, Merck Eurolab GmbH, D-60487 Frankfurt

N,N,N',N',-Tetramethylethylendiamin (TEMED), Bio-Rad,D-80939 München

Triton X-100, Merck Eurolab GmbH, D-60487 Frankfurt

Trypan-blue dye 0,4%, GibcoBRL, Life Technologies GmbH, D-76131 Karlsruhe

Urea, Sigma, St.Luois, MO, USA

Methods:

Unless otherwise noted, all preparation, extractions and incubations were proceeded in microcentrifuge tubes (0,2 ml; 1,5 ml; 2 ml). Small volumes were pipetted with automatical pipettes (Gilson/ Abimed: P2, P10, P20, P200, P1000, and P5000). All buffers and solutions were made from commercially available chemicals of analytical grade and purified (Millipore MilliQ-Plus) or double distilled water and were autoclaved or sterile filtered prior to use.

Human volunteers and animals:

Human blood samples:

All experiments regarding human subjects have the approval of an ethical commission (approval date: 11/ 1997; Dept. of Psychiatry, Univ. of Frankfurt, PD Dr. L. Fröhlich, Prof. Dr. K. Maurer). All venous blood samples were collected between 8.00 am and 11.00 am in 9 ml NH_4-heparin monovettes in order to prevent fluctuations due to circadian rhythmic. Samples were stored in the blood collection system with slight agitation until isolation. To prevent bursting of red blood cells, which elevates the extracelluar K^+ levels, blood samples were collected slowly.

Blood collection system:	
Monovettes (NH_4-heparin)	Sarstedt
Adapters	Sarstedt
Butterflies (Wing-Flo)	Braun

Non-demented control blood donors:

Blood samples were taken from healthy subjects less than 35 years old, termed young, and healthy persons over 60 years, termed old group. Subjects with psychiatric and neurodegenerative disorders or acute infections were excluded. In addition, subjects with pathological distribution of lymphocyte subpopulations in flow cytometric analysis according to the SENIEUR protocol (Ligthardt *et al.*, 1984) were rejected from the study. Most of the elderly patients were taking drugs against cardiovascular disorders. None of the volunteers received psychotropic medication, drugs with known effects on the immune system, or antioxidative agents.

AD-patients:

Blood samples were taken from patients diagnosed with possible DAT or probable DAT. Patients were randomized, blinded and blood samples collected by the clinicians of the Gedächtnis Ambulanz of University Frankfurt. Patients were rated by Mini Mental State Examination Scores (MMSS), Syndrom-Kurz Test (SKT) and Brief Cognitive Rating Scale (BRCS). Patients with acute infections were rejected from the study. Some of the AD-patients received anti-dementia drugs mainly nootropics or acetylcholine esterase inhibitors. None of the patients received drugs with known effects on the immune system or antioxidative agents.

Animals

All animals were housed in cages with a maximum of 10 animals per cage. Food and water were free accessible, room temperature and humidity was kept constantly. A light/ dark cycle of 12 hours is installed in the animal housing. All experiments described in this thesis are in concordance with the German law and had been approved by the Regierungspräsidium Darmstadt (committee regarding experimental animals, approval date 01/1998).

Cages	Altromin
Standardized diet for mice and rat	Altromin

NMRI mice

Young (3-4 months) and old (23-25 months) female NMRI mice used in this thesis were from Harlan Winkelmann or kind gifts from Aventis Pharma (Frankfurt, Germany). The latter were obtained at an age of 12 months and maintained at the Biocenter's animal care facility until use. All animals were housed in plastic cages with water and food ad libitum and were maintained on a 12-hour light/dark cycle. Mice with skin lesions, spleenomegaly and macroscopically visible tumors were excluded. For *ex vivo* studies, mice were treated daily with 100 mg/kg EGb761 *per os* in 0.2% agarose or with vehicle alone for 14 days. All experiments were performed in accordance with the German animal right regulations.

Transgenic mice:

For experiments with transgenic mice, C57BL/6J mice of both sexes were used. Three different types of transgenic mice were generated by N. Touchet and C. Czech (Rhone

Poullenc/ Aventis Pharma, Vitry-Sur-Seine, France) in cooperation with IFFA Credo, Transgenic Alliance (France) and kindly donated to our laboratory.

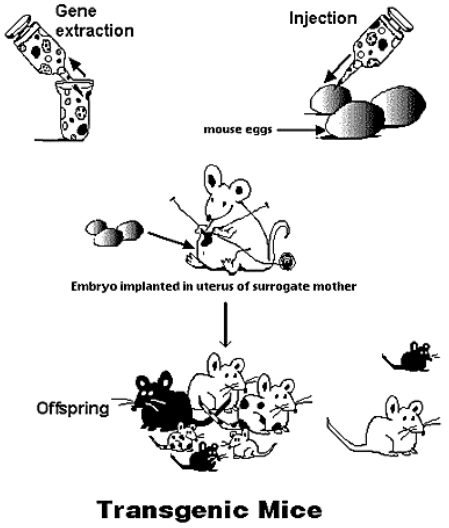


Figure 7. Generation of transgenic mice (taken from http://www.accessexcellence.org/AB/GG/transgenic.html).

Genetic engineered mice were expressing a human wildtype Presenilin-1 (PS1 wt) transgene or a PS1 transgene expressing either a single pathogenic mutation at codon M146 (PS1 M146L) or multiple FAD-specific mutations M146L + H163R + A246E + L286V + C410Y (PS1 M5), since the combination of different PS1 mutations shows additive effects on $A\beta_{1-42}$ secretion (Citron *et al*., 1998), or mutant human APP (APP_{695}SDL; described by Moussaoui *et al*., 2000). Transgene expression is under the control of the human HMG-CR-promotor which represents a housekeeping-type promotor that shows a strong and ubiquitous expression pattern with high expression in neurons (Czech *et al*., 1997; Czech *et al.*, 1998). APP expression was under the control of βPDGF promotor (Czech *et al*., 2000). APP transgenic and non-transgenic animals were littermates or subsequent generation siblings and are therefore strain matched. PS1 transgenic mice showed normal growth and development, and revealed no apparent behavioral changes compared to littermate controls. At an age of 18, 6 months, respectively, plaque formation is observed in brains of APP_{695}SDL or APP_{695}SDLxPS1 M146L transgenic mice. In later experiments, mice from our own breeding facilities were used. Genotyping had been investigated by genomic PCR (see molecular biology section of this chapter). Moreover, double transgenic were generated by crossing PS1 M146L with APP_{695}SDL mice.

Female NMRI mice	Harlan Winkelmann
Transgenic BL/6 mice	gift from C.Czech Aventis, France

Cellbiological methods:

General cell culture:

All here described experiments with primary cells and cell lines were conducted under sterile conditions in a laminar flow box. Cells were kept at 37°C, 94% humidity and 5% CO_2 in sterile culture vessels.

For short-term culture, human, murine PBMC, murine spleenocytes or Jurkat leucimic T cells were seeded at 5 x 10^5 cells/ml in culture medium. Cell suspension was maintained in RPMI supplemented with 10% fetal calf serum (FCS), 1% antibiotics (Penicillin (10.000 IU/ml), Streptomycin (10.000 IU/ml) and 2 mM GlutaMax) as culture medium. Cells were washed with phosphate buffered saline (PBS).

RPMI-culture medium:	
-10% FCS (2 hours heat inactivated at 56°C)	Gibco BRL Life Tech.
- Penicillin/ Streptomycin	Gibco BRL Life Tech.
→ in RPMI	Gibco BRL Life Tech.
10 x PBS	Gibco BRL Life Tech.
Culture vessels and -flasks	Iwaki/ Corning/ Greiner
Sterile filters 0,2 µm pores	Iwaki

Cells were investigated directly (native cells/ PBMC) or after short-term culture (activated cells = lymphoblasts) as indicated. For short-term culture cells were seeded at density of 0.5 x 10^6 cells/ml in complete medium supplemented with 5 µg/ml PHA-L. After 48 h of incubation at 37 °C and 5% CO_2, cells were washed once in PBS, seeded in complete medium containing 50 U/ ml of human recombinant IL-2 and incubated for 72 h to predispose to apoptosis according to the method of Zheng *et al* (1995). Activated lymphocytes consisted to nearly 100% of $CD3^+$ cells and expressed CD95 to over 96% as determined in FACS analysis.

Leucoagglutinin (PHA-L; source: *Phaseolus vulgaris*, red kidney bean)	Sigma
Interleukin-2 (IL-2)	Boehringer Mannheim/ Roche

Determination of cell number and vitality

To resolve the cell number, cells were resuspended with care and mixed with trypan dye solution (10:1). An aliquot of the stained cell suspension is subjected to a Neubauer chamber. The arithmetical mean from different counting sections is determined and the cell number calculated in respect to the chamber factor and the dilution.

Neubauer-chamber	Merck
Trypan blue dye	Gibco BRL LifeTech

Cryoconservation of cells

Cells were washed with PBS, collected by centrifugation and all supernatant removed. Cells were resuspended in freezing medium, containing DMSO, in order to prevent harm by ice crystal formation during storage under freezing conditions. Cells were subjected quickly on ice for several minutes and than stored overnight in a freezer (-20°C). The following day, the cells were subjected in an –80°C freezer overnight and can than stored for an independent time in liquid nitrogen.

To thaw cells, cryotubes containing the desired cells were rapidly subjected from the freezer or the nitrogen tank to a prewarmed (37°C) water bath for one or two minutes, until the solution is thawed. The cells were immediately diluted in a large amount of prewarmed complete culture medium, and washed several times by gentle centrifugation. Finally the cells are seeded in a culture vessel.

Freezing medium:	
-50% FCS	Gibco BRL Life Tech
-10% DMSO (dimethylsulfoxide)	Sigma
→ in RPMI	
Cryotubes	Greiner

Isolation of human PBMC

Freshly collected, heparinized venous whole blood was diluted with the same amount of PBS and carefully layered on 2 parts of Ficoll Hypaque in a 15 or 50 ml centrifuge tube. PBMC were separated from red blood cells, granulocates and platelets by gradient centrifugation at 2000 rpm (400 x g) for 20 minutes at room temperature. The Ficoll Hypaque solution consists of polymerized sugars with an exact density of 1.07 g/cm^3 that is identical with that of vital lymphocytes and monocytes (constituents of the PBMC). Therefore, PBMC collect at the

interphase between the Ficoll solution and the blood serum (see Figure 8). The more dense cellular blood components are collected at the bottom of the tube, the less dense parts like platelets remain in the supernatant. The PBMC from the interphase is carefully collected with a sterile pipette, washed twice in PBS, counted with trypan staining and resuspended in culture medium.

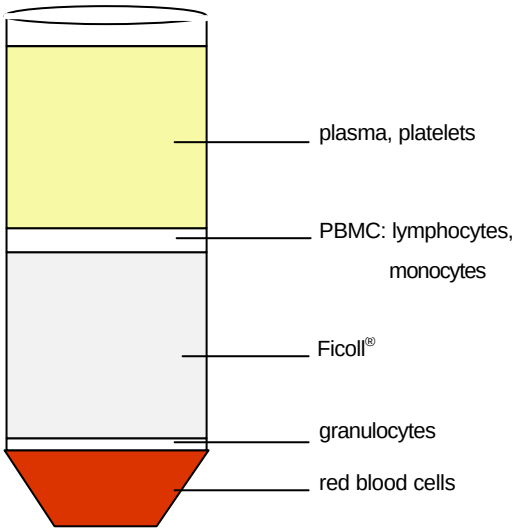


Figure 8. Separation of cellular blood components by gradient centrifugation on Ficoll-Hypaque.

Tubes	Greiner
Ficoll-Hypaque	Biochrom
PBS	GibcoLifeTech
Culture medium:	
RPMI 1640	
1% penicillin/ streptomycin	
10% fetal calf serum (FCS)	all GibcoLifeTech

Isolation of $CD4^+$ or $CD8^+$ lymphocytes

PBMC were isolated as described above. Cells were resuspended in cold PBS supplemented with 10 mM EDTA at concentration of 10^7 cells/ml. Appropriate magnetic-coupled antibody is added and the mixture incubated for 15 minutes at 4°C. Separation columns are placed in the magnetic field and washed twice with PBS/EDTA. Labeled cells were loaded onto the

column and eluted by gravity flow. Isolated T cell subsets were cultured under standard conditions.

PBS/EDTA:	
10 mM EDTA	Merck
→ in PBS	Gibco BRL LifeTech
Magnetic-labeled antibodies (anti CD4/ anti CD8)	
Midi MACS separation columns	
Magnetic device (Midi)	all MACS Miltenyi Biotec

Isolation and preparation of murine PBMC and spleen lymphocytes:

Mice were killed by cervical dislocation, venous blood was collected and anticoagulated with heparin. PBMC were isolated by centrifugation on Ficoll hypaque as described for human cell preparation (see there). T cells were isolated from the spleen with magnetic beads according to the manufacturer's instructions (Dynal, Sweden). In brief, spleen cell suspensions were prepared by mechanical dissociation of individual spleens in PBS supplemented with 10% FCS, penicillin (10.000 IU/ml) and streptomycin (100 µg/ml; PBS-FCS). Red blood cells were lysed in hypotonic buffer for 2 minutes. Cells were washed twice, mixed with magnetic beads specific for B cells and incubated with shaking for 20 minutes at 4°C. T cell content of spleen cells was determined by CD3-staining to be over 80%.

Pan B magnetic beads	Dynal (Sweden)
Magnetic stand for 15 ml tubes and microcentrifuge tubes	Dynal (Sweden)
Tubes	Greiner
Anti-murine CD3	Pharmingen
PBS-FCS:	
1 x PBS	GibcoLifeTech
Penicillin/ Streptomycin	GibcoLifeTech
10% fetal calf serum (FCS)	GibcoLifeTech
Hypotonic lysis buffer:	
155 mM NH_4Cl	Merck
10 mM $KHCO_3$	Merck

Whole blood cultures:

Blood was collected in NH_4-Heparin vacuum syringes. One volume of blood was diluted in 9 volumes of RPMI (1:10) supplemented with penicillin (100 U/ ml) and streptomycin (100 µg/

ml). The cell suspension was cultured in 96-well plates. Cells were stimulated with either PHA-L (10 µg/ml) or 12-O-tetradecanoyl 12-phorbol 13-acetate (PMA) and Ionomycin (50 ng/ml, 200 ng/ ml, respectively) or remained untreated (basal) as indicated.

Whole blood culture medium:	
100 U/ml penicillin	
100 µg/ml streptomycin	
→ in RPMI	all Gico BRL LifeTech
Leucoagglutinin (PHA-L; source: *Phaseolus vulgaris*, red kidney bean)	Sigma
12-O-tetradecanoyl 12-phorbol 13-acetate (PMA)	Sigma

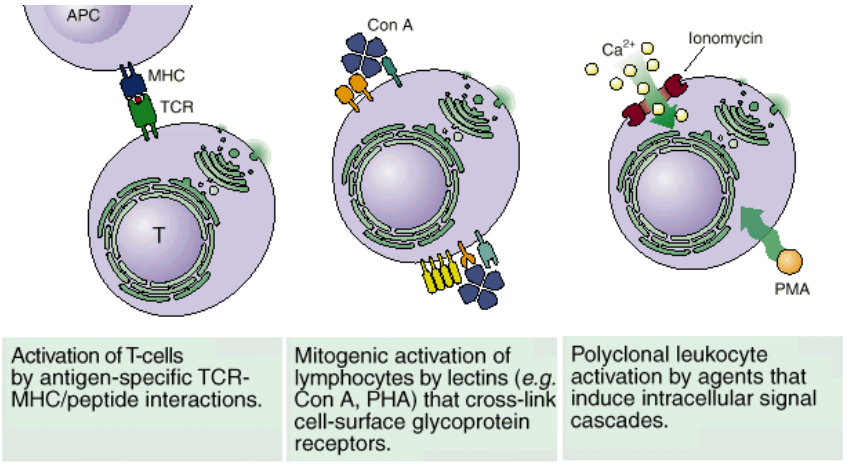


Figure 9. *In vivo* activation of T cells by TCR-MHC/peptide ligation. This event can be mimicked *in vitro* by TCR binding with mitogens (e.g. PHA) or phorbolester (PMA) and Ca^{2+} ionophore (Ionomycin).

Induction of apoptotic cell death:

Lymphocyte death was induced with the following stimuli for up to 24 hours: 10^{-7} M dexamethasone, 10 mM 2'-deoxy-D-ribose, 100 or 500 nM staurosporine or 0.4 ng/ ml agonistic CD95-antibody (clone 2R2) or 50 ng/ml Fas-ligand. As control, spontaneous *in vitro* apoptosis, that occurs when primary cells are removed from the organism, was always determined, as well. Withdrawal of the growth factor IL-2 leads lymphoblasts into the so-called activation induced cell death (AICD).

Jurkat cells were incubated with Fas-ligand (0-100 ng/ml) for 3 or 24 hours as indicated. When announced, murine spleen cells were incubated with EGb761 or other antioxidants 1 to 2 hours prior to apoptosis induction.

dexamethasone (dissolved in DMSO)	Sigma
2'-deoxy-D-ribose	Sigma
staurosporine (dissolved in DMSO)	Sigma
DMSO	Merck
CD95 antibody	Boehringer Mannheim/ Roche
Fas-ligand and potentiator kit	Upstate Biotechnology (Biozol)
Gingko biloba extract (EGb761, dissolved in PBS)	Schwabe AG, Karlsruhe

Analytical cellbiological methods:

Flow cytometry with a fluorescence-activated cell-sorter (FACS)

Flow Cytometry involves the use of a beam of laser light projected through a liquid stream that contains cells, or other particles, which when struck by the focussed light give out signals, which are picked up by detectors. These signals are then converted for computer storage and data analysis, and can provide information about various cellular properties. The term "flow cytometry" derives from the measurement (meter) of single cells (cyto) as they flow past a series of detectors. The fundamental concept is that cells flow one at a time through a region of interogation where multiple biophysical properties of each cell can be measured at rates of over 1000 cells per second. These biophysical properties are then correlated with biological and biochemical properties of interest. The high through-put of cells allows for rare cells, which may have inherent or inducible differences, to be easily detected and identified from the remainder of the cell population.

In order to make the measurement of biological/biochemical properties of interest easier, the cells are usually stained with fluorescent dyes which bind specifically to cellular constituents. The dyes are excited by the laser beam, and emit light at longer wavelengths. Detectors pick up this emitted light, and these analogue signals are converted to digital so that they may be stored, for later display and analysis.

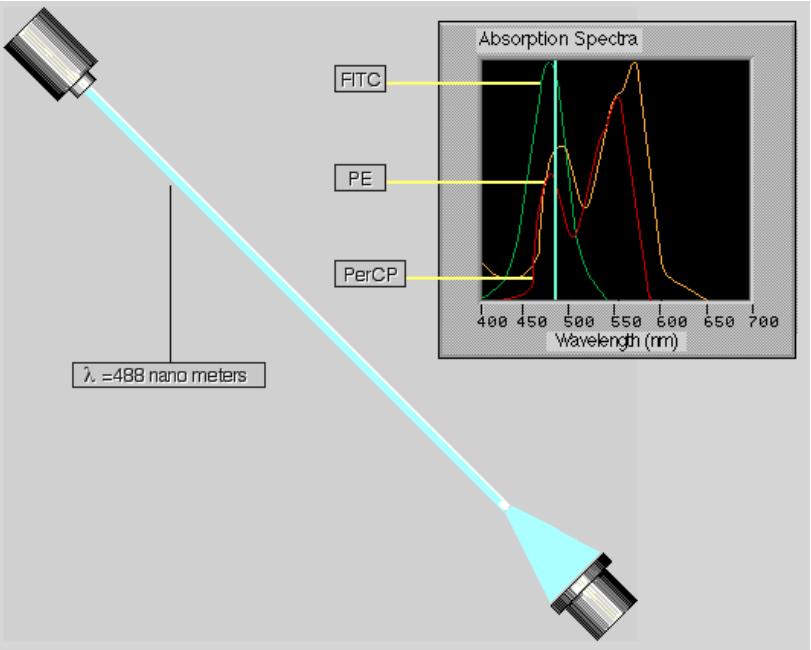


Figure 10. All used fluorochromes and dyes can be excited by light of 488 nm.

Many larger flow cytometers are also "cell sorters", instruments which have the ability to selectively deposit cells from particular populations into tubes, or other collection vessels. These selected cells can then be used for further experiments, cultured, or stained with another dye/antibody and reanalysed. In order to sort cells, the instrument's electronic interprets the signals collected for each cell as it is interogated by the laser beam, compares the signal with sorting criteria set on the computer. If the cell meets the required criteria, an electrical charge is applied to the liquid stream which is being accurately broken into droplets containing the cells. This charge is applied to the stream at the precise moment the cell of interest is about to break off from the stream, then removed when the charged droplet has broken from the stream. As the droplets fall, they pass between two metal plates, which are strongly positively or negatively charged. Charged droplets get drawn towards the metal plate of the opposite polarity, and deposited in the collection vessel, or onto a microscope slide, for further examination.

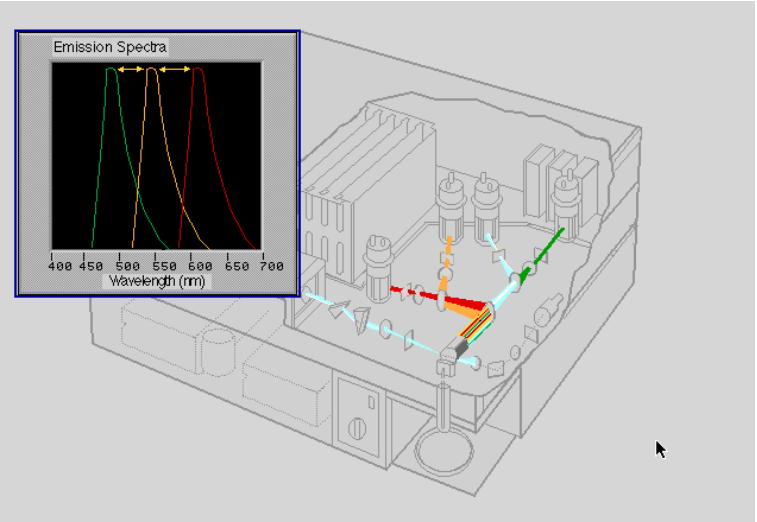


Figure 11. A single laser flow cytometer with five parameters of detection. Two detectors detect the light scatter, and three photomultiplier tubes (PMTs) detect the fluorescent signals. The bandpass filters are set up for optimal detection with the fluorochrome: FITC (Fluoresceinisothiocyanate in FL-1), PE (R-phycoerythrin in FL-2) and PerCp (Peridin chlorophyll protein) or CyChrome (both in FL-3).

Following acquisition of data on the flow cytometer, analysis is undertaken to find out how many cells from the sampled population meet a criteria of interest. For example, does a population of cells express one molecule, but not another. The data can be displayed in a number of different formats, each having advantages and disadvantages. The common methods are histogram, or dot plots. Once excited, the emitted light is directed to one of three fluorescent detectors (FL1, FL2 and FL3). There are a series of fixed (i.e. can't be changed) light filters that direct a particular wavelength light to a designated detector. All FITC (green) emissions go to FL1, all red to FL2 and all far red to FL3. In addition to these fluorescent emissions the FACS can also collect information on reflected laser light. There are two additional detectors the measure forward and right angle light scatter. Forward light scatter (FSC) measures that ability of a particle (cell) to deflect light from its path. Small cells deflect little light while large cells will deflect more. Consequently this parameter is a rough approximation of size. Side scatter (SSC) measures the ability of a particle to redirect light at a 90° angle. Cells with a lot of cellular structure (i.e. granulocytes) will redirect more light than a cell with little intracellular structure (red blood cells).

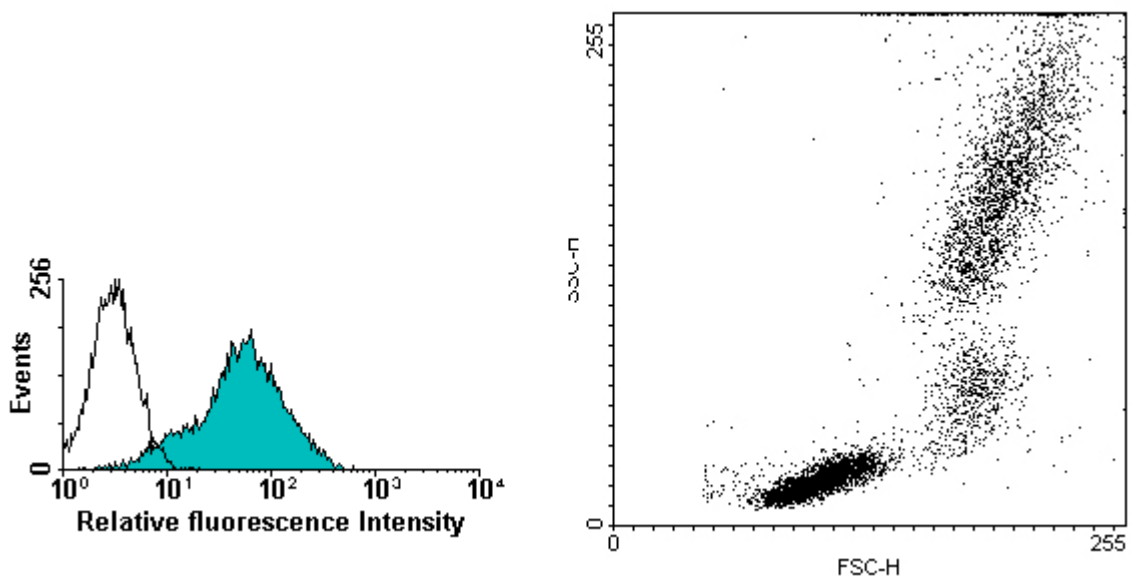


Figure 12. A representative histogram, displaying relative fluorescence intensity in relation to the number of events, and a representative dot plot, plotting one parameter e.g. FSC against another like SSC, for instance. The right figure represents a typical dot plot of lysed whole blood. Smallest cells low in SSC are lymphocytes (e.g. T-, B-, and NK-cells). The cell population in the right lower corner are the monocytes, the cells with the approximately same size and high in granulate are the granulocytes.

The simplest way of displaying flow cytometry data is the frequency histogram. Frequency histograms display relative fluorescence or scattered light signals plotted against the number of events. The simplicity of this type of display is the main attraction. To see the relative levels of other parameters which were collected at the same time , one needs to use one of the forms of bivariate displays namely dot, density or contour plots. In these type of displays, one parameter is plotted against another in an X versus Y axis display.

Fluorescence activated cell sorter (FACS, flow cytometer) FACSCalibur™	
CellQuest™ analysis software	
FACSComp™ software	
SimulTest™ software	all Becton Dickinson
Analysis computer Power Macintosh 7600/132	Apple Macintosh

Fluorochromes:

Fluoresceinisothiocyanate (FITC):

FITC is a fluorochrome with a molecular weight of 389 kDa and an absorption maximum at 495 nm (Figure 13). Its excitation by 488 nm light leads to a fluorescence emission around 520 nm. Using a 530 ± 15 nm bandpass filter will give optimum detection for this fluorochrome. The isothiocyanate derivative is the most widely used for conjugation with antibodies. FITC has a high quantum yield (efficiency of energy transfer from absorption to emission fluorescence) and approximately half of the absorbed photons are emitted as fluorescent light. The number of FITC molecules per conjugate partner (antibody) is usually in the range of three to five molecules (Pharmingen, 1999).

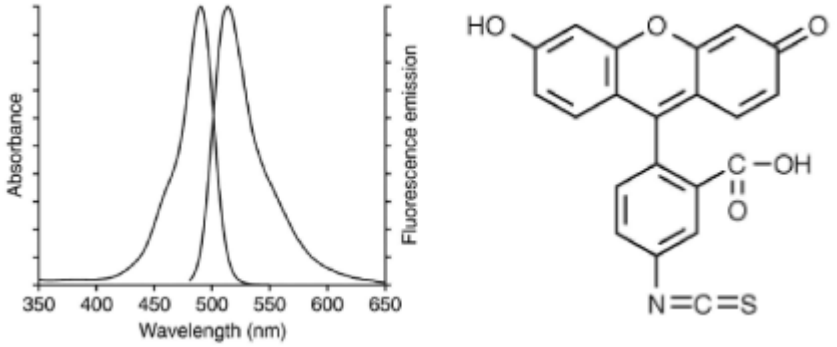


Figure 13. Spectra and formula of fluorescein-isothiocyanate (FITC)

R-phycoerythrin (PE):

PE is an accessory photosynthetic pigment found in red algae. *In vivo,* its functions is to transfer light energy to chlorophyll during photosynthesis. *In vitro,* it is a 240 kDa protein with 34 phycoerythrobilin fluorochromes per molecule. The large number of fluorochromes per PE molecule make R-phycoerythrin an ideal pigment for flow cytometry applications. Its absorption maximum is 564 nm (Figure 14). When excited by 488 nm light, its fluorescence emission maximum is approximately 576 nm. In average, one molecule PE is conjugated with one antibody molecule (Pharmingen, 1999).

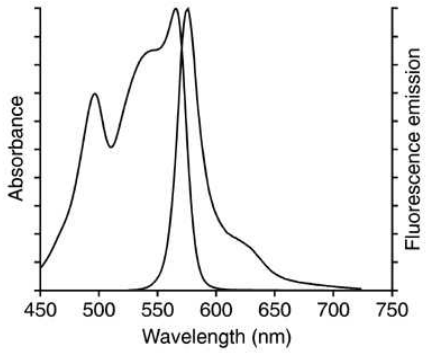


Figure 14. Absorption and emission spectra of PE.

Peridin chlorophyll protein (PerCp):

PerCp is a component of the photosynthetic apparatus found in the dinoflagellate, *Glenodinium sp*. PerCp is a protein complex with a molecular weight of approximately 35 kDa. When excited by light at 488 nm from an argon-ion laser, PerCp has a excitation maximum around 490 nm, with an emission spectrum, which peaks at 677 nm. The emitted light is collected in the fluorescence-3 (FL-3) channel (Pharmingen, 1999).

Cy-Chrome^{TM}:

CyChrome is a tandem conjugate system, with an absorption maximum of approximately 650 nm, which combines PE and a cyanine dye (MW 1.5 kDa). When excited by 488 nm light, the excited fluorochrome PE is able to transfer its fluorescence energy to the cyanine molecule, which than fluoresces at a longer wavelength. The resulting fluorescent maximum is approximately 650 nm. Using a 650 longpass filter will give optimum detection for this fluorochrome. The efficiency of the light energy transfer between the two fluorochromes is rather high, as less than 5% of the absorbed light is lost as fluorescence by PE. An average of one CyChrome molecule is coupled per molecule antibody (Pharmingen, 1999).

Electronic compensation for multi-color immunofluorescence staining:

Monoclonal antibodies conjugated to FITC, PE, PerCp, or CyChrome and DNA dyes (PI, 7-AAD) are used in combination to analyze multiple antigenic determinants within a cell population. FITC emission is detected as green signal by the FL-1 detector, PE and PI measured as an orange signal by the FL-2 detector and PerCp, CyChrome or 7-AAD is measured as a violet signal by the FL-3 detector. However, a significant amount of orange fluorescence is present in the FITC emission, some green fluorescence is present in the PE

emission, and similar for the other fluorochrome and dyes, Especially PI has a broad emission and can be detected in all fluorescence channels. This spectral overlap, if uncorrected, will lead to a fluorochrome signal picked up by an inappropriate detector. Compensation is the process of correction for this overlap; it is the electronic subtraction of unwanted signal to remove the effects of spectral spillover. Through compensation, the fluorescence measurement of a cell sample stained with one fluorochrome is electronically forced to be identical to unstained cells, with regard to the two remaining, inappropriate detectors.

Calibrite™ Kit — Becton Dickinson

Cell preparation and staining for Flow Cytometry:

Staining of whole blood or whole blood cultures:

Whole blood cultures were transferred into an 1.2 ml 96-well plate, centrifuged at 300 x g, and the supernatants were discarded. Whole blood was stained with the appropriate antibodies following the manufacture's manual. In brief, 1 µl of each antibody was added to blood cell pellets and incubated for 10 minutes at room temperature. Adding 500 µl of hypotonic buffer for 15 minutes lysed red blood cells. Since PMA/ Ionomycin treatment stabilizes the red blood cell membrane, 1 ml CellLyse was needed to completely lyse the erythrocytes. Cells were pelleted by 300 x g and additionally washed with 500 µl CellWash®.

Anti-human antibodies:
FITC-conjugated
isotype control IgG_1 (clone X40)
anti-CD3 (clone SK7)
anti-CD8 (clone SK1)
anti-CD69 (clone L78)
anti-HLA-DR (clone)
anti-IL-2 (clone 25723.11)
anti-TNF-α (clone 5344.111)
anti-BrdU (clone B44) — all Becton Dickinson

isotype control IgG_{2b} (clone 27-35)
isotype control IgG_1
anti-Bcl-2 — all Pharmingen/ BD

anti-phospho-tyrosine (clone 4G10) — Santa Cruz biotechnology, USA

PE-conjugated
isotype control IgG_1

anti-CD4 (clone SK3)	
anti-CD8 (clone SK1)	
anti-CD19 (clone 4G7)	
anti-CD16+ anti-CD56 (clones B73.1; MY31,respectively)	
anti-CD25	
anti-CD95	
anti-IL-2 (clone 25723.11)	
anti-IFN-γ (clone 6401.1111)	all Becton Dickinson
anti-TNF-α	Sigma
PerCp-conjugated	
isotype control IgG_1	
anti-CD3 (clone SK7)	
anti-CD8	
anti-murine antibodies:	
FITC-conjugated	
k isotype control IgG_1 (clone R3-34)	
anti-CD3e (clone 145-2C11)	
anti-CD4 (clone RM4-5)	
PE-conjugated	
rat isotype control IgG_1	
anti-CD8 (clone 53-6.7)	
CyChrome-conjugated	
anti-CD3 (clone 17A2)	all Pharmingen/ BD
TrueCount™ beads	all Becton Dickinson
DNA-dyes	
propidium iodide	Sigma
7-AAD (dissolved in DMF)	Molecular Probes
CellWash™	
10 x CellLyse™	
10 x CellPerm™	
10 x CellFix™	all Becton Dickinson
10 x PermWash™	
PermFix™	
Brefeldin A (Golgi Plug™)	all Pharmingen/ BD

Samples were assayed with FACSCalibur using Simulset software (all Becton Dickinson). Forward (FSC) and side (SSC) angle scattered light gating excluded dead cells, granulocytes and monocytes. For each sample, 10,000 cells were acquired.

The lymphocyte subset were quantified by staining with anti-CD3-FITC, anti-CD19-PE, and anti-CD16-PE + anti-CD56-PE in order to explore the percentage of T-, B-, and NK-lymphocytes, respectively. Staining with anti-CD4-FITC and anti-CD8-PE determined T lymphocyte subsets. Early T cell activation was resolved by staining with anti-CD69-FITC or IgG_1-isotype control.

All activated cells were stained with CD3-PerCp and CD8-PE in order to gate all $CD3^+$ T cells and to differentiate between the subsets. Findings of Rostaing *et al*. (Rostaing *et al*., 1999) and unpublished data of our group revealed that the CD4 antigen is down regulated after PHA activation while the CD8 surface density remains unchanged. Therefore we electronically gated the $CD3^+CD8^+$ cells (T cytotoxic/ suppressor cells) and the $CD3^+CD8^-$ cells ($CD3^+CD4^+$ or T helper cells) and assumed that all $CD8^-$ cells are $CD4^+$.

Quantification of lymphocyte number:

Absolute numbers of lymphocytes were determined with TrueCount® beads and CD3-PerCp, CD8-PE, and CD4-FITC according to the manufacture's manual. In brief, 50 µl of whole blood and 1µl antibody reagent containing fluorochrome conjugated antibodies against anti-CD3, anti-CD8, and anti-CD4 (TriTest®) were pipetted into an TrueCount® tube containing a specific amount of beads and incubated for 15 minutes at room temperature. 250 µl CellLyse® were added for additional 15 minutes in order to lyse red blood cells. Samples were measured by flow cytometry with settings appropriate for suspension containing lysed red blood cells without wash. For each sample, 100,000 events were acquired. Cell population and beads were gated and cell count calculated in relation to the bead's number.

Detection of cytokine expressing cells:

Whole blood cultures were placed in an incubator for 22 hours at 37°C. For cytokine detection, cells were incubated with Brefeldin A at a concentration 10 µg/ml for the last 16 hours of stimulation, to inhibit cytokine secretion. Cultures were surface stained as described above, followed by fixation and permeabilization with FACSPerm®. Intracellular cytokine staining was performed with anti-cytokine mAbs specific for IL2, IFN-γ, and TNF-α or an isotype control antibody (1 µl of each mAb per 30 µl whole blood) in FACSPerm® for 30 minutes at room temperature.

Determination of proliferation:

Whole blood cultures were incubated for 72 hours with or without stimulation at 37°C. 24 hours before harvest, Brom-3'desoxy-uridine (BrdU) was added at a final concentration of 20 µM to the cultures. Cells were stained according to the method of Gaines *et al*. (1996) using an anti-BrdU-antibody or isotype control. In brief, surface receptors were stained as described above. After red blood cell lysis and an additional washing-step, leukocytes were transferred into a 96-microtiter roundbottom plate and fixed with 100 µl 1% paraformaldehyde (CellFix®) overnight at 4°C. Cells were permalized by washing with saponin-containing buffer (PermWash®). Cells were resuspended in PBS containing 10 mM $MgCl_2$ and 50 U DNase-I and incubated at 37°C for 45 minutes. DNA digest was stopped by centrifugation at 4°C, cells washed and resuspended in 50 µl PermWash®. Blood cells were stained with 1 µl of an anti-BrdU-antibody or IgG_1-isotype control for 30 minutes at room temperature in the dark. After washing out excessive antibodies, cells were resuspended in CellWash® and analyzed by flow cytometry.

Brom-3'desoxy-uridine (BrdU)	Sigma
DNase-I	Boehringer Mannheim/ Roche
$MgCl_2$	Sigma

Determination of phosphorylated tyrosine residues:

T cells were stimulated and samples prepared according to the method of Hubert *et al.* (1997). In brief, 500 µl diluted whole blood was transferred into microcentrifuge tubes and at brought to 37°C in a heatblock with gentle agitation. Cells were activated by addition of PHA-L (10 µg/ml) for 3 minutes, rapidly chilled on ice, and centrifuged at 500 x g. Pellets were resuspended in freezing medium, containing 10% DMSO and 50% FCS in RPMI medium. Samples were stored at –20°C until analysis. For sample preparation, cells were rapidly thawed at 37°C, transferred into an 1.2 ml 96-well plate and washed two times with CellWash®. Red cell lysis was not necessary since freezing the cells already destroyed red blood cells' membranes. After surface staining, cells were fixed with 4% paraformaldehyde (CellFix®) for 20 minutes on ice, permeabilized with 1% saponine (PermWash®), stained with 1 µl of anti-phosphotyrosine antibody or isotype control (IgG_{2b}), and analyzed by flow cytometry. The ratio between mean fluorescence intensity of the stimulated and unstimulated cells was calculated.

Analysis of intracellular Bcl-2

For intracellular staining, wholeblood was surfaced stained with each 1 µl of PE- and PerCp-conjugated antibodies for 15 minutes, lysed with CellLyse^{TM} for 10 minutes, washed with CellWASH^{TM}, and fixed with PermFix overnight at 4°C. After fixation cells were washed and resuspended in PermWash to permeabilize the leukocytes. Staining of Bcl2 was done with 1 µl of the corresponding antibody or isotype control for 45 minutes at room temperature. Finally, cells were washed with PermWash and resuspended in 200 µl CellWASH.

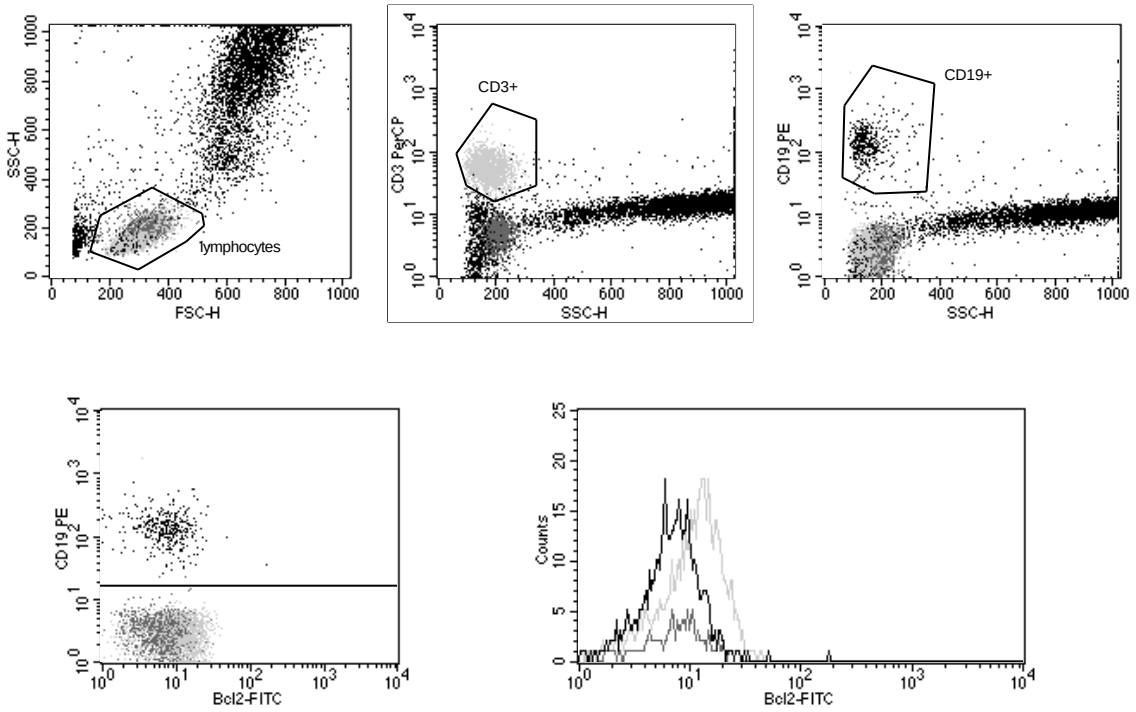


Figure 15. Gating strategy for determining intracellular Bcl-2. Lymphocytes were gated by FSC/SSC and CD3-PerCp/SSC or CD19-PE/SSC and double positive cells displayed in a FL-1 (Bcl-2-FITC) histogram. NK lymphocytes (dark gray) remain after subtracting CD3 (light gray) and CD19 (black) cells.

Detection of apoptotic cell death

Staining with propidium iodide

Propidium iodide as well as its analogous ethidium bromide intercalates between the bases in double stranded DNA. When stacked between the bases, interactions between the π-electrons of the dye and the DNA bases occur, resulting in an altered emission maximum (Figure 16).

The emitted fluorescence intensity is dependent on the length of the DNA molecule; the more basepairs the molecule consists of, the stronger is the fluorescence intensity subsequent to appropriate excitation.

According to Nicoletti and coworkers (Nicoletti *et al.*, 1991) apoptosis can be detected by lysing the cells in hypotonic buffer and staining of the DNA content. Sub-G_0-DNA content, indicative for apoptotic nuclei, is determined by flow cytometry in FL-2.

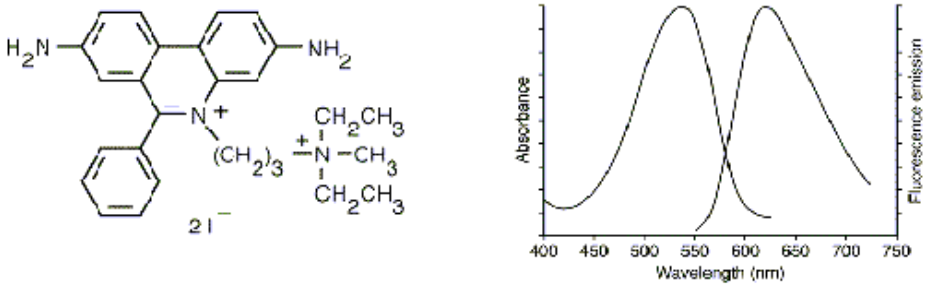


Figure 16. Molecular structure of propidium iodide. It posses the common planar conformation with conjugated bindings as the most DNA intercalating substances. Absorbance and emission spectra of propidium iodide. In contrast to ethidium bromide, propidium iodide can be excited by an argon laser (488 nm) and detected in FL-2.

Lymphocytes were harvested by centrifugation at various time points and the pellets were resuspended in lysis buffer containing propidium iodide. Samples were stored at 4°C for 1-2 hours before flow cytometry analysis using Cell Quest software. In order to avoid to detect RNAs that are smaller than chromosomal DNA and occur in the very low sub-G_0-DNA content, events with very low fluorescence intensity are excluded by electronically gating.

Lysis buffer:	
0.1% sodium citrate	Merck
0.1% Triton X-1000	Sigma
50 µg/ml propidium iodide	Sigma

Vital cell populations displaying a very small percentage of apoptotic subG_0 nuclei (PBMC derived from healthy controls, for instance) exhibits a huge G_0/G_1-peak and smaller G_2-peak when subjected in a histogram plot (Figure 17). The G_0/G_1-peak represents the cells in the G_0-/G_1-phase, respectively. Doubled DNA contents owned by cells in the G_2-phase are displayed

as G_2-peak. In between those two peaks, there is a population, displaying cells that are in the DNA-synthesis (S) phase. Usually, primary cells from healthy volunteers provide a representative large G_0/G_1-peak and a relatively small though well detectable G_2-peak. In contrast, immortalized cell lines or tumor cell lines (Jurkat leucimic lymphoblasts, for instance) are permanently entering the cell cycle, therefore display a relatively increased G_2-peak.

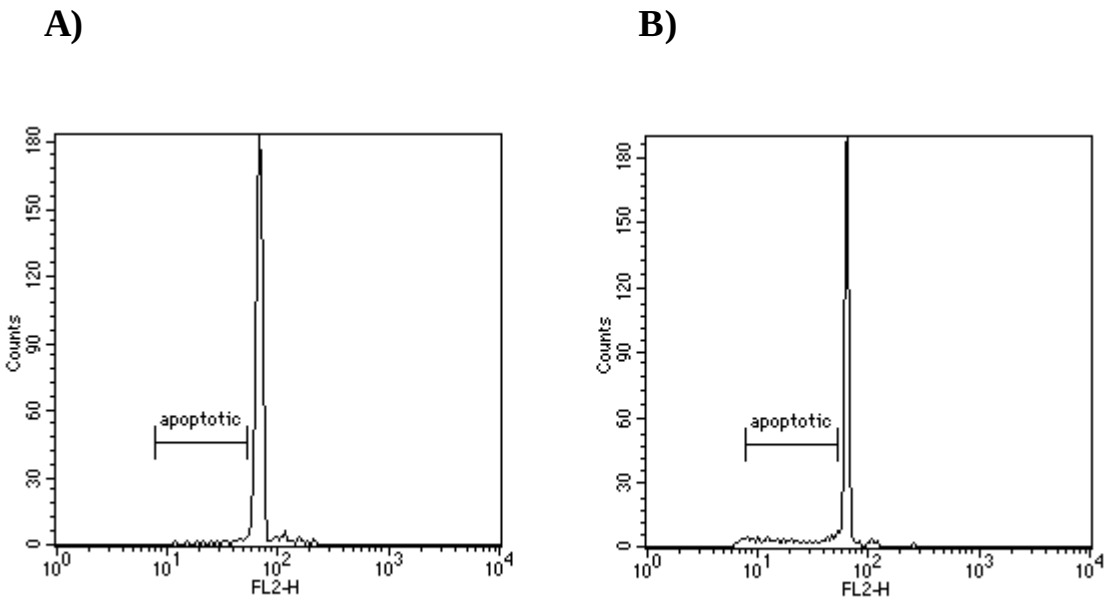


Figure 17. representative histogram of PI-stained (according to Nicoletti *et al*., 1991) PBMC of young individual. A) Basal apoptotic levels of freshly isolated PBMC; B) dRib-induced apoptosis in human PBMC after 24 hours incubation.

Staining with 7-aminoactinomycin (7-AAD)

Experiments, exploring the apoptotic behavior of lymphocyte subsets, were performed with the DNA dye 7-Aminoactinomycin (7-AAD; described by Schmid *et al*., 1994), that can be detected in FL-3; Figure 18), since 7-AAD posses a very distinct emission profile in contrast to PI, that provides no conflict with the other cytometer channels and permits the detection of two other fluorochrome (FITC and PE, for instance) within the same sample. In order to explore the apoptotic levels of different lymphoycte subsets, the isolated PBMC samples were stained with 7AAD, diluted in DMF, and monoclonal antibodies coupled with FITC, and another coupled with PE.

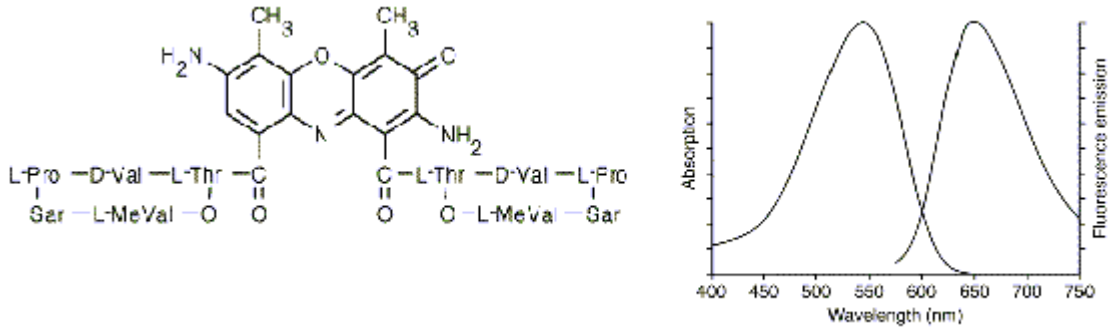


Figure 18. Molecular formula and absorption and emission spectra of 7-AAD.

An additional advantage of 7-AAD compared to PI-staining is that 7-AAD can not only detect apoptotic cells, but also necrotic/ dead cells (Figure 19).

For 7-AAD-staining, the dye was dissolved at a concentration of 1.5 mM in N,N-dimethylformamide (DMF) and added at a concentration of 6 µM to the cell suspension for 15 minutes at room temperature. Cells were washed by centrifugation, resuspended in PBS, and immediately analyzed by flow cytometry. 7-AAD fluorescence intensity was plotted against forward scatter (FSC) and cells in the area between autofluorescence and full G_0/G_1-DNA content were termed apoptotic.

7-AAD dye:

7-Aminoactinomycin (7-AAD; dissolved in DMF)	Molecular Probes
N, N-dimethylformamide (DMF)	Merck

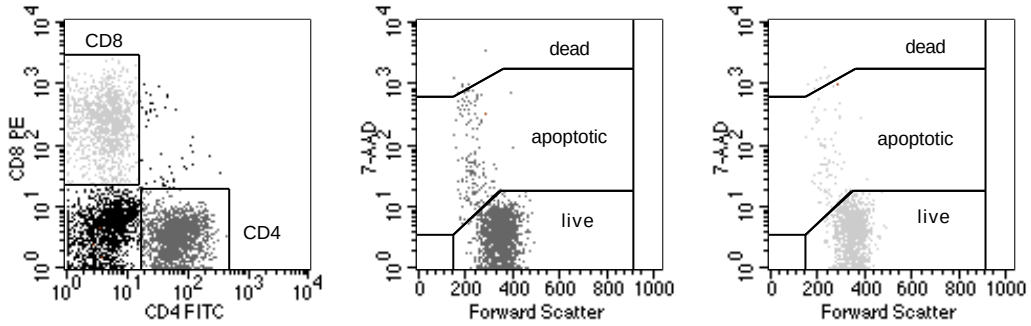


Figure 19. To determine apoptosis of single subsets cells were gated in a dot plot (left panel) displaying FL-1 (CD4-FITC) against FL-2 (CD8-PE) and $CD4^+$ (middle) and $CD8^+$ (right panel) gated cells were plotted against 7-AAD fluorescence intensity

PBMC were triple stained with FITC-conjugated anti-CD3 or anti CD4 and PE-conjugated anti-CD19, anti-CD16+56, anti-CD8 or anti-CD14, and the nuclear dye 7-AAD. 1 µl of each antibody and 20µg/ml 7-AAD were used. Staining was performed in 96-well-MTP for 20 minutes at room temperature in culture medium. Cells were washed and resuspended in CellWASH™.

DNA ladder

Cells were harvested by centrifugation (200 x g, 5 minutes) and washed with PBS. Cell pellets were incubated in 50 mM TRIS (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, 1% SDS and 20 µg/ml proteinase K at 55°C overnight. After digestion of RNA with RNase A (10 µg/ml) nucleic acids were extracted with phenol/chloroform and precipitated with 70% ethanol as standard procedure. Genomic DNA was electrophoresed in a 1% agarose gel and stained with ethidium bromide.

Lysis buffer for DNA-ladder:	
50 mM TRIS (pH 7.5)	
100 mM NaCl	
1 mM EDTA	
1% SDS	
20 µg/ml proteinase K	all Merck
RNase A	Roche/Boehringer Mannheim
phenol (equilibrated with TRIS buffer pH 7.4)	Sigma
chloroform	
ethanol	
agarose	all Merck
ethidium bromide solution (10 mg/ml)	Gibco BRL LifeTech

Genetic engineering:

DNA sources:

Oligonucleotidprimer were purchased from MWG Biotech (Ebersberg, Germany) or ARK Scientific (Darmstadt, Germany). For sequences of the numbered oligonucleotides described under genetic engineering, see appendix.

Human PS1 in pcDNA3 was a kind gift from C.Haass (München, Germany) to H. Hartmann of our laboratory.

cDNA constructs were generated using standard molecular biology techniques (Sambrook, Fritsch, and Manitais, 1989). Enzymes for genetic engineering of plasmid or genomic DNA were purchased from New England Biolabs (Frankfurt, Germany) and utilized as recommended by the supplier. DNA fragments were extracted from agarose gel using GlassMax DNA isolation Kit or CONCERT^{TM} Rapid Gel Extraction System (both GibcoBRL LifeTech).

For cDNA cloning, the constructs were transformed into competent cells of the bacterial strain Top 10 (Invitrogen, Groningen, The Netherlands) prepared by the $CaCl_2$ method (according to Sambrook, Fritsch, and Maniatis, 1989).

Plasmid DNA was purified from bacterial cultures by using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). Large-scale preparations of plasmid DNA were prepared with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). Total RNA was isolated from human PBMC or human brain with the RNAgents Kit (Promega, Heidelberg, Germany). Oligonucleotides and genomic or plasmid DNA were stored in sterile H_2O at 4°C or –20°C. RNA was stored in DEPC-H_2O at –80°C.

RT-PCR cloning of human Presenilin-2 (PS-2) and alg-3

Human brain samples from the frontal lobe of a non-demented person were obtained from the Institue of Psychiatry (London/ UK; Eckert *et al*., 2000). The brain sample was frozen in liquid nitrogen immediately after autopsy with a post-mortem delay of 8-33 hours and stored at –80°C. All vessels and instruments were rinsed thoroughly with RNaseAWAY® (Merck, Frankfurt, Germany). About 200 mg tissue from one human brain was homogenized (Potter: B.Braun Biotech Int., Melsungen, Germany) with 1ml Denaturating Solution (Promega,

Heidelberg, Germany) in a glass teflon homogenizer and total RNA was extracted according to RNAgents protocol. For first strand synthesis, 1 µg of total RNA and either 1 µg of oligo dT_{15} primer (oligonucleotide # 1) were annealed and than incubated with reverse transcriptase (M-MuLV Reverse Transcriptase, MBI Fermentas, Heidelberg, Germany) according to the supplier protocol.

PS-2 cDNA comprising the complete coding sequence of PS-2 and alg-3, a shorter fragment of PS-2 (Vito *et al*., 1996) were amplified from the obtained single stranded cDNA. Reactions were performed with 0.5 U *PfuI*-polymerase (NatuTec, Frankfurt, Germany), 0.25 µM of each dNTP and 3 µM of each PCR primer in a final volume of 50 µl of 1 x *PfuI* reaction buffer per reaction (standard procedure).

sense primer (PS-2: oligonucleotide # 2; alg-3: oligonucleotide # 3)	MWG Biotech AG
antisense primer (oligonucleotide # 4)	MWG Biotech AG
deoxynucleotides (dNTPs; 10 mM each)	GibcoBRL LifeTech
PfuI polymerase (5 U/µl)	NatuTec
10 x reaction buffer	NatuTec

cycling parameters (Perkin Elmer Gene Amp PCR System 9700; standard procedure):

segment	cycles	temperature	time
1	1	94°C	3 minutes
2	20	94°C	30 seconds
		55°C	30 seconds
		68°C	45 seconds
3	1	68°C	10 minutes

The primers were designed to give defined cohesive termini upon digest with *KpnI* and *XbaI* (New England Biolabs, Frankfurt, Germany). Furthermore, a Kozak consensus sequence (CCACC) and a startcodon (ATG), where necessary, was initiated in sense oligonucleotides prior to the first coding base triplett. Subsequent to a digest with *KpnI* and *XbaI*, the cohesive fragment was ligated into a linearized pcDNA3 vector (Invitrogene)according to standard procedures. PS-2-pcDNA3 and alg-3-pcDNA3 were sequenced by the dideoxynucleotide method.

KpnI + 10 x reaction buffer 2	New England Biolabs
XbaI + 10 x reaction buffer 2	New England Biolabs
Ligase + reaction buffer (containing ATP)	New England Biolabs
pcDNA3 vector	Invitrogene

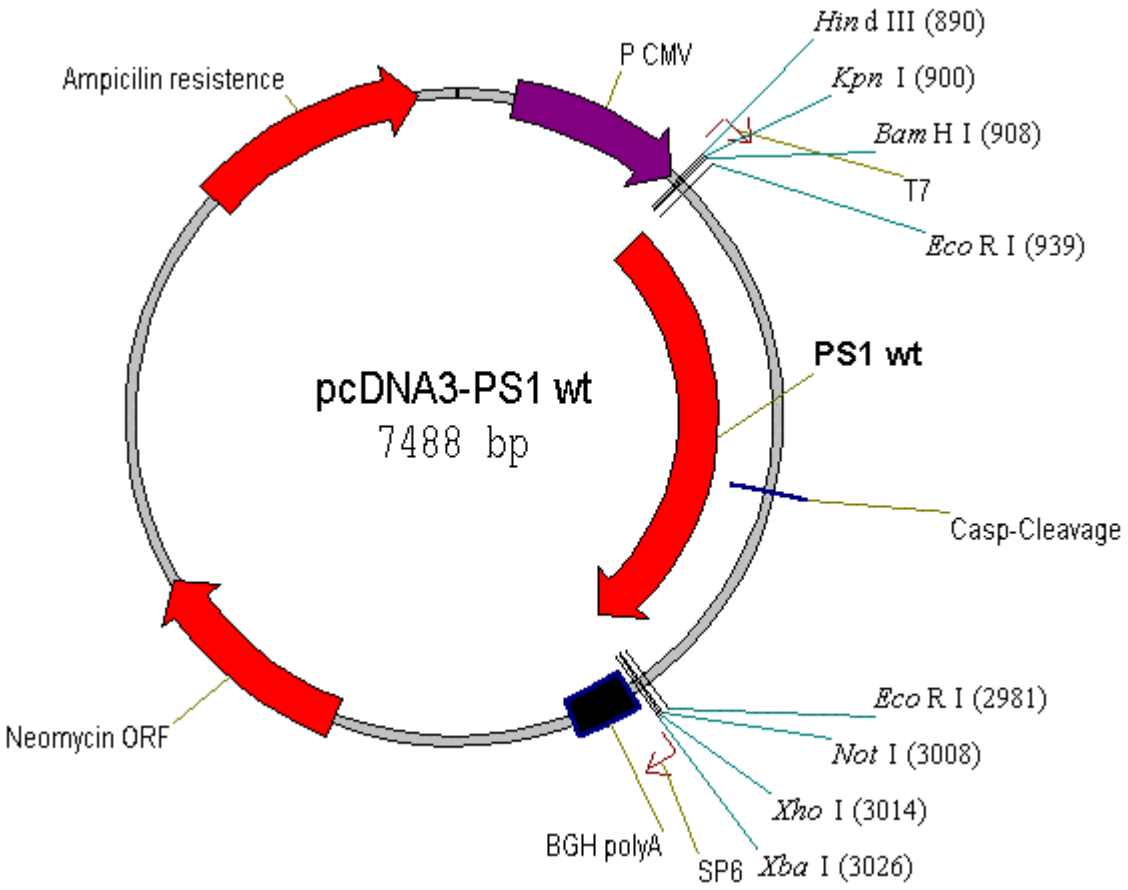


Figure 20. PS-2-pcDNA3 vector. The cloned PS-2 cDNA is under the control of the CMV promotor, subsequent to a stop codon there is a polyA site. The vector contains a neomycin resistance for selection in eukaryotic cells, and an ampicillin resistance for selection in prokaryotes. All other vectors generated here are analogously.

RT-PCR cloning of human Presenilin-1s (PS-1s) and Presenilin-1Cas (PS-1Cas)

PS-1s and PS-1Cas were amplified from PS-1-pcDNA3 using appropriate primers (PS-1s: oligonucleotide # 5 and oligonucleotide # 6; PS-1Cas: oligonucleotide # 7 and oligonucleotide # 6). The annealing temperature was 54°C (PS-1s) and 50.7°C (PS-1Cas), respectively.

For cloning strategy, see above generation of PS-2 and alg-3.

ApoE-genotyping

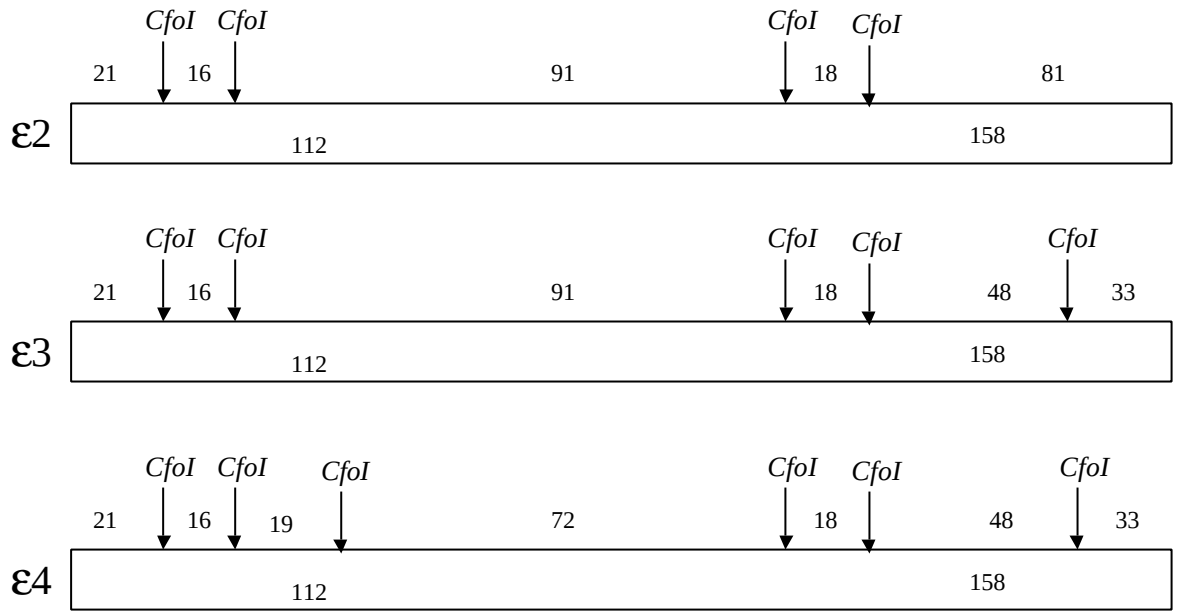


Figure 21. Restriction map of three principal alleles e_2, e_3, and e_4 of apoE gene. Codons 112 and 158 contain polymorphic nucleotides 3745 and 3883. Cutting sites for *CfoI* are arrowed. Numbers between cutting sizes indicate fragment sites in base pairs.

Genomic DNA from whole blood of AD-patients was isolated, and 10 µl of obtained DNA subjected to PCR using standard procedures (oligonucleotide # 8; oligonucleotide # 9), according to Wenham *et al*., 1991). The amplificate was digested overnight with 25 U *CfoI* at 37°C and analyzed in 20% polyacrylamide gel.

High pure PCR template preparation kit	Boehringer Mannheim/ Roche
HotStarTaq Polymerase	Qiagen
CfoI and 10 x buffer	Boehringer Mannheim/ Roche
10 bp ladder	Promega

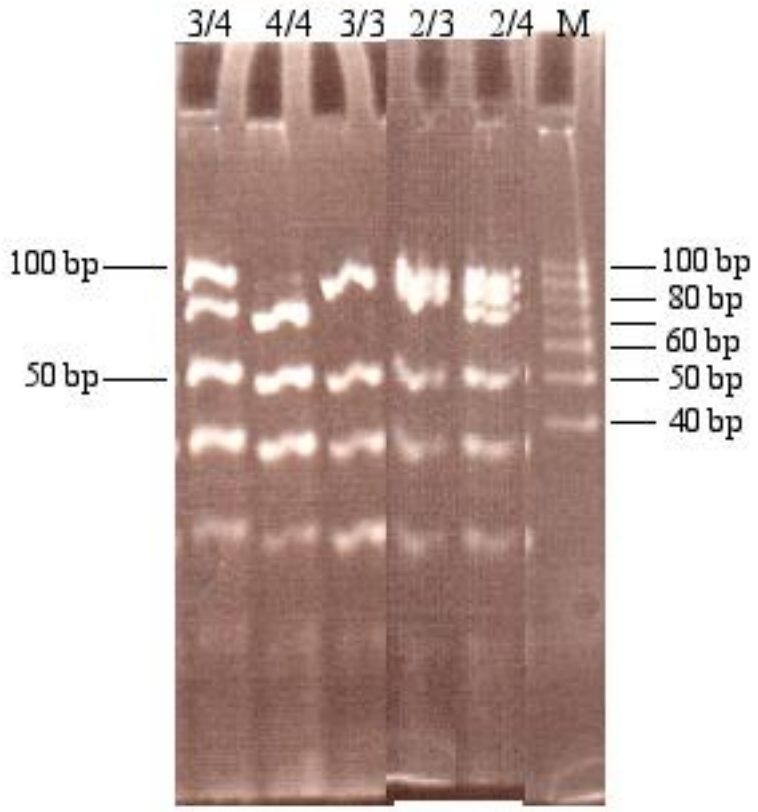


Figure 22. Polyacrylamide gel stained with ethidium bromide and viewed through UV light. *CfoI* restriction fragments from five possible *apo*e genotypes: *apo*e 3/3, 16 bp, 18 bp, 21 bp, 33 bp, 48 bp, 91 bp; *apo*e 2/3, 16 bp, 18 bp, 21 bp, 33 bp, 48 bp, 81 bp, 91 bp; *apo*e 3/4, 16 bp, 18 bp, 21 bp, 33 bp, 48 bp, 72 bp, 91 bp; *apo*e 4/4, 16 bp, 18 bp, 21 bp, 33 bp, 48 bp, 72 bp; *apo*e 2/4, 16 bp, 18 bp, 21 bp, 33 bp, 48 bp, 72 bp, 81 bp, 91 bp. 10 bp step ladder. Sizes in basepairs.

Genotyping of the offspring from transgenic mice

Genomic DNA from suspected transgenic mice was isolated from about 1 cm ends of mouse tails. PCR was performed using standard procedures and primers for human ps-1 (oligonucleotide # 10; oligonucleotide # 11), human app (oligonucleotide # 12; oligonucleotide # 13), and MFH (murine fetal hemoglobin; oligonucleotide # 14; oligonucleotide # 15; annealing temperature for all reactions 53°C) as an internal control for genomic DNA. PCR was analyzed in an 1% agarose gel.

Taq Polymerase + 10 x reaction buffer Gibco BRL LifeTech

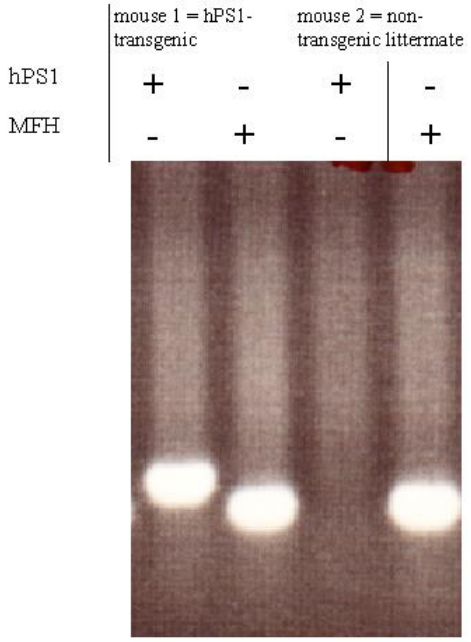


Figure 23. Ethidium bromide stained agarose gel viewed through UV light. 1st and 2nd lane, mouse transgene for human PS-1 (PCR with Primers for human PS1 and MFH, respectively); 3rd and 4th lane, non-transgenic littermate mouse (no amplificate with PS-1 primers but PCR product with MFH control primers).

Sequences of oligonucleotides

oligonucleotide # 1: TTTTTTTTTTTTTTT

oligonucleotide # 2: CGGGGTACCCCGATGCTCACATTCATGG

oligonucleotide # 3: CGGGGTACCCCGATGGTGTGGACGG

oligonucleotide # 4 TGCTCTAGAGCATCAGATGTAGAGCT

oligonucleotide # 5: CGGGGTACCCCGATGAATATGGCAGAAGG

oligonucleotide # 6: TGCTCTAGAGCACTAGATATAAAAT

oligonucleotide # 7: CGGGGTACCCCGATGCCTCATCGC TCT

oligonucleotide # 8: TCCAAGGAGCTGCAGGCGGCGCA

oligonucleotide # 9. ACAGAATTCGCCCCGGCCTGGTACACTGCC**A**

oligonucleotide # 10: TAATTGGTCCATAAAAGGC

oligonucleotide # 11: GCACAGAAAGGGAGTCACAAG

oligonucleotide # 12: GTAGCAGAGGAGGAAGAAGTG

oligonucleotide # 13: CATGACCTGGGACATTCTC

oligonucleotide # 14: GATCATGACCGCCGTAGG

oligonucleotide # 15: CATGAACTTGTCCCAGGCTT

Proteinchemical methods:

Western blotting procedure:

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, protein samples were supplemented with 2 x SDS-sample buffer. For PS1 detection, samples were run under reducing conditions, but without boiling the sample since aggregation of presenilins is likely to occur under heat. Usually 10-30 µl samples were electrophoresed in parallel with a rainbow molecular mass marker on vertical slab SDS-polyacrylamide gels. Homogenous gels (0.5 mm) were cast as indicated, according to the protocol of Biorad Bulletin 1156 (1984).

Electrophoresis was started at 100 V until the buffer front entered the separating gel; voltage was than set up to 200 V. The running time was about 4 hours.

1 x SDS-sample buffer
62.5 mM Tris-HCl, pH 6.8 at RT
2% w/v SDS
10% glycerol
50 mM DTT

Running buffer:
25 mM Tris-HCl, pH 8.3 at RT,
192 mM glycine
0.1% (w/v) SDS.

Stacking gel :

40% acrylamide
2% bis-acrylamide
Tris-HCl, pH 6.8
10% SDS
H_2O
TEMED
APS

Running gel:
40% acrylamide
2% bis-acrylamide
Tris-HCl, pH 8.8
10% SDS
H_2O
TEMED
APS all Merck

For analysis of human PS1 transiently expressed in Jurkats or in transgenic mice, Western blotting was performed with 16% polyacrylamide gels as described elsewhere (Sych *et al.*, 2000). R27 is a polyclonal antibody that was raised in rabbit and reacts with several epitopes (N- and C- terminal) in the loop region. 231 reacts with the N-terminus. Both antibodies were a kindly gift of B. Yankner (Children Hospital, Havard Medical School, Boston, USA) .

The transfer cassette is assembled according to the manufacturer's instructions and the transfer was run at 120V and 350 mA for 1.5 hours. The blotting apparatus is chilled in an ice bath. After transfer, the membrane is placed in blocking solution on a rocker platform for at least 60 min at RT and subsequently incubated with primary antibody diluted in Blocking Solution on a rocker plate 1 h at RT or overnight at 2-8°C. After extensive washing with PBS-Tween, the membrane is incubated with the secondary antibody for 60 minutes. Chemiluminescent detection was performed with ECL according the supplier instructions.

Transfer buffer:
12 mM Tris-HCl, pH 8.3 at RT,
96 mM glycine,
20% (v/v) methanol. Merck
Chill to 2-8°C prior to use.

PBS-Tween:
0.01% (v/v) Tween 20 in Dulbecco's PBS

Blocking Solution:
2% (w/v) nonfat dry milk in PBS-Tween

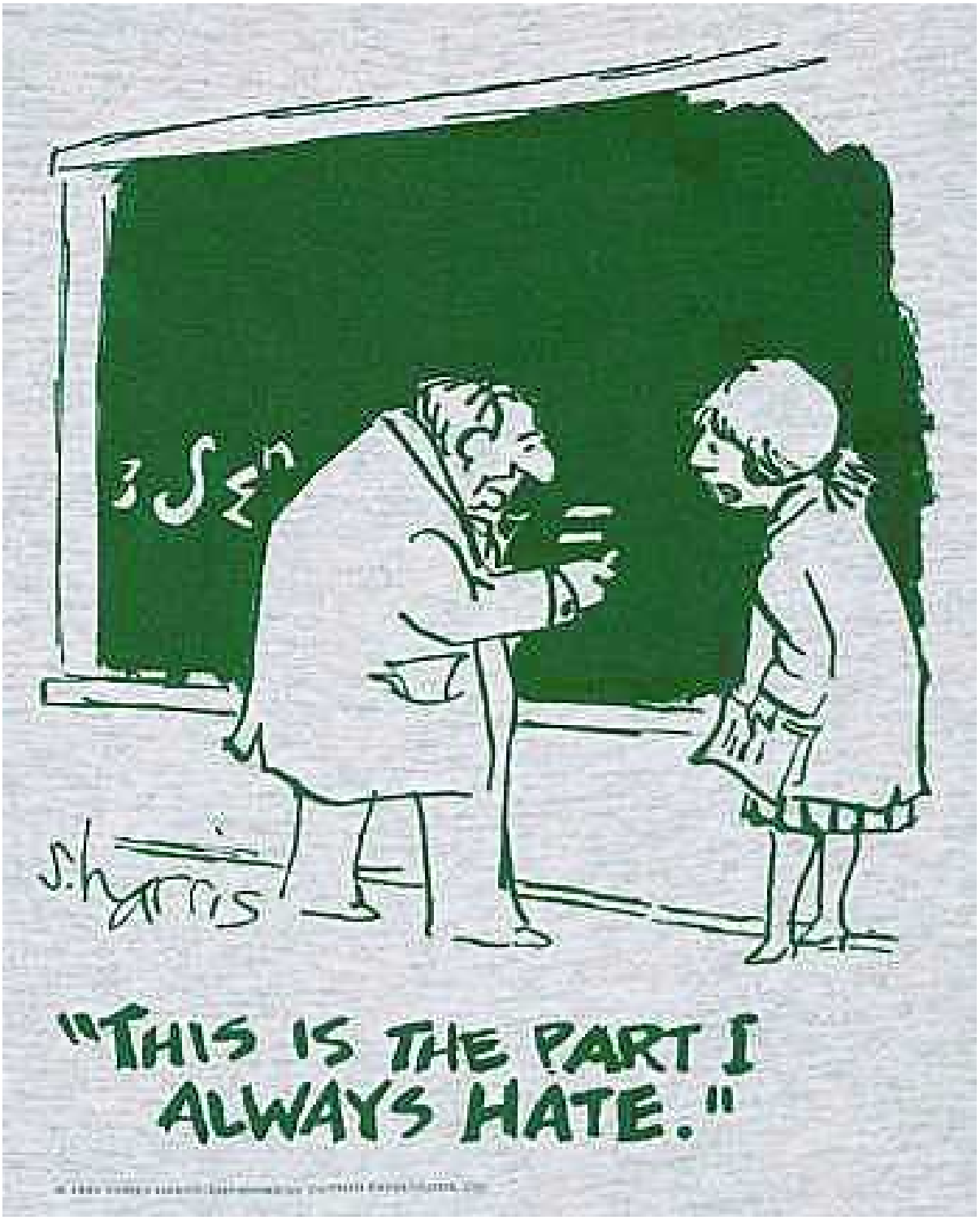
Quantitfication of Bcl-2

The content of Bcl-2 was determined from peripheral lymphocytes ($1x10^6$ cells) with an ELISA assay (Oncogene, USA) according to the manufacture's manual.

Statistical evaluation of the data:

Statistical analysis was performed by Student's paired/ unpaired t-test, correlation or one-way or two-way ANOVA followed by post-hoc tests (Tukey, Bonferroni) calculations (Prism 2.01, GraphPad Software, Inc, San Diego, USA). All data presented are means ± S.E.M. Results were termed significant if $p<0.05$.

Results



Age-related changes in apoptotic cell death of human peripheral blood cells

Basal and spontaneous apoptosis are increased in lymphocytes from elderly subjects

Using PI staining, the sub-G_1-DNA content in lymphocytes from 'healthy' elderly and young controls was quantified. Figure 27 shows the histogram plots of PI-stained lymphocytes of two representative subjects. Student's t-test revealed that the percentage of basal apoptotic nuclei in freshly isolated lymphocytes was significantly elevated in aged subjects compared to young controls (means ± S.E.M.: old: 1.35 ± 0.196% vs. young 0.84 ± 0.113%; *p<0.05; Figure 24a).

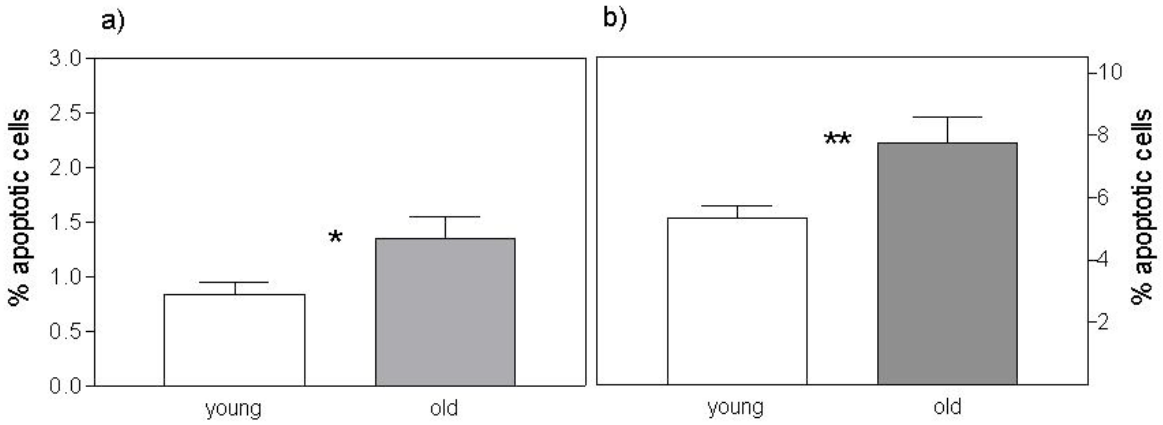


Figure 24. a) basal levels of apoptotic nuclei in freshly isolated PBMC. Apoptosis was determined by propidium iodide staining as described in methods. Cells derived from aged humans (over 60 years old) display significantly more basal apoptotic nuclei (1.35 ± 0.196%, n = 34) than younger controls (up to 35 years old; 0.84 ± 0.113%; n = 32, *p<0.05, Student's t-test). b) spontaneous apoptosis in PBMC of young and old humans: cells were cultured for 24 hours in absence of stimuli. In aging, significantly more apoptotic cells accumulate under this condition. PBMC from old subjects displayed 8.06 ± 0.85% apoptotic cells while young subjects showed only 5.52 ± 0.43% apoptotic nuclei **p<0.01.

In addition, we found a significant higher content of cells undergoing spontaneous *in vitro* apoptosis after 24 hours in the group of old subjects in relation to young ones (Figure 24b, **p<0.01; old 8.06 ± 0.85%; young: 5.52 ± 0.43%). Moreover, when all investigated subjects were pooled for regression analysis, increasing age positively correlated with percentage of

apoptotic cells by regression analysis. Spontaneous apoptosis after 24 hours increased 2.1-fold (slope = 0.061 ± 0.02) between 15 and 93 years of age (Figure 25, dotted line (- - -)). There was a significant (***p < 0.001, n = 85) correlation between age and the portion of apoptotic cells.

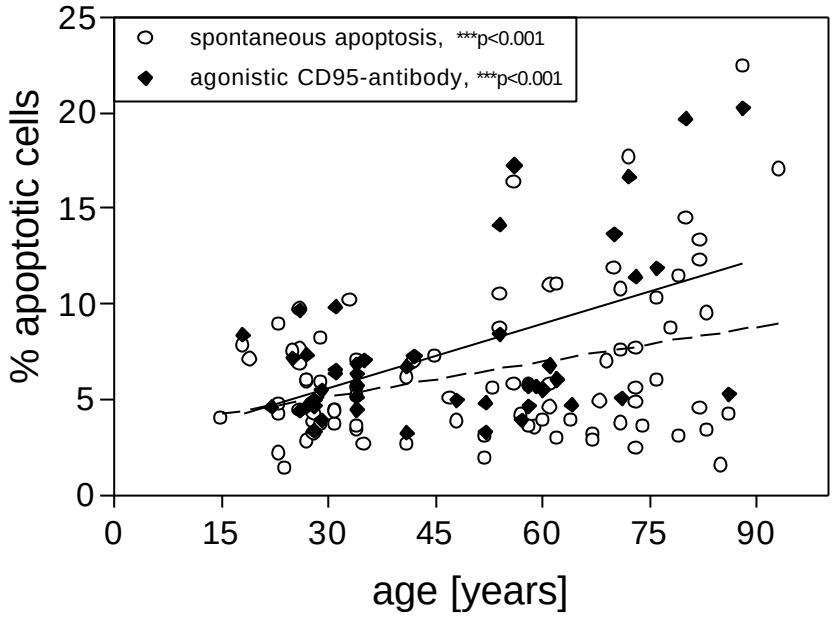


Figure 25. Spontaneous and CD95 induced apoptosis in PBMC after 24 hours incubation: Lymphocyte apoptosis correlates with increasing age. PBL from subjects ranging from 17 to 82 years of age were cultured 24 h in vitro and spontaneous apoptosis (open circles = O) was determined by flow cytometry analysis. % apoptotic cells was correlated with gender age (r= 0.34, ***p < 0.001, n = 85). The percent increase per year is 0.06 ± 0.03 %. Black rhombs (◆) symbolize apoptosis of cells cultured for 24 hours with an agonistic CD95-antibody. In addition, correlation with age is also highly significant (r = 0.50, ***p < 0.001, n = 46). CD95 induced apoptosis increases even more with age than the spontaneous *in vitro* apoptosis does (slopes of the linear regression analysis: 0.122 ± 0.04; 0.061 ± 0.02, respectively).

To ascertain that these alterations in apoptotic cell death were not linked to alterations in the distribution of PBMC subpopulations, the same experiments were performed with activated lymphocytes consisting to over 95% of $CD3^+$ cells. Lymphocytes, which are undergoing proliferation, can be triggered to programmed cell death by withdrawal of interleukin-2 (IL-2). IL-2 is the most critical determinant in this process (21). Activated lymphocytes have been shown to be more sensitive to apoptosis than non-treated native cells. Basal apoptotic levels

of activated T-cells correlated significantly with the donor age (*p<0.05, n=52; Figure 26). In addition, spontaneous *in vitro* apoptosis after 24 hours (under these conditions also called AICD: activation-induced cell death, that is triggered by IL-2 treatment followed by withdrawal) did as well significantly correlate with age (*p<0.05, n=45; Figure 26).

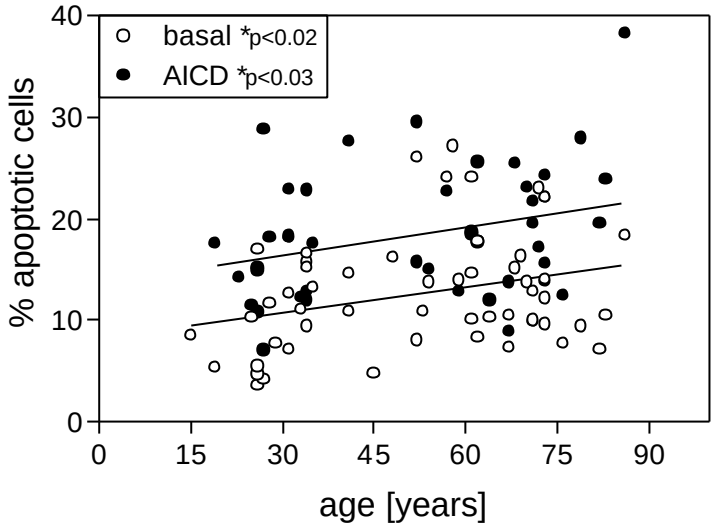


Figure 26. Basal apoptotic levels and activation-induced cell death (AICD) of activated lymphoblasts correlate significantly with the age of the donor. Open circles (○) show basal apoptotic levels after lymphocyte activation as described in methods. Correlation with subjects' age is significant (*p<0.02; n = 52). Closed circles (●) display AICD of activated lymphoblasts after 24 hours withdrawal of IL-2 (*p<0.03; n = 45).

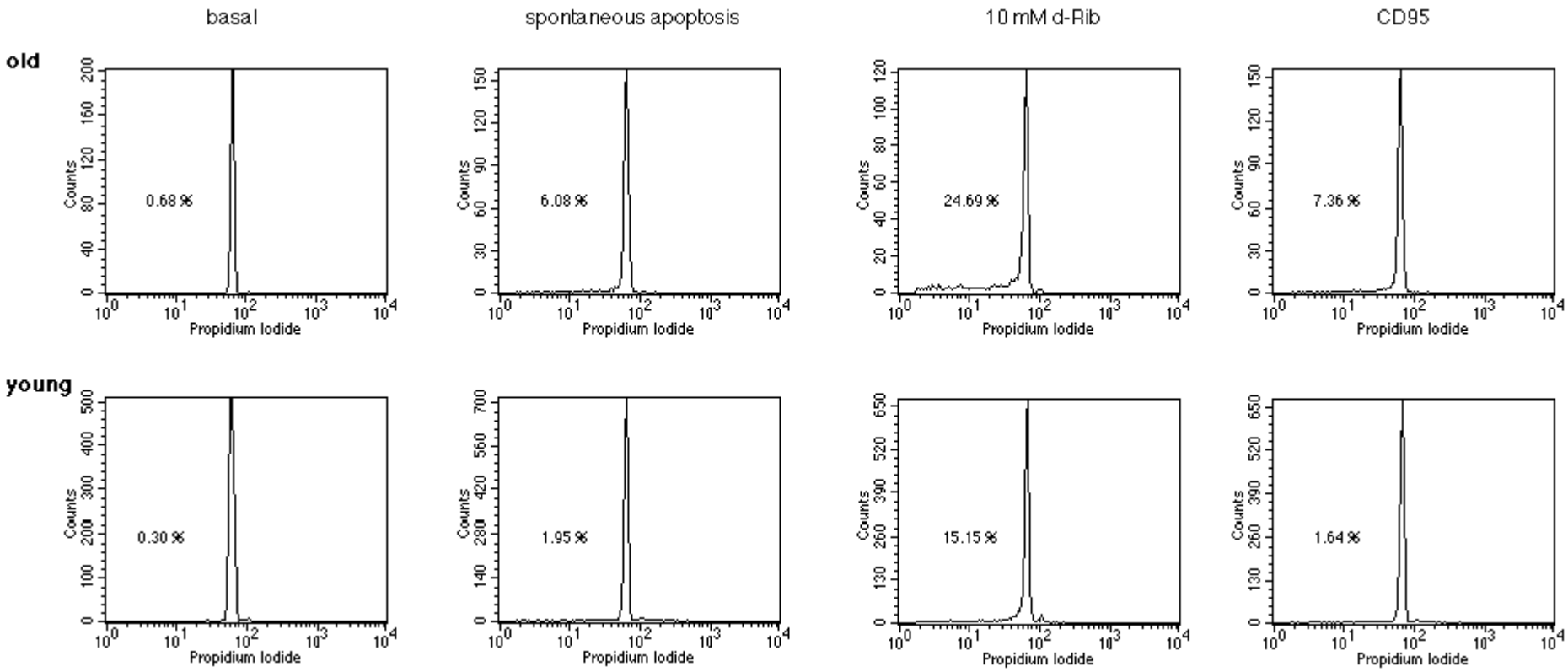


Figure 27. Histogram plots of propidiume-iodide stained and lysed PBMC from each an old (upper level) and a young (lower level) individual. Percentage of sub-G_1-DNA-content (% apoptotic cells) under different conditions is indicated. First column shows basal apoptotic levels of freshly isolated PBMC, the second displays the spontaneous *in vitro* apoptosis after 24 hours in culture, and the last two columns exhibit dRib- and CD95-induced apoptosis after 24 hours.

PBMC from aged donors are more vulnerable to induction of apoptosis by oxidative stress

Oxidative stress was induced with 2-deoxy-D-ribose (dRib, (Barbieri *et al.*, 1994)). Cells were incubated for 24 hours at 37°C, harvested, propidium iodide stained and analyzed by flow cytometry. In the presence of 10 mM dRib, the sub G_1-DNA content was elevated in both groups compared to spontaneous apoptosis but PBMC of elderly subjects did react significantly more sensitive to this apoptotic stimulus than the younger group.

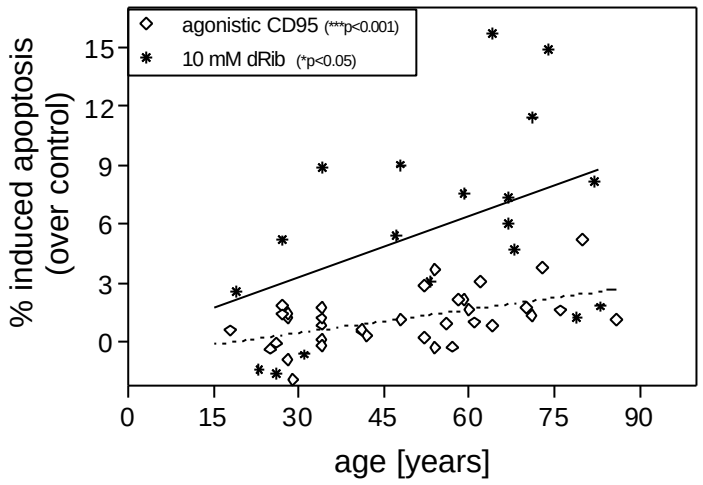


Figure 28. Spontaneous *in vitro* apoptosis was subtracted from CD95- and dRib-induced apoptosis resulting in the percentage of apoptosis induced by the agent. This so called induced apoptosis over control was correlated with age of subjects. Black stars (*) represent dRib- induced apoptosis, open rhombs (à) symbolize CD95-induced apoptosis.

After subtraction of spontaneous apoptosis, a significant increase of vulnerability to ROS-induced apoptosis was determined (Figure 28; correlation *p<0.05 and Figure 29: old: 7.92 ± 1.8% vs. young: 3.0 ± 1.2%, *p<0.05), indicating an age-related impairment to cope with oxidative stress. In addition, higher concentrations of 2-deoxy-D-ribose (50 mM) could not induce higher levels of apoptotic cells in old volunteers (old: 7.22 ± 1.20 vs. young: 6.85 ± 1.45; n = 37 per group; p = 0.42).

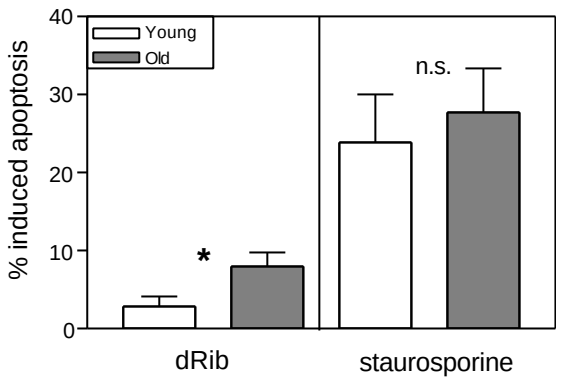


Figure 29. Induced apoptosis over control (spontaneous apoptosis) after 24 hours by dRib (10 mM) and staurosporine (100 nM) in groups of young and old subjects (dRib: young = 3.00 ± 1.187% vs. old = 7.92 ± 1.741%, n = 11; *p<0.05; staurosporine: 23.71 ± 6.331% vs. 27.65 ± 5.634%; n.s.; n = 11, respectively).

Functional CD95 expression is higher in lymphocytes of elderly subjects

Incubation for 24 hours with an agonistic monoclonal anti-CD95 antibody that acts like a Fas-Ligand, showed significant changes in the induction of apoptosis in native cells between young and old genders (6.19 ± 0.26%; 11.05 ± 1.78%, respectively, ***p<0.001). Apoptotic cells correlated significant with donor age after incubation with a CD95 antibody (***p<0.001, Figure 28, black line). However, in young donors the number of apoptotic cells is not altered after CD95 treatment compared to spontaneous apoptosis (n.s.; paired t-test). In contrast, PBMC from aged donors are far more sensitive to apoptosis with agonistic CD95 antibody (paired t-test gave: ***p<0.001). By subtracting the spontaneous apoptosis in each subject the CD95 induced apoptosis is determined, that increases significantly with aging (Figure 28).

Apoptosis induced by staurosporine does not correlate with aging

To examine whether the age-related increase in apoptosis is specifically associated with oxidative stress or not, and/or with the higher expression of activation receptors, we also induced programmed cell death in PBMC by staurosporine. Though, induction of cell death with staurosporine is very common throughout literature, the mechanism are still not fully

elucidated (Krohn *et al.*, 1998). However, it seems that ROS are not directly involved in apoptosis initiation, possibly caspases are activated directly by staurosporine. Incubation of PBMC with 100 nM staurosporine showed a dramatic increase in the percentage of apoptotic cells compared with the untreated controls undergoing spontaneous apoptosis (***$p<0.001$). However, no significant effect could be determined related to aging (Figure 29). Staurosporine induces apoptosis independently from the donor's age. In summary, the data indicate that there are differences in efficacy to induce age-related changes in apoptosis by different stimuli.

Elevated expression of activation markers in aging

In order to explore the mechanism for the increased susceptibility to apoptosis in PBMC from aged donors, we determined the expression of activation markers HLA-DR, CD95 (Apo, Fas) and CD25 (Interleukin-2-receptor). Quantitation of T-lymphocytes expressing HLA-DR, CD95 and CD25 (IL2-Rα) was performed by direct immunofluorescence in wholeblood followed by FACS analysis (Figure 30). $CD3^+$ T-cells from young donors (up to 35 years) showed a significantly (***$p<0.0001$) smaller portion of HLA-DR-positive cells (5.9 ± 0.4%) than $CD3^+$-T-lymphocytes from aged subjects (10.8 ± 0.97%). $CD3^+$ cells from young donors expressed significantly less CD95 receptors than elderly subjects over the age of 60 years (young: 34.2 ± 2.3% vs. old: 46.4 ± 2.6%). No significant changes with age concerning the expression of CD25 could be detected (young: 13.3. ±0.7% vs. old: 14.8 ±1.3%).

The portion of cells displaying CD95 correlates, as expected, significantly with the percentage of CD95-agonistic-antibody induced apoptosis (data not shown), confirming the age-related increase in CD95-induced cell death.

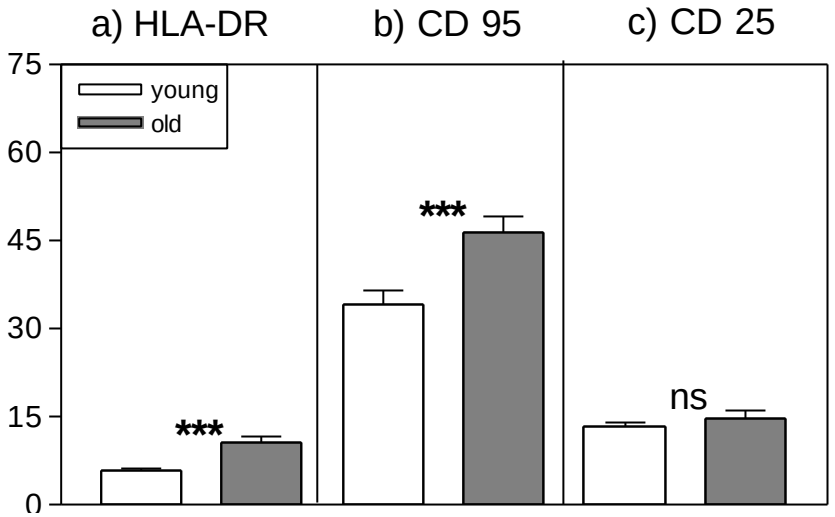


Figure 30. Determination of surface expressed activation markers of $CD3^+$-lymphocytes from young (n = 32) and old genders (n = 34). T-cells from young donors displayed a significantly (*p<0.0001) smaller portion of HLA-DR-positive cells (5.9 ± 0.4%) than T-lymphocytes from older subjects (10.8 ± 0.97%). $CD3^+$ cells from young donors expressed significantly less CD95 receptors than elderly subjects (young: 34.2 ± 2.3% vs. old: 46.4 ± 2.6%). No significant changes with age concerning the expression of CD25 could be determined (young: 13.3. ± 0.7% vs. old: 14.8 ± 1.3%).**

The more the peripheral lymphocytes express CD95 and CD25 at basal levels, the higher is the percentage of cells undergoing apoptosis initiated by glutathione depletion (10 mM and 50 mM dRib) as shown by correlation (Figure 31), indicating that these lymphocytes are more susceptible to oxidative stress.

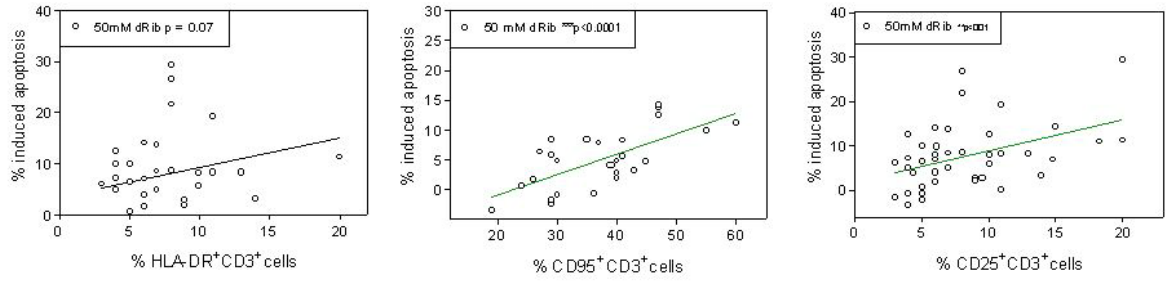


Figure 31. Correlation of dRib-induced (50 mM) apoptosis and basal expression of activation markers. Correlation with a) HLA-DR (n.s. p = 0.07, n= 33); b) with CD95 (*p<0.0001; n = 25); and c) with CD25 (**p<0.01, n = 41).**

Elevated levels of Bcl-2 in lymphocytes from aged humans

Overexpression of Bcl-2 counteracts apoptotic processes (6) probably by activating anti-oxidative mechanism. To investigate the link between apoptosis and oxidative stress in peripheral human lymphocytes we determined the basal content of Bcl-2 in freshly isolated PBMC by ELISA. There was a positive correlation between the content of Bcl-2 and the percentage of basal apoptotic cells (Figure 32; *p<0.05; n= 18). Moreover, the amount of Bcl-2 seems to increase with aging (Figure 32 inset).

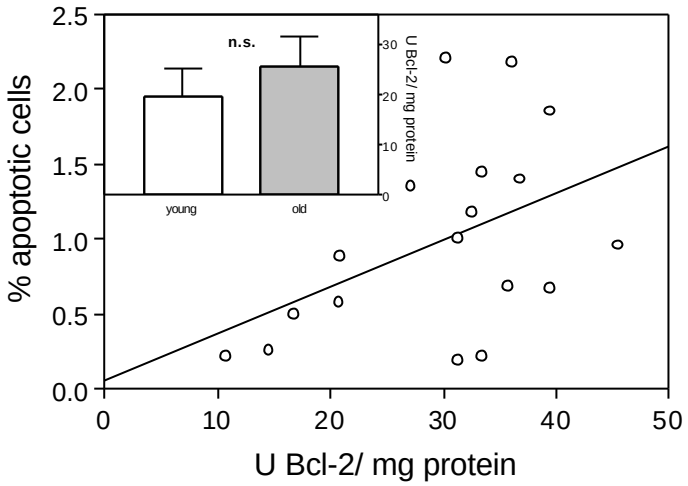


Figure 32. Basal expression of Bcl-2 seems to increase with aging (inset) in human PBMC. Peripheral cells of young subjects displayed 19.6 ± 5.66 U Bcl-2/ mg protein, PBMC of aged volunteers expressed 25.8 ± 5.67 U Bcl-2/ mg (n = 9 per group). Content of Bcl-2 correlates significantly (*p<0.03; n=18) with basal percentage of apoptotic cells in freshly isolated PMBC from young and old donor.

Increased apoptosis in Alzheimer's disease

Apoptosis in peripheral blood mononuclear cells of patient with DAT

Earlier results of our group indicate elevated basal levels in PBMC from patients suffering from sporadic DAT (Eckert et al., 1998; 1999; 2001). Moreover, spontaneous and dRib-induced apoptosis was similarly altered in this cohort of patients. Apoptotic cell death was determined by an ELISA kit sensitive for histones which become apparent when nucleosomes occur during apoptosis.

One aim of the present thesis was to reproduce and extend these results with flow cytometric methods in a second cohort of sporadic AD patients. Furthermore, the mechanisms underlying the altered apoptotic behavior of PBMC from AD-patients should be elucidated.

Heparinized blood samples of DAT-patients were collected by our clinical cooperation partners from the 'Gedächtnis-Ambulanz des Universitätsklinikums Frankfurt am Main'. The PBMC were isolated from the blood samples within 1 hour later as described. Staining of apoptotic nuclei was performed with PI and analyzed by flow cytometry. Samples of matched elderly non-demented controls were run in parallel.

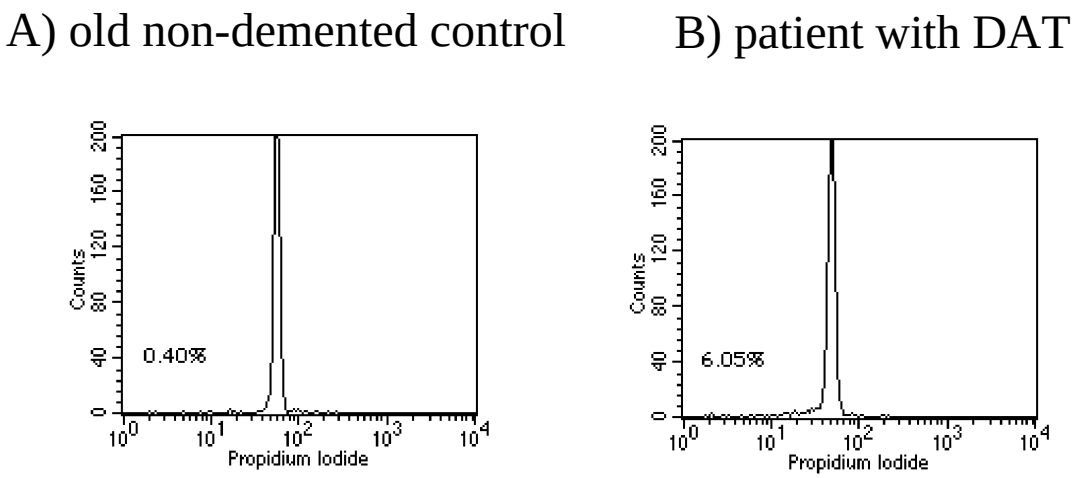


Figure 33. Representative PI-stained basal PBMC (immediately after isolation) from an old non-demented control and an AD-patient.

A significant increase in the basal apoptotic levels was determined in PBMC from AD-patients compared to age-matched controls (Figure 33). Demented patients showed about the doubled amount of apoptotic cells after isolation of PBMC compared with cells from control

persons. Moreover, when all data of demented patients were pooled and plotted against the Mini Mental State Examination Score (MMSS) there was a significant correlation with increasing apoptotic nuclei (*p<0.05).

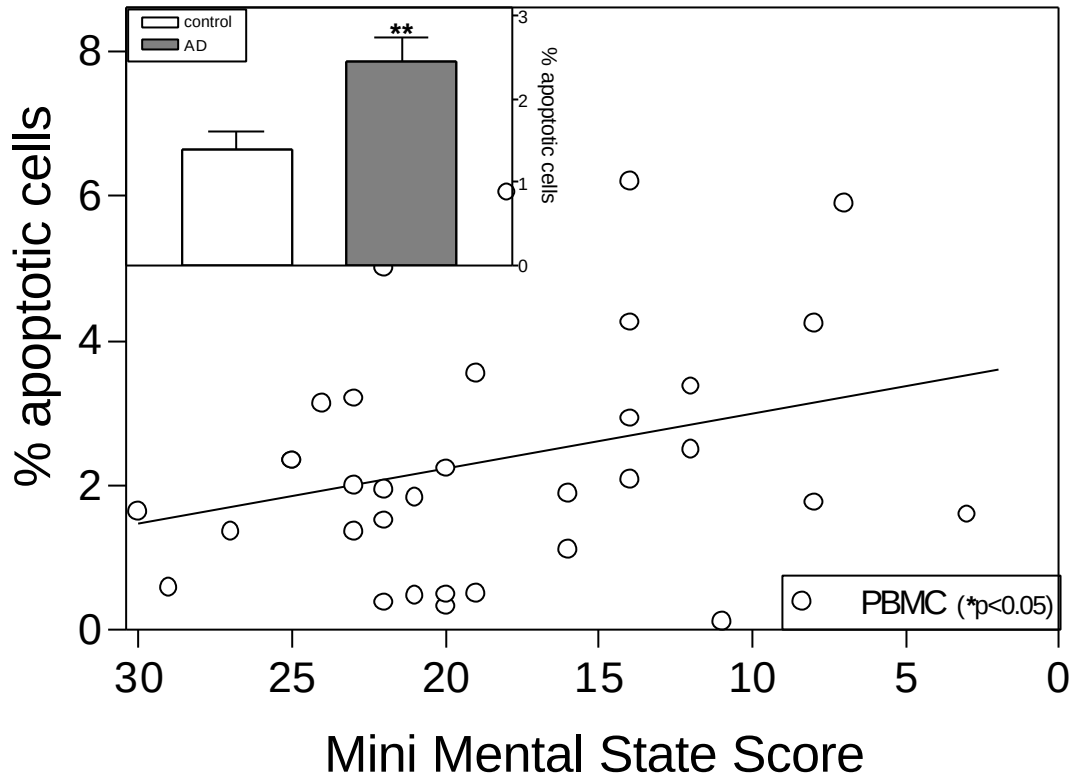


Figure 34. Whole freshly isolated PBMC were stained with Propidiume Iodide as described by Nicoletti *et al*., 1993. Basal levels of apoptotic peripheral cells correlate significantly with severity of disease (Mini Mental State Score; n = 34, *p<0.05). Compared with old non-demented controls, AD-patients provide increased basal levels of apoptotic cells (inset; control: 1.35 ± 0.196%vs. AD: 2.37 ± 0.287%; n = 34 per group; **p<0.01).

Analysis of apoE genotype showed an increased percentage of basal apoptotic cells from AD patients carrying APOE4 (Figure 35). However, due to the very small number of E4 positive subjects these changes are not statistically significant. PBMC of AD-patients and elderly controls were incubated for 24 hours in order to determine the spontaneous apoptosis. In addition, samples were cultured in the presence of 10 mM or 50 mM dRib, 0.1 µM dexamethasone, 50 ng/ml Fas-ligand or an agonistic CD95-antibody, respectively. Samples were processed as described and apoptosis detected by PI-staining. The results are

summarized in Figure 35. Treatment with 50 mM dRib showed elevated levels of apoptotic nuclei in AD (AD: 13.86 ± 1.625% vs. control: 8.43 ± 1.554%; *p<0.05; n = 13 per group), however, since massive necrosis did already occur at this dose of dRib, this treatment was omitted in further experiments. In contrast, dRib at a concentration of 10 mM clearly induced apoptosis. This concentration was the standard condition for the following experiments.

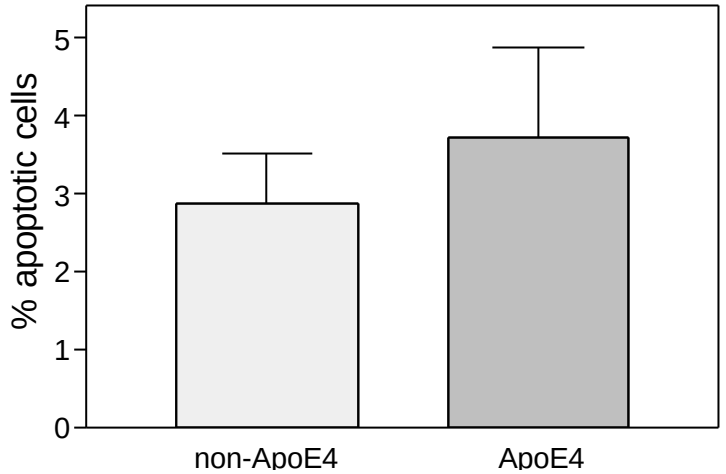


Figure 35. Patients with apoE4 genotype show elevated levels of basal apoptotic cells (3.72 ± 1.163%; n = 11) compared with patients that are not ApoE4-positive (2.87 ± 0.636%; n = 19). However, those changes are not significant, probably due to a too low number of included subjects included.

Significant differences were detected in spontaneous and Fas-receptor mediated apoptosis. Furthermore, ROS- (*via* dRib) and Fas-ligand induced apoptosis correlated significantly with the MMSS of each individual, indicating an increased vulnerability to ROS and an enhanced functional expression of Fas-receptor as the dementia proceeds.

	AD-patients	old controls	PBMC			
	mean age; number of patients; mean MMSS	mean age; number of volunteers	% apoptotic cells from AD-patients	% apoptotic cells in age-matched controls	significance (AD vs. controls)	correlation with MMSS
basal	73.4±3.5 years; n=34, 18.8±1.12 MMSS	71.5±4.6 years; n=34	2.37 ± 0.287%	1.35 ± 0.196%	**p<0.03	*p<0.05
spontaneous	72.2±3.1 years; n=22, 19.1±1.48 MMSS	74.0±4.9 years; n=25	8.10 ± 0.793%	5.01 ± %	**p<0.01	not done.(n.d.): due to too small number to calculate proper correlation analysis
dRib (10 mM)	74.7±6.1 years; n=10, 19.0±1.94 MMSS	73.2±3.1 years; n=11	7.23 ± 1.964%	7.92 ± 1.740	n.s.	n.d.
dexamethasone	74.1±4.8 years; n=11, 18.9±1.34 MMSS	73.7±3.1 years; n=15	5.32 ± 1.215%	6.57 ± 2.107%	n.s.	n.d.
Fas-L	77.6±5.8 years; n=15, 19.3±1.78 MMSS	73.8±6.0 years; n=16	13.64 ± 3.528%	7.40 ± 0.980%	*p<0.05	n.d.

Table 2. Percentage of PI-stained PBMC from patient with DAT and elderly controls.

Changes in lymphocyte's subsets in aging and AD

In order to explore, whether those changes in apoptotic cell death were due to, it is necessary to determine if the constituents of the PBMC (T-, B-, and NK-lymphocytes) were changed in AD-patients. Moreover, the lymphocyte subsets might have a different apoptotic behavior that should be analyzed.

The PBMC consist mainly of T-cells (varying from 80 to 60% in relation to the volunteer's age). About two thirds of the T lymphocytes are T helper cells ($CD4^+$). Therefore, changes in the percentage of T lymphocytes, and/ or their subsets T helper- or T suppressor cells, could influence the number of apoptotic cells in the whole PBMC.

First, the changes of PBMC constituents were determined in 'healthy aging'.

Absolute number of total T cells, as well as the number of $CD4^+$- and $CD8^+$-lymphocytes decreases with aging

To determine the absolute amount of T lymphocytes with respect to their subsets, an aliquot of heparinizied blood samples was quantified by flow cytometry using TrueCount® beads and antibodies against CD3, CD8, and CD4. Only CD3-PerCp-positive cells were electronically gated and a minimum of 100,000 cells was acquired The number of CD3-positive cells significantly decreases in aging (CD3, young: 1888 ± 147 cells/µl; old: 1236 ± 153 cells/µl; n = 17-21; $^{**}p<0.005$). The loss of cells observed in aged subjects seems to be distributed among both subsets (CD4, young: 1109 ± 103 cells/ µl; old: 763 ± 84 cells/ µl; n = 17-21; $^{*}p<0.012$ and CD8, young: 601 ± 53 cells/µl; old: 353 ± 68 cells/µl; n = 17-21; $^{**}p<0.005$; Figure 36A). However, the number of $CD8^+$ cells seems to be more affected by aging than the T helper lymphocytes ($CD4^+$).

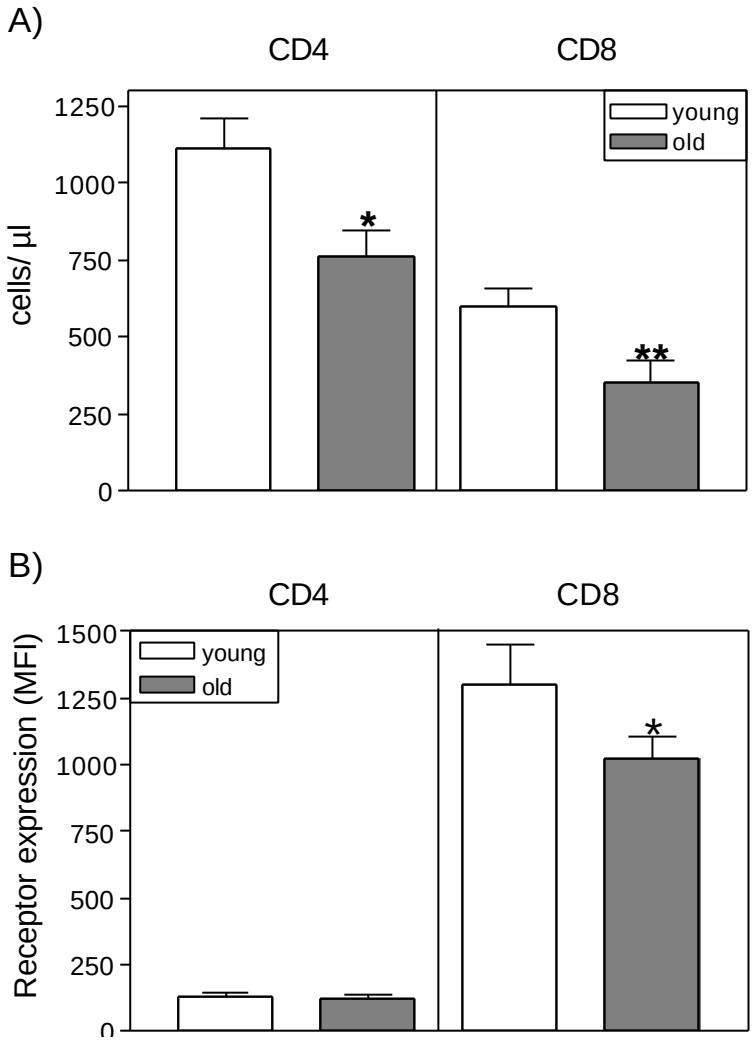


Figure 36. (A) The absolute number of CD4 and CD8 lymphocyte is significantly reduced in blood samples from aged donors. Cell counts were determined by using the TrueCount® kit, providing a defined number of beads in the staining tube. (B) The density of the costimulatory receptors CD4 and CD8 on lymphocytes from young and old subjects. Mean fluorescence intensity (MFI) determined, as the mean channel number is proportional to the density of the measured receptor. No changes concerning CD4-receptor density were observed between lymphocytes from young and old donors. In contrast, a significant decrease of CD8-receptor was determined in lymphocytes from aged donors when compared with young ones. Soluble CD8-receptor is known as an activation marker.

In addition, the mean fluorescence intensity (MFI) that is proportional to receptor surface density was measured. The density of the CD3- and CD4-receptor was not altered between the group of young and old donors (CD3, young: 182 ± 17; old: 160 ± 7, CD4, young: 130 ± 10;

old: 122 ± 10; n = 11-14). Interestingly, the density of CD8-receptor on each single T suppressor cell was significantly decreased in the aged group (CD8, young: 1300 ± 151; old: 1025 ± 81; n = 11-14; *p<0.05; Figure 36B).

To explore the changes of subsets within the whole lymphocyte population, the percentage of T-, B-, and NK (natural killer)-lymphocytes were surveyed and plotted against the donors' age. The percentage of CD3-positive cells within the gated lymphocyte population, as determined by gating in FSC/ SSC, decreases with age (n = 54; **p<0.02; r^2 = 0.15; Figure 37A). Excitingly, the ratio of T helper to T suppressor-lymphocytes, termed CD4/CD8-ratio, is not significantly changed in old individuals (young: 1.91 ± 0.18; old: 1.68 ± 0.21; n = 20-22; figure 2B). The slight reduction of the CD4/CD8-ratio in the aged group becomes evident, looking at the absolute number of $CD4^+$ and $CD8^+$ cells. The loss of $CD8^+$ cells is more dominant than the loss of $CD4^+$ in aging (Figure 36A). However, there is no significant correlation of CD4/CD8-ratio with donor's age; n = 53; r^2 = 0.004. The percentage of B lymphocytes was not significantly affected by the volunteer's age (n = 53, r^2 = 0.006; data not shown). Focusing on the NK subset, there was a significant increase of the percentage of $CD16^+$ and $CD56^+$ cells within the lymphocyte gate (n = 53; ***p<0.001; r^2 = 0.19; Figure 37C). No changes concerning appearance of the forward-side scatter plot could be observed. In both age groups, lymphocytes could be clearly identified and were sharply separated by gating from monocytes and granulocytes.

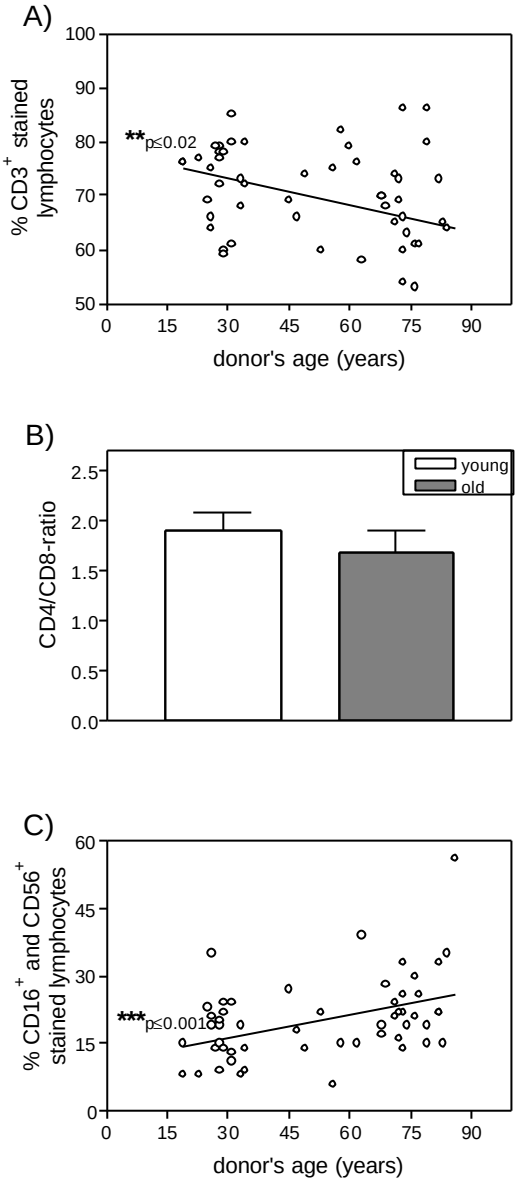


Figure 37. Lymphocyte subset distribution in aging. The percentage of all FSC-SSC-gated lymphocytes was determined and plotted against donor's age. (A) The percentage of CD3 positive cells significantly decreases with increasing age of the blood donor. (B) However, the subset distribution of the $CD3^+$ T cells, expressed as CD4/CD8-ratio, did not differ significantly between the two age groups. (C) The percentage of natural killer (NK) lymphocytes significantly increases with increasing donor's age.

In aging, the content of T lymphocytes ($CD3^+$) decreases and the percentage of NK cells ($CD16^+CD56^+$) increases significantly with the donor's age (Table 3). In addition, the ratio of the T cell subsets ($CD4^+$, T helper/inducer and $CD8^+$, T cytotoxic/suppressor) remains unchanged with aging.

	young		old					
	number of volunteers	mean percentage of positive cells	number of volunteers	mean percentage of positive cells	significance	correlation with age	r^2	number of volunteers
lymphocyte subsets								
CD3	n = 20	72.91 ± 1.543%	n = 25	65.96 ± 2.162%	**p<0.009	**p<0.003	0.1464	n = 54
T lymphocyte subsets								
CD4	n = 20	41.38 ± 1.122	n = 16	39.19 ± 2.801%	n.s.	n.s.	-	n = 40
CD8		23.65 ± 1.034%		25.76 ± 1.716%	n.s.	n.s.	-	
CD4/CD8-ratio		1.852 ± 0.130		1.682 ± 0.214	n.s.	n.s.	-	
CD19	n = 22	11.77 ± 0.862%	n = 25	10.88 ± 1.175%	n.s.	n.s.	-	n = 53
CD16+ CD56		16.55 ± 1.452%		24.36 ± 1.895%	**p<0.002	***p<0.0006	0.1860	
activation markers on $CD3^+$ cells								
HLA-DR	n = 20	5.90 ± 0.435%	n = 24	10.96 ± 0.995%	***p<0.0001	***p<0.0001	0.3166	n = 50
CD95	n = 17	34.24 ± 2.343%	n = 22	46.36 ± 2.718%	***p<0.001	***p<0.0006	0.2228	n = 44
CD25		13.29 ± 0.690%		15.05 ± 1.300%	n.s.	n.s.	-	n = 44
absolute determination of cell number								
CD3/µl	n = 17	1888 ± 147	n = 21	1236 ± 153	**p<0.006	**p<0.005	0.1908	n = 35
CD4/µl		1109 ± 103		763 ± 83.5	*p<0.02	*p<0.03	0.1158	
CD8/µl		601 ± 53		353 ± 67.9	**p<0.005	**p<0006	0.1770	
mean fluorescence intensity of surfaced expressed receptor								
CD3	n = 10	181.6 ± 16.65	n = 14	160.4 ± 7.32	n.s.	n.s.	-	n = 18
CD4		130.3 ± 10.31		121.9 ± 9.90	n.s.	n.s.	-	
CD8		1300 ± 151		1025 ± 80.90	*p<0.05	*p<0.04	0.1902	

Table 3. Determination of lymphocyte subset distribution and percentage of T cells expressing activation markers in aging.

The latter experiments showed that lymphocyte subsets change with aging: there is a decrease of T lymphocytes and an incraese of NK cells. Interestingly, in AD patients we observed a significant decrease in the absolute number of $CD3^+$ cells with increasing cognitive impairment (*$p<0.02$; Table 4). Therefore, a significant involvement of T cell mediated immunity in more demented patients can be considered, since most of the patients displaying quite high MMSS already showed this tendency.

The reduction of TCR associated CD3 in AD, though statistically insignificant, was analyzed by back gating of the $CD8^+$ cells. When only $CD8^+$ gated cells of DAT-patients were displayed in 2D plot, it became evident, that those cells that are low in CD8 display lower number of CD3, as well (data not shown).

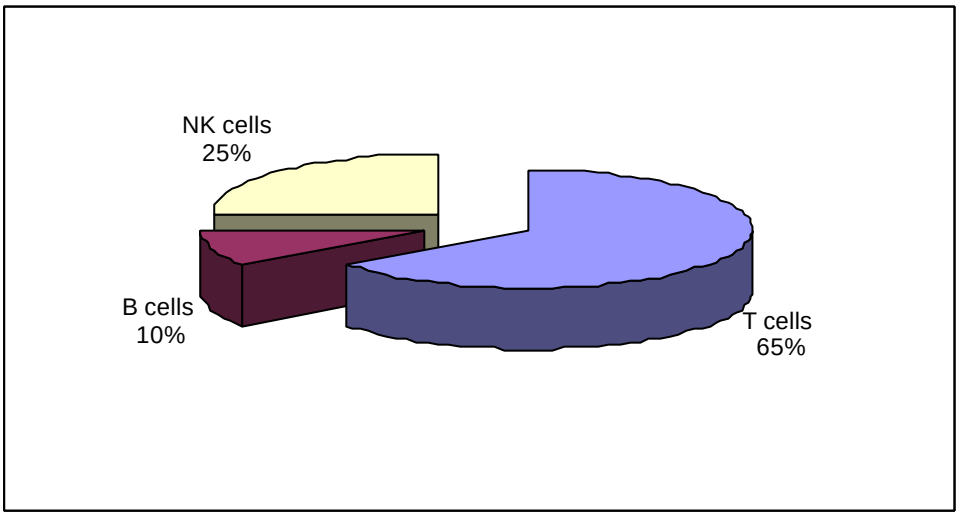


Figure 38. Average contents of the lymphocyte content in the PBMC in aged non-demented persons.

No changes in the percentage of T-, B-, and NK-cells could be determined in whole blood samples of AD-patients. In addition, the absolute number of T cell per µl whole blood did not differ in demented persons (Table 4). However, one of these cell types must account for the elevated of apoptosis found in PBMC in demented subjects. Therefore, apoptosis was determined for each subset. First, the method was validated by determining changes in human aging.

	AD-patients		elderly controls					
	mean age; mean MMSS	mean percentage of positive cells	mean age; number of volunteers	mean percentage of positive cells	significance	correlation with MMSS	r^2	number of patients
lymphocyte subsets								
CD3	n = 23	69.79 ± 1.865%	n = 25	65.96 ± 2.162%	n.s.	n.d.	-	n = 23
T lymphocyte subsets								
CD4	n = 39	60.06 ± 2.971%	n = 16	50.95 ± 3.156%	*p<0.05	n.s.	-	n = 39
CD8		24.31 ± 1.785%		33.51 ± 2.111%	**p<0.003	n.s.	-	
CD4/CD8-ratio		2.31 ± 0.237		1.68 ± 0.214	*p<0.03	**p<0.008	0.1477	
CD19	n = 23	10.34 ± 0.832%	n = 25	10.88 ± 1.175%	n.s.	n.d.	-	n = 23
CD16+ CD56		23.32 ± 1.830%		24.36 ± 1.895%	n.s.	n.d.	-	n = 23
activation markers on $CD3^+$ cells								
HLA-DR	n = 10	9.83 ± 1.182%	n = 24	10.96 ± 0.995%	n.s.	n.d.	-	n = 10
CD95	n = 9	49.22 ± 5.443%	n = 22	46.36 ± 2.718%	n.s.	n.d.	-	n = 9
CD25		19.13 ± 3.712%		15.05 ± 1.300%	n.s.	n.d.	-	
absolute determination of cell number								
CD3/µl	n = 26	1285 ± 69.0	n = 21	1236 ± 153	n.s.	*p<0.02	0.2012	n = 21
CD4/µl		796 ± 40.0		763 ± 83.5	n.s.	*p<0.02	0.2317	
CD8/µl		365 ± 38.5		353 ± 67.9	n.s.	n.s.	-	
mean fluorescence intensity of surfaced expressed receptor								
CD3	n = 12	135.80 ± 10.740	n = 14	160.4 ± 7.32	n.s.	n.d.	-	n = 12
CD4		119.40 ± 9.939		121.9 ± 9.90	n.s.	n.d.	-	
CD8		841.0 ± 87.45		1025 ± 80.90	n.s. (p=0.067)	n.d.	-	

Table 4. Distribution of lymphocyte subsets, percentage of activated, absolute number and receptor expression of T cells in AD-patients and elderly non-demented controls.

Apoptosis in lymphocytes' subsets

Based on these data, the apoptotic behavior of lymphocyte subset from AD patients or aged controls should be determined, in order to explore, whether the altered vulnerability to apoptosis is all cells or specifically distributed in the different lymphocytes subsets.

To determine apoptotic levels of lymphocyte subsets, the detection method had to be adapted. Propidiume iodide provides a too strong fluorescence that is detected not only in one fluorescence channel (FL-2) of the flow cytometer but also partially overlaps with the two other channels (FL-1 and FL-3). Therefore, the following experiments were performed with the DNA dye 7-Aminoactinomycin (7-AAD; described by Schmit *et al*., 1994), that holds an emission maximum at 650 nm and is purely detectable in FL-3. Moreover, 7-AAD posses a very distinct emission profile, that provides no conflict with the other cytometer channels and permits the co-detection of two other fluorochrome (FITC and PE, for instance) within the same experiments. In order to explore the apoptotic levels of T-, B-, and NK-cells, the isolated PBMC samples were stained with 7AAD and monoclonal antibodies against CD3 or CD4, coupled with FITC, and against CD19, CD16+CD56, or CD8, coupled with PE.

In contrast to PI-staining according to Nicoletti *et al.*, 1993, 7-AAD staining allows the differentiation between apoptotic and necrotic/ dead cells.

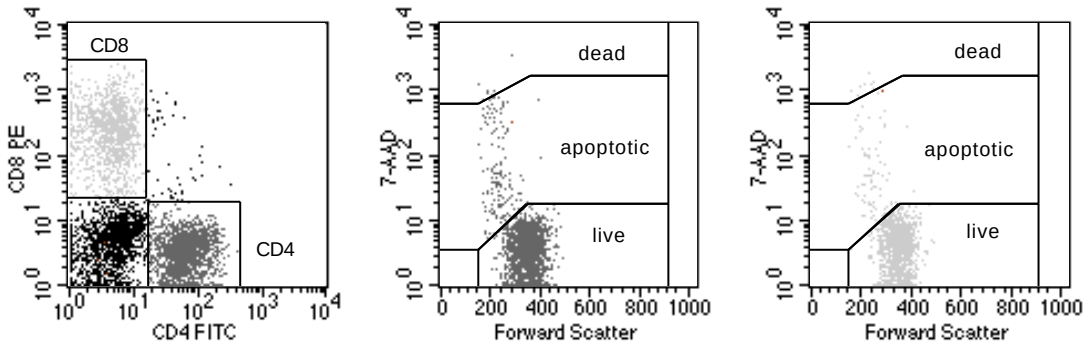


Figure 39. Determination of apoptotic cells with triple staining with 7-AAD and monoclonal antibodies against CD4 and CD8. CD4 and CD8 positive cells, respectively, were gated and displayed as dot plot showing 7-AAD fluorescence versus forward scattered light. Photo multiplier detectors were set, that autofluorescence of 7-AAD of live, intact cells were set in channel numbers between 10^0 and 10^1. A higher 7-AAD fluorescence is evident in apoptotic cells, due to membrane damage. Cells that display the whole DNA content, like permeabilized PI-stained nuclei, were termed dead.

7-AAD detects the same percentage of apoptotic cells as PI does.

To measure accurately the apoptotic population within the PBMC subsets, we used the method of single-cell analysis by flow cytometry. In order to demonstrate that PI and 7-AAD detect the same amount of apoptotic cells, the percentage of apoptotic cells detected with 7-AAD was plotted against the amount of apoptosis determined with PI. Whole PBMC, magnetic isolated CD4 and CD8 were single stained with 7-AAD or PI in two different tubes and analyzed. The correlation is shown in Figure 40 ($y = (0.88 \pm 0.06)x + (2.65 \pm 1.14)$; $r^2 = 0.833$; $n = 51$).

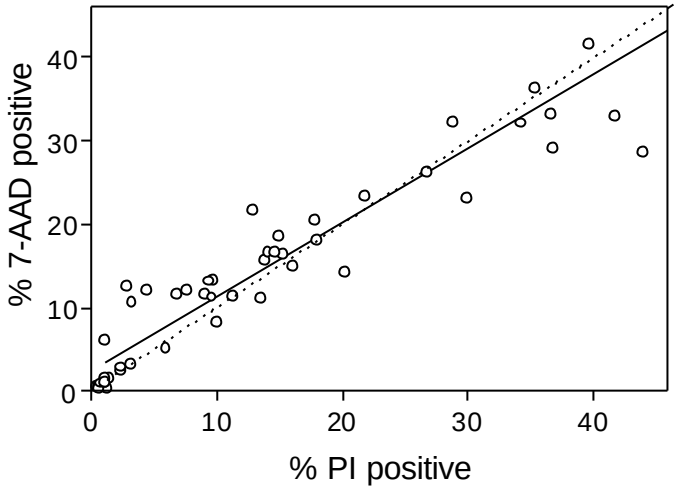


Figure 40. Apoptotic cell death was induced in PBMC from healthy volunteers and apoptosis detected in two separate experiments by PI- and CD4-FITC/7-AAD- or CD8-PE/7-AAD-staining. The content of apoptotic cells found by PI-staining could be reproduced by the new method using 7-AAD as DNA dye. Dotted line shows ideal diagonal. Black line represent the linear regression of the experiment ($n = 51$, $r^2 = 0.833$; $p<0.0001$).

Not only discrimination between CD4 and CD8-positive cells was made possible by the new method, but also the remaining lymphocyte subsets (B- and NK-lymphocytes) could be resolved.

To proove the reproducibility of this method, the comparison of apoptotic lymphocyte behavior in aging was performed by using 7-AAD to determine the physiological apoptotic changes in lymphocyte subsets in healthy aging.

Differences in apoptosis of human lymphocyte subsets in 'healthy' aging.

Basal levels of all lymphocyte subsets were elevated in samples derived from the aged group, indicating that all types of lymphocytes are affected by aging. It is noteworthy that B lymphocytes displayed large variances from both young and aged volunteers. Significant changes in the B cell subsets are therefore unlikely to occur for this relatively small cohort.

Spontaneous apoptosis, in addition, was altered in all lymphocyte subsets with aging. However, it seems that T cells are less vulnerable to spontaneous apoptosis than B cells. ROS-induced cell death mediated by GSH depletion is increased in T and NK cells from aged persons. Interestingly, B cells seem to be less vulnerable to dRib with increasing age, but this was probably due to significantly increased levels of necrotic cells occurring in the aged cohort, reducing apparently the amount of apoptotic cells (data not shown). Apoptotic cell death by staurosporine-, dexamthasone- and Fas-ligand-treatment was not inducible in B and NK lymphocytes. Induced levels of apoptotic cells calculated by subtracting the spontaneous apoptosis gave differences near zero or negative results, therefore these data were omitted and not displayed in the table.

Dexamethasone and staurosporine induced cell death is comparable with that found for the T cell subsets (Table 8). There were no changes with aging.

The next table displays a summary of the percentage of apoptotic cells determined by triple staining with 7-AAD, anti-CD3-FITC (T cells) and anti-CD19-PE (B cells) or anti-CD16+ anti-CD56-PE (NK cells), respectively (Table 6).

Bcl-2 content of lymphocyte subsets

In order to explore whether the antiapoptotic-acting proto-oncogene Bcl-2 is involved in the apoptotic process observed with aging in lymphocyte's subsets, the amount of Bcl-2 was determined at a single cell level by flow cytometry.

In concordance with the findings, using an ELISA against Bcl-2 (see Figure 32), there were no significant changes in Bcl-2 expression in the different lymphocyte's subsets concerning aging (CD3, young: 6.05 ± 0.798; n = 9 vs. old: 5.71 ± 0.503; n = 16; CD19, young: 6.00 ± 0.908; n = 5 vs. old: 5.83 ± 0.637; n = 9; CD56+16, young: 4.34 ± 0.645, n = 5 vs. old: 4.36 ± 0.434; n = 9; all data are mean fluorescence intensity (MFI); Figure 41).

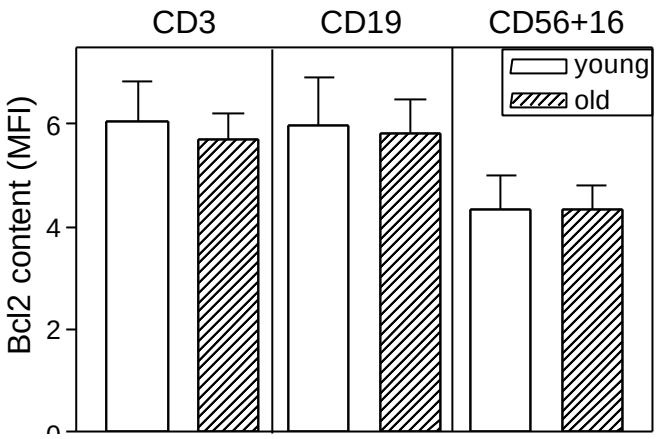


Figure 41. Content of Bcl2 in human lymphocytes. No changes between cells from young and aged volunteers could be detected.

When correlating the percentage of apoptotic cells of each individual with the content of Bcl2 in the corresponding subset, a significant correlation becomes evident for B cells. The more B cells are apoptotic in a sample, the higher is the level of intracellular Bcl-2 of the same sample. No significant correlation has been found in T and NK cells.

	P value	sig.	n	r^2
T cells	0.2392	n.s.	18	0.03189
B cells	0.0325	*	12	0.3003
NK cells	0.1289	n.s.	12	0.1289

Table 5. Correlation of apoptotic cells with Bcl-2 content in different lymphocyte subsets.

	young	old	T lymphocytes				B lymphocytes				NK lymphocytes			
	number of volunteers	number of volunteers	% apoptotic cells from young persons	% apoptotic cells in aged persons	significance (young vs. aged)	correlation with age; r^2 (number of subjects)	% apoptotic cells from young persons	% apoptotic cells in aged persons	significance (young vs. aged)	correlation with age; r^2 (number of subjects)	% apoptotic cells from young persons	% apoptotic cells in aged persons	significance (young vs. aged)	correlation with age; r^2 (number of subjects)
basal	n = 9	n = 11	0.67 ± 0.114%	1.08 ± 0.208%	n.s. p = 0.07	*p<0.04; 0.0749 (22)	2.89 ± 0.850%	5.03 ± 1.607%	n.s.	n.s. (13)	0.71 ± 0.223%	1.47 ± 0.273%	*p<0.04	n.s. (22)
spontaneous	n = 9	n = 7	1.52 ± 0.292%	3.02 ± 0.467%	**p<0.006	**p<0.06; 0.2230 (22)	11.33 ± 5.382%	13.96 ± 2.897%	n.s. p=0.08	n.s. p = 0.06 (21)	9.43 ± 1.835%	11.74 ± 2.059%	n.s.	n.s. (20)
dRib	n = 9	n = 11	8.57 ± 1.122%	17.56 ± 3.277%	*p<0.02	*p<0.04; 0.1782 (20)	37.19 ± 8.999%	25.56 ± 7.176%	n.s.	n.s.	13.31 ± 2.997%	25.51 ± 4.205%	*p<0.02	*p<0.02 (18)
Dex	n = 9	n = 12	4.17 ± 0.805%	4.02 ± 0.508%	n.s.	n.s.	-				-			

Table 6. Apoptosis in different lymphocyte subsets in aging.

Apoptosis in lymphocyte subsets (T-, B-, and NK-cells) from AD patients

Apoptosis of lymphocyte subsets from AD patients were determined as described and established above (see: Differences in apoptosis of human lymphocyte subsets in 'healthy' aging.). When correlated with the severity of the cognitive impairment, only basal apoptotic levels of T lymphocyte correlated with the MMSS (*p<0.05; Table 9), but not B- and NK-cells. Comparing the data with that of elderly non-demented controls, T cells showed elevated levels of basal apoptotic cells, however due to the small number of included patients yet not significant. Interestingly, natural killer cells show a clear increase in basal apoptosis (Figure 42).

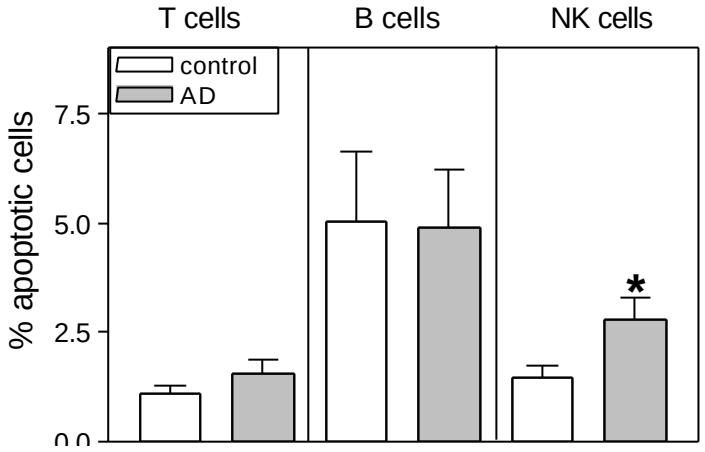


Figure 42. Comparison of all AD-patients with age-matched controls. Basal levels of apoptotic T lymphocytes are increased in demented persons, though here not significantly (possible due to the smaller number of included subjects compared with Figure 34). B lymphocytes display quite high apoptotic levels, in general. There is no difference between AD-patients and non-demented persons. In concordance with findings of Solerte *et al*., 1998; 2000; Araga *et al*., 1991, NK lymphocytes seems to be basely activated in AD-patients, since they show significantly increased levels of apoptosis immediately after isolation (*p<0.05). All data are summarized in the following table (Table 7).

Levels of spontaneous and agent-induced apoptosis determined after 24 hours in vitro is summarized inTable 7. It becomes clearly evident, that only T cells from AD patients show changes in cell death in correlation with the MMSS. B cell apoptosis is not effected by AD, but basal apoptotic levels of natural killer lymphocytes are altered in demented persons.

	AD-patients	old controls	T lymphocytes				B lymphocytes				NK lymphocytes			
	number of patients; mean MMSS	number of age-matched controls	% apoptotic cells from AD-patients	% apoptotic cells in age-matched controls	significance (AD vs. controls)	correlation with MMSS; r^2	% apoptotic cells from AD-patients	% apoptotic cells in age-matched controls	significance (AD vs. controls)	correlation with MMSS	% apoptotic cells from AD-patients	% apoptotic cells in age-matched controls	significance (AD vs. controls)	correlation with MMSS
basal	n=16; 19.3±1.5 MMSS	n = 11	1.54 ± 0.223%	1.08 ± 0.208%	n.s. (p=0.08)	*p<0.03; r^2 = 0.2762	4.89 ± 1.782%	5.03 ± 1.607%	n.s.	n.s.	2.78 ± 0.528%	1.47 ± 0.273%	*p<0.023	n.s.
spontaneous	n=7; 17.7±2.5 MMSS	n = 7	3.77 ± 0.268%	3.02 ± 0.467%	n.s.	n.d.	24.80 ± 7.723%	13.96 ± 2.897%	n.s.	n.d.	8.60 ± 1.734%	11.74 ± 2.059%	n.s.	n.d.
dRib	n=7; 17.7±2.5 MMSS	n = 11	18.83 ± 5.039%	17.56 ± 3.277%	n.s.	n.d.	33.55 ± 5.018%	25.56 ± 7.176%	n.s.	n.d.	18.42 ± 5.402%	25.51 ± 4.205%	n.s.	n.d.
Dex	n=7; 17.7±2.5 MMSS	n = 12	3.55 ± 0.776%	4.02 ± 0.508%	n.s.	n.d.	-				-			

Table 7. Apoptosis in lymphocyte subsets from AD-patients and elderly controls.

Bcl-2 content in lymphocytes from AD patients

The amount of the anti-apoptotic protein Bcl2 was measured by flow cytometry in the different subsets of AD patients by triple staining. Elevated, however insignificant, intracellular expression of Bcl2 could be determined in T cells of AD patients when compared with non-demented elderly controls.

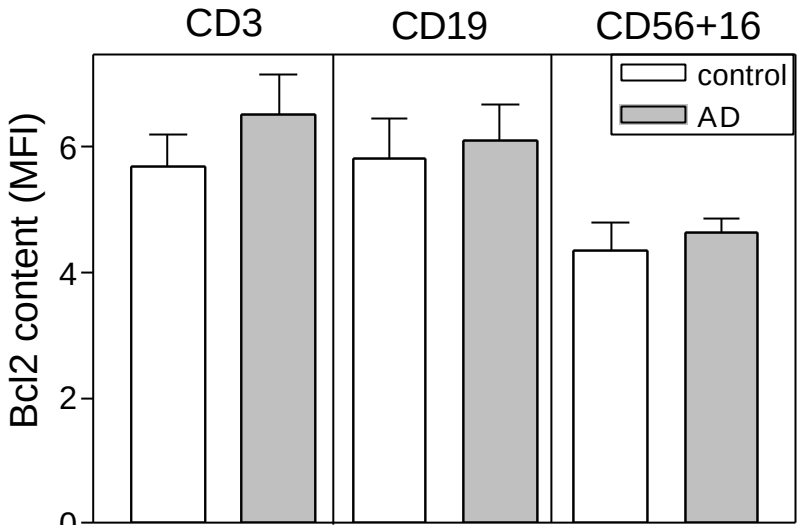


Figure 43. Amount of intracellular Bcl2 in T, B and NK cells of AD patients and elderly controls. T cells of AD patients show a trend to increased levels (CD3: AD, 6.52 ± 0.635; n = 11; control, 5.71 ± 0.503; n = 16). B and NK cells showed no changes in Bcl2 levels (CD19: AD, 6.12 ± 0.554; n = 11; control, 5.83 ± 0.637 n = 9; CD16+56: AD, 4.63 ± 0.227; n = 9; control, 4.36 ± 0.434; n = 9).

To explore whether changes of Bcl2 expression in lymphocytes are involved in the progression of AD, the amount of Bcl2 of each cell type was correlated against the Mini Mental State Score of each individual. Interestingly, only T cells (dotted line) show a significant relation to the cognitive capacity of the patients (CD3: *$p<0.03$, $r^2 = 0.346$, n = 11). No changes were detectable in B and NK cells.

Increased $CD4^+/CD8^+$-ratio in AD that correlates with the severity of the dementia

An additional subset within the PBMC are the T cell subsets, or CD4 and CD8. The following diagramm shows a normal distribution pattern of an aged non-demented individual.

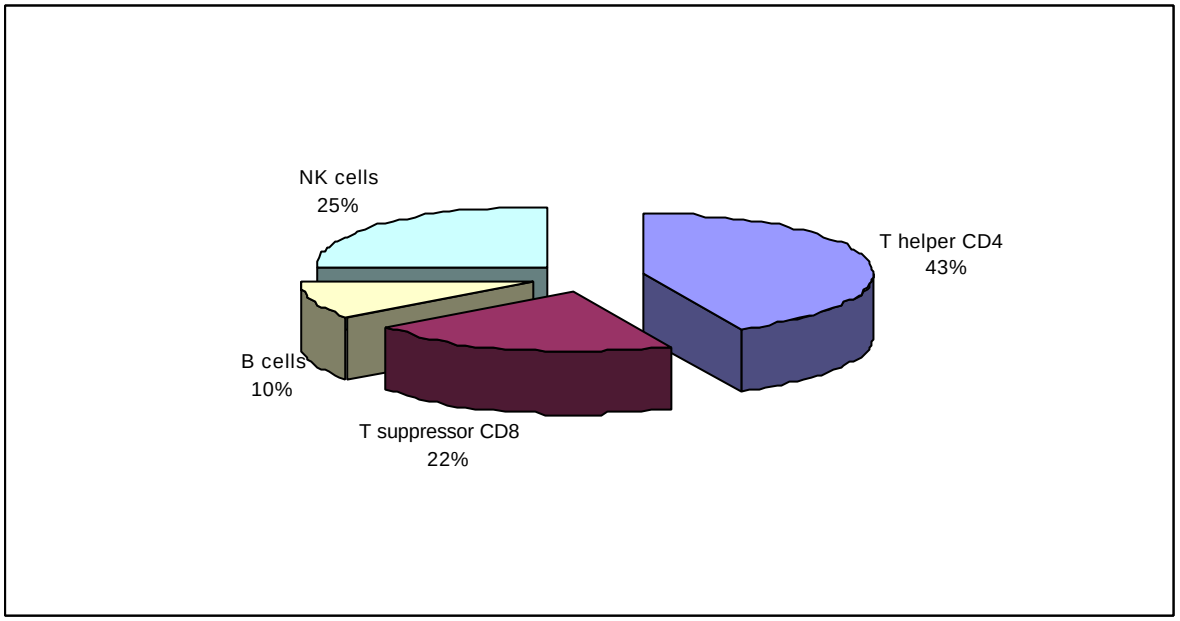


Figure 44. Constituents of PBMC including the T Cell subsets CD4 and CD8 of an average elderly human.

There was a significant difference in the ratio of the T cell subsets, the CD4/CD8-ratio, which correlates significantly with the severity of the dementia (**$p<0.008$). These data are in concordance with other findings (Robinson Agramonte *et al.*, 2001; Lombardi *et al.*, 1999; Shalit *et al.*, 1995; Hu *et al.*, 1995; Pirttila *et al.*, 1992; Leffell *et al.*, 1985).

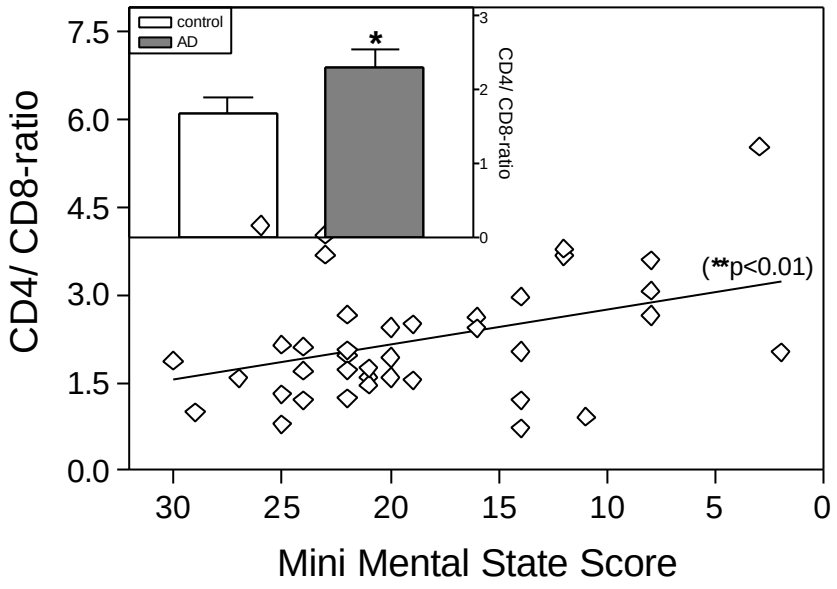


Figure 45. The percentage of $CD4^+$ and $CD8^+$ lymphocytes was determined in whole blood samples of elderly controls and AD-patients. There is highly significant correlation of the CD4/CD8-ratio with the MMSS of each individual patient (n = 39, **p<0.01). Compared with old controls, the ratio is significantly increased in demented patients (controls: 1.68± 0.214; n = 16 vs. AD: 2.31± 0.237; n = 39; *p<0.05).

The absolute number of determined T helper and T cytotoxic/ suppressor cells did not differ in demented persons, but there was a positive correlation between the MMSS and the number of $CD3^+$ and $CD4^+$ cells per aliquot whole blood (*p<0.02). The fluorescence intensity of stained receptors determined by flow cytometry is proportional to the number of surface expressed receptors per cell. Here, a tendency to a decreased number of CD8-receptors was determined in T cells from persons with DAT. Based on these data, it can be speculated, that in dependence to the progression of the cognitive decline (measured as MMSS) changes in the T cell subsets occur. The ratio of the T helper to the T suppressor cells significantly increases and so does the number of activated T cells (cells displaying HLA-DR and CD95). Moreover, number of T cells and expression of the CD8 receptor is altered in more severe demented persons. Therefore, it should be controlled whether the apoptotic changes in AD are due to by determining the content of apoptotic cells for each single subset.

First, changes of T lymphocyte subset apoptosis were determined in ‘healthy’ aging.

Changes of apoptosis in T cell subsets, CD4 and CD8.

In concordance with earlier findings (see Age-related changes in apoptotic cell death of human peripheral blood cells), increasing basal levels of apoptotic cells within the CD4 and the CD8 subset were found in aging (Figure 46 and Table 8), indicating an involvement of both subsets in the aging process. Here again, we could detect large variances in the cells from the aged group, indicating that immunosenescence affects each person individually.

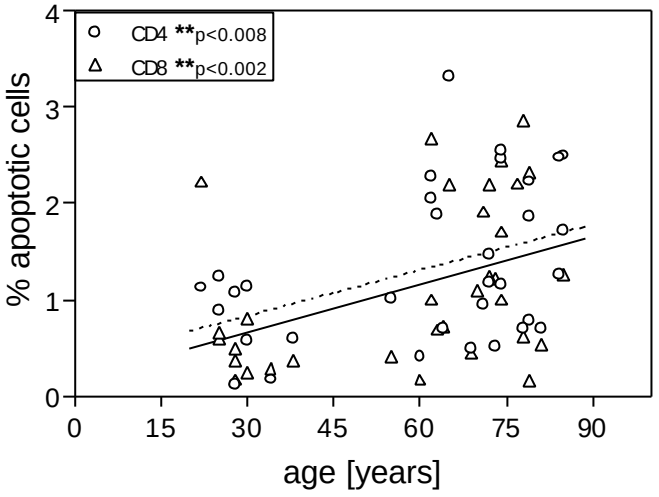


Figure 46. 7-AAD positive cells plotted vs. the age of each blood donors resulted in significant correlation.

Spontaneous and dRib-induced apoptosis were increased in T lymphocyte subsets from elderly persons. However, some data are only close to statistical significance ($p = 0.06 – 0.08$). That might be due to the small number of volunteers included, since the aim of this study was mainly to validate and establish the method and to reproduce the former data obtained by PI-staining in healthy aging.

CD95 mediated cell death was increased in both subsets. It is evident from our data that T helper are more susceptible to this apoptotic pathway since $CD4^+$ lymphocytes express more CD95/Fas-receptor compared to $CD8^+$ (data not shown).

In concordance with earlier results we found no difference in cell death after staurosporine (data not shown) and dexamethasone treatment (Table 8).

	young	old	$CD4^+$ lymphocytes				$CD8^+$ lymphocytes			
	number of volunteers	number of patients	% apoptotic cells from young persons	% apoptotic cells in aged persons	significance (young vs. aged)	correlation with age; r^2 (number of subjects)	% apoptotic cells from young persons	% apoptotic cells in aged persons	significance (young vs. aged)	correlation with age; r^2 (number of subjects)
basal	n = 11	n = 21	0.93 ± 0.312%	1.43 ± 0.161 %	n.s. p=0.06	*p<0.05; r^2= 0.0871 (34)	0.78 ± 0.190%	1.45 ± 0.221 %	*p<0.03	*p<0.04; r^2= 0.1602 (34)
spontaneous	n = 9	n = 10	3.07 ± 0.223%	5.98 ± 0.990%	*p<0.05	*p<0.02; r^2= 0.1190 (24)	2.20 ± 0.242%	3.50 ± 0.795%	n.s. p=0.08	**p<0.01; r^2= 0.2152 (24)
dRib	n = 6	n = 23	7.64 ± 3.805%	15.64 ± 2.478 %	*p<0.05	*p<0.05; r^2= 0.1084 (28)	7.10 ± 2.425%	14.83 ± 2.974%	*p<0.04	n.s. p=0.06
Dex	n = 9	n =24	3.33 ± 0.748%	3.70 ± 1.302%	n.s.	n.s.	4.33 ± 1.718%	6.03 ± 2.309%	n.s.	n.s.
Fas-L	n = 5	n =6	7.03 ± 1.294%	12.16 ± 2.110%	n.s. p=0.08	n.d.	4.36 ± 0.708%	8.82 ± 2.933%	n.s.	n.d.

Table 8. Apoptosis in T lymphocyte subsets in physiological aging.

Density of Bcl-2 in human T cell subsets in aging

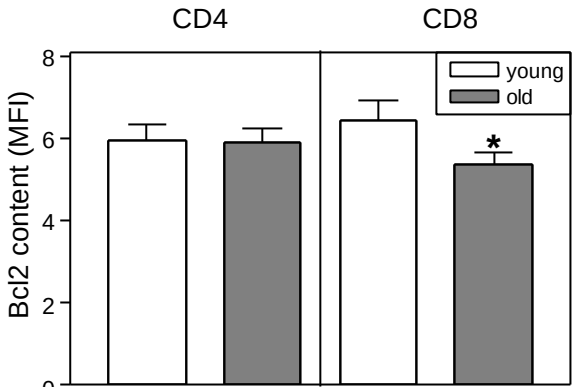


Figure 47. There was a significant reduction of Bcl-2 levels in $CD8^+$ cells in the aged cohort (CD8, young: 6.45 ± 0.476, n = 11 vs. old: 5.37 ± 0.298, n = 15), while there were no changes detectable in the T helper subset (CD4, young: 5.93 ± 0.423; n = 11 vs. old: 5.88 ± 0.382, n = 15).

Only $CD8^+$ lymphocytes showed altered Bcl-2 expression in aging. $CD4^+$ lymphocytes are not affected (Figure 47). Determining the amount of Bcl-2 in all $CD3^+$ cells, there was a slight reduction, possibly due to diminished mean fluorescence intensity (MFI) of Bcl-2 observed for the T suppressor cells. No changes with aging were determined in B and NK cells (Figure 41). Excitingly, when the number of apoptotic cells per subsets was plotted against the MFI of Bcl-2 for each subset, there was a positive correlation for the T-helper subset (Figure 48; *$p<0.05$; $r^2 = 0.316$; n = 10). It could be speculated, that there is no significant dependency of $CD8^+$ lymphocyte's apoptosis on the Bcl-2 content of the sample.

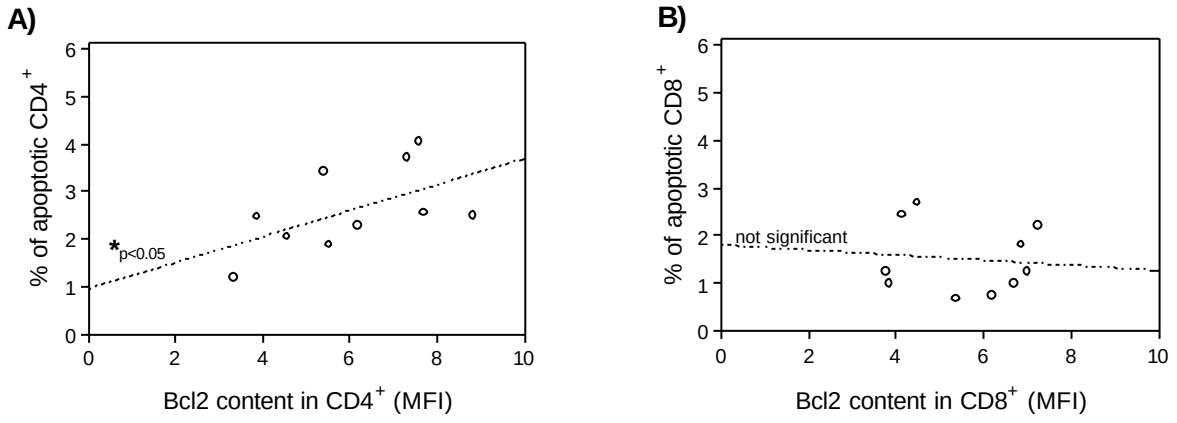
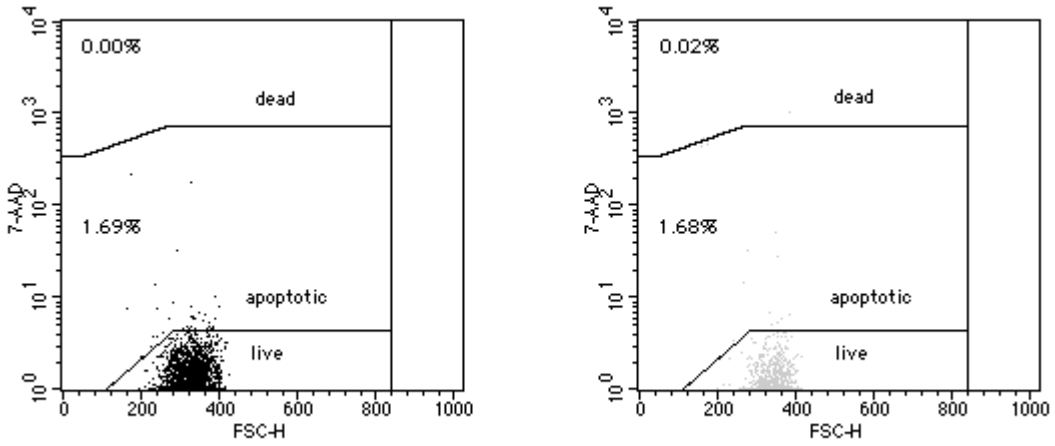


Figure 48. Expression of Bcl-2 significantly correlated with the percentage of apoptotic cells in $CD4^+$ lymphocytes. Interestingly, no association was seen in $CD8^+$ cells.

Altered apoptosis in T cell subsets of AD patients

Clues that T cells of AD patients show impaired apoptotic behavior and Bcl2 expression were shown in earlier findings of this thesis. Moreover, since AD patients show an increased CD4/CD8-ratio it seemed to be reasonable to further explore apoptosis in the CD4 and CD8 subsets of T cells.

A) old non-demented control



B) patient with DAT

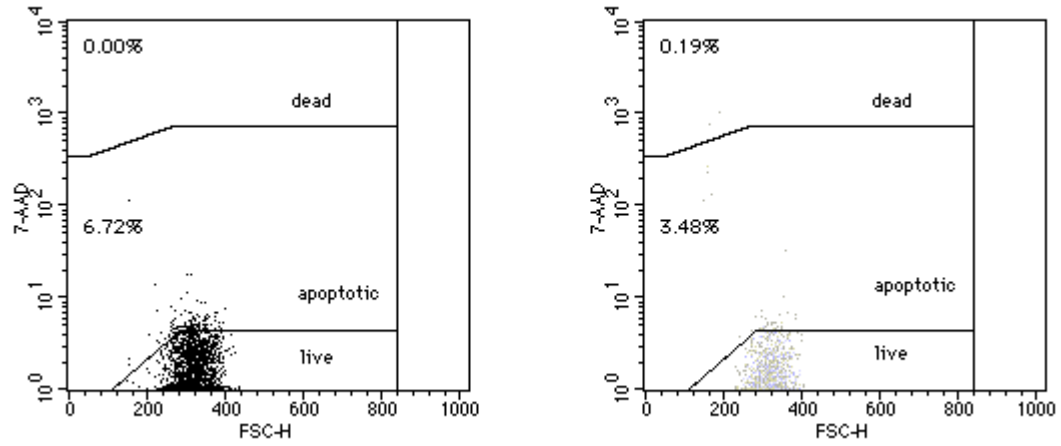


Figure 49. Representative dot-plots from 7-AAD stained freshly isolated PBMC from an old (A) and an AD-patient (B). Basal apoptosis is dramatically increased in the CD4 subset, but the CD8 lymphocytes are affected as well.

Basal apoptosis was determined in PBMC of AD patients triple stained with antibodies raised against CD4 and CD8 and the DNA dye 7-AAD. Representative dot plots of CD4- and CD8-positive T cells of an AD patient and an elderly control are shown above (Figure 49).

Basal percentage of apoptotic T cells were enhanced in demented patients (Inset Figure 50; CD4: AD, 2.29 ± 0.247%; control, 1.43 ± 0.161%, **p<0.003; CD8: AD, 1.63 ± 0.306%; control, 1.45 ± 0.221%; n = 21 in each group). A clear increase is evident in $CD4^+$ cells from AD patients. Interestingly, when all analyzed patients were pooled and plotted against decreasing MMSS, there was a significant correlation for the CD8 subset (black line), indicating an enhanced vulnerability of $CD8^+$ T cells with increasing dementia.

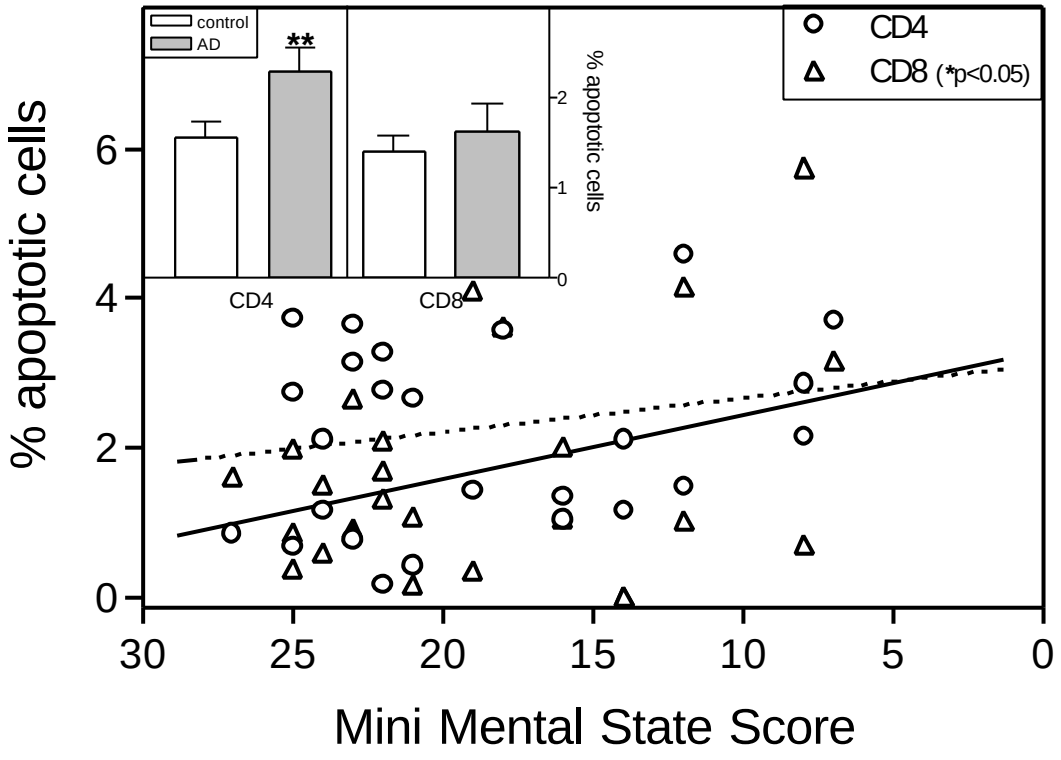


Figure 50. Percentage of basal apoptosis of T helper lymphocytes (CD4) and cytotoxic T suppressor cells (CD8) from AD-patients. CD8 cells show an enhanced vulnerability with increased severity of the disease (*p<0.05; n = 21). CD4 cells do not show a significant correlation with MMSS. Compared with cells derived from elderly non-demented persons, the content of apoptotic cells is significantly increased in T helper lymphocytes. In contrast, in this cohort of AD-patients the percentage was nor elevated in CD8 cells, although this parameter increases with dementia's severity. This seems to be due to the relatively high distribution of patients with quite high Mini Mental State Scores (mean MMSS: 19.38 ± 1.27).

In addition, patients that carry the apoE4 gene show increased basal apoptotic levels in both T cell subsets (CD4: non-ApoE4, 1.87 ± 0.437%, n = 12; ApoE4, 2.41 ± 0.398%, n = 9; CD8: non-ApoE4, 1.39 ± 0.340%, n = 12; ApoE4, 1.78 ± 0.366%, n = 9).

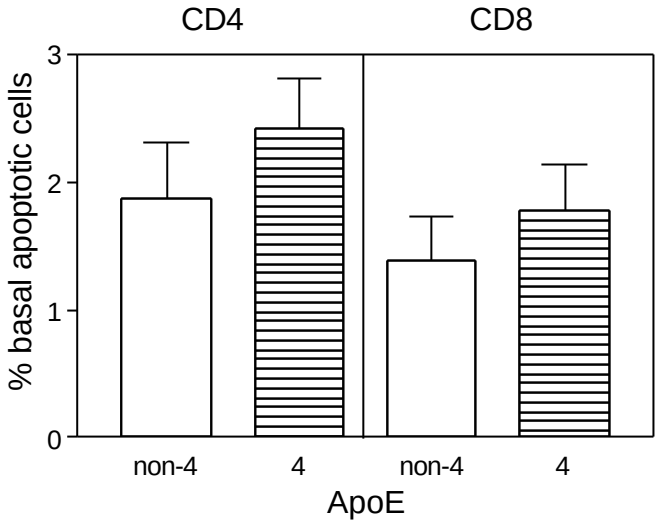


Figure 51. AD patients positive for ApoE4 display enhanced basal levels of apoptotic cells in CD4 and CD8 T cells.

Spontaneous and agent-induced apoptosis of T cell subsets are summarized in the following table. In AD, $CD8^+$ cells become more vulnerable against spontaneous and Fas-L induced apoptosis as the diseases proceeds. T helper cell apoptosis, induced with oxidative stress or Fas-L, correlates significantly with MMSS. Dexamethasone and staurosporine (data not shown) showed no effects on cell from AD patients that are altered from that of aged controls.

	AD-patients	old controls	$CD4^+$ lymphocytes				$CD8^+$ lymphocytes			
	number of patients; mean MMSS	number of volunteers	% apoptotic cells from AD-patients	% apoptotic cells in age-matched controls	significance (AD vs. controls)	correlation with MMSS	% apoptotic cells from AD-patients	% apoptotic cells in age-matched controls	significance (AD vs. controls)	correlation with MMSS
basal	n = 26; 18.9 ± 1.15 MMSS	n = 21	2.26 ± 0.264 %	1.43 ± 0.161 %	**p<0.003	n.s.	1.68 ± 0.285 %	1.45 ± 0.221 %	n.s.	*p<0.05
spontaneous	n = 10; 17.0 ± 2.2 MMSS	n = 10	8.78 ± 1.369 %	5.98 ± 0.990%	n.s. (p=0.053)	n.d.	3.73 ± 0.975 %	3.50 ± 0.795%	n.s.	n.d.
dRib	n = 12; 17.6 ± 1.86 MMSS	n = 23	12.84 ± 2.027 %	15.64 ± 2.478 %	n.s.	n.d.	9.45 ± 2.359 %	14.83 ± 2.974%	n.s.	n.d.
Dex	n = 10; 17.4 ± 2.02 MMSS	n = 24	5.99 ± 1.655%	3.70 ± 1.302%	n.s. (p=0.15)	n.d.	4.37 ± 1.340 %	6.03 ± 2.309%	n.s.	n.d.
Fas-L	n = 5; 17.0 ± 3.03 MMSS	n = 6	25.80 ± 7.043 %	12.16 ± 2.110%	*p<0.04	n.d.	11.15 ± 2.902 %	8.82 ± 2.933%	n.s.	n.d.

Table 9. Summary of apoptotic cells determined by 7-AAD staining of PBMC from AD-Patients and age-matched controls.

Bcl-2 content in lymphocytes derived from AD patients

A clear reduction of Bcl-2 content can be observed in CD4 and CD8 T cells of AD patients with increased severity of cognitive impairment. For $CD8^+$ lymphocytes, there is a significant correlation with decreasing MMSS. Thus, when all examined AD patients where compared with aged healthy volunteers, $CD4^+$ cells showed elevated Bcl-2 levels, while $CD8^+$ are not altered. Possible explanation might be the high number of AD patients in this cohort with a relatively high MMSS that show high Bcl-2 expression. In the progression of the diseases, the Bcl-2 content is clearly reduced (CD4, $p = 0.065$, $r^2 = 0.117$; CD8, $^{**}p<0.01$, $r^2 = 0.258$; $n = 21$).

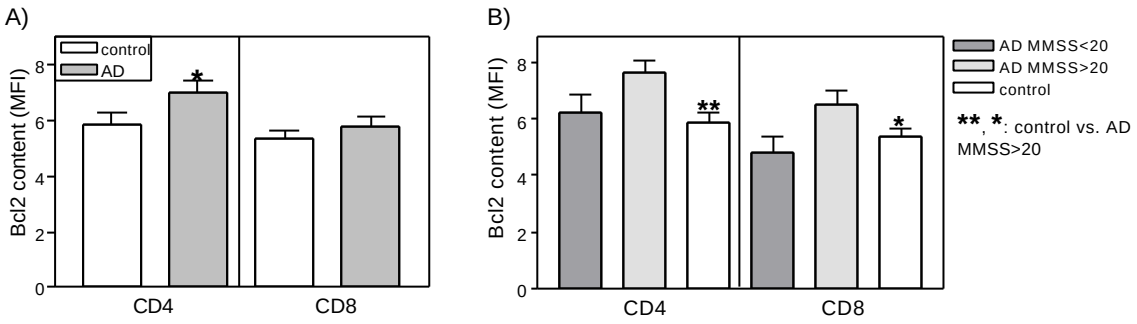


Figure 52. A) Comparison of Bcl-2 expression of AD patient with elderly controls. B) Splitting of the AD patient group into two with the MMSS of 20 as cut-off reveals that Bcl2 expressing is significantly incraesed only in mild AD cases. With increasing dementia the Bcl2 content of T lymphocytes decreases.

Interestingly, when the percentage of apoptotic cells is plotted against the mean fluorescence intensity a significant correlation occurs for $CD4^+$ lymphocytes (CD4, $^{**}p<0.008$, $r^2 = 0.262$; $n = 22$), a similar pattern as already seen in aging (see Figure 48).

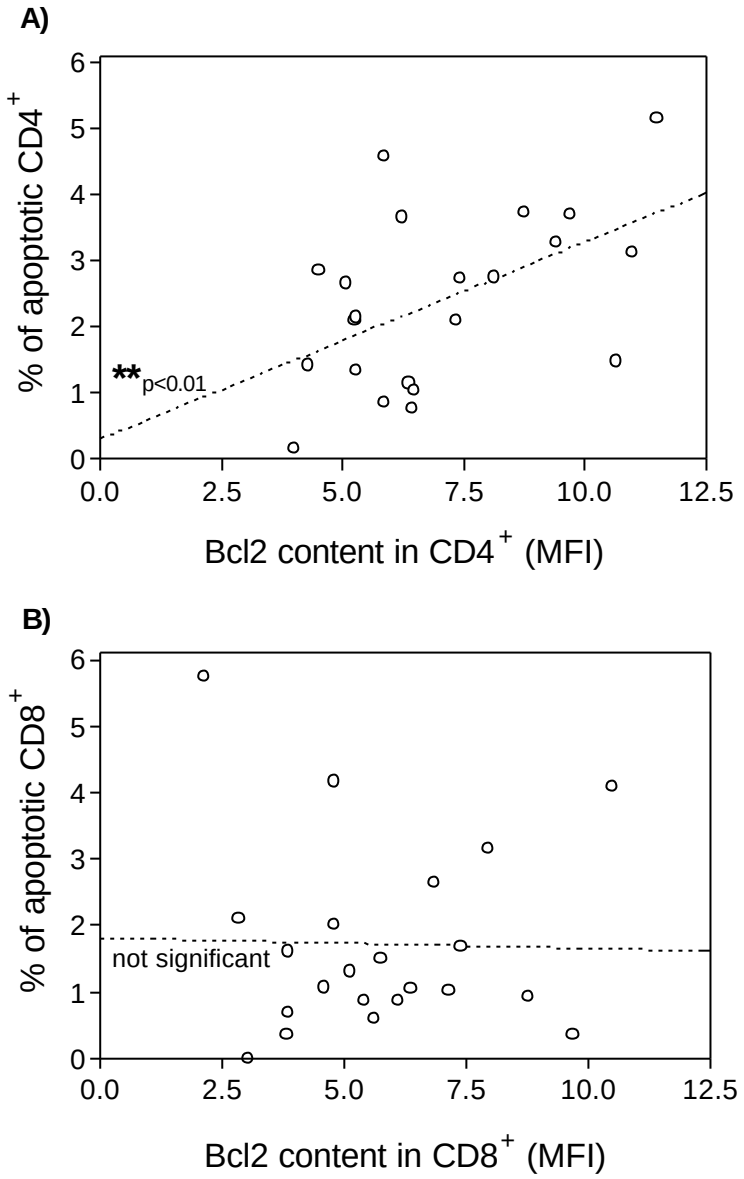


Figure 53. Matching with the data derived young and aged non-demented volunteers, the number of apoptotic cells in CD4 lymphocytes of AD patients correlates the Bcl-2 content with. No correlation can be observed in $CD8^+$ cells.

Is the observed increased basal apoptosis of T cells from AD patients due to activation-induced cell-death, a result of T cell activation?

The results of the above part of this thesis show that the T lymphocytes are mainly responsible for the enhanced cell death observed in AD peripheral leukocytes. In this part, the underlying mechanism be determined.

As mentioned in the introduction, apoptosis is important for termination of T cell activation. After immune response, great number of T lymphocytes will die by programmed cell death. Some cells will survive as memory T lymphocyte, showing enhanced recall indelibility. This is a physiologically occurring phenomenon. To test whether this mechanism is accountable for T cell apoptosis in AD, an experimental model to analyze several events of T cell apoptosis was established.

Establishment of *in vitro* T cell activation in aging

T cell activation can be mimicked *in vitro* by mitogens, e.g. PHA-L. Lectins like PHA-L bind to glycolysated proteins, as the TCR. TCR engagement induces the normal cascades of activation, summarized in the following figure (Figure 54). First event that was measured is phosphorylation of tyrosine residues 3 minutes after PHA treatment to analyze the amount of autophosphorylation of TCR associated proteins and members of subsequent kinase cascades. 4 hours after start of mitogen treatment, expression of the early activation marker CD69 was assesed. Expression of some T cell relevant cytokines was determined after over night incubation and finally proliferation of the leukocytes was explored after 3 days in culture.

Tyrosine phosphorylation subsequent to TCR engagement declines with the donor's age

After TCR engagement the number of phosphorylated tyrosines raises as a consequence of the induction of kinase cascades. Whole blood cultures were stimulated with PHA-L (10 µg/ml) or remained untreated for 3 minutes at 37°C with gentle agitation. Cells were transferred rapidly on ice in order to stop the reactions. Intracellular staining was performed as described. A ratio was calculated from the mean fluorescence intensity of PHA-L stimulated and the untreated cells.

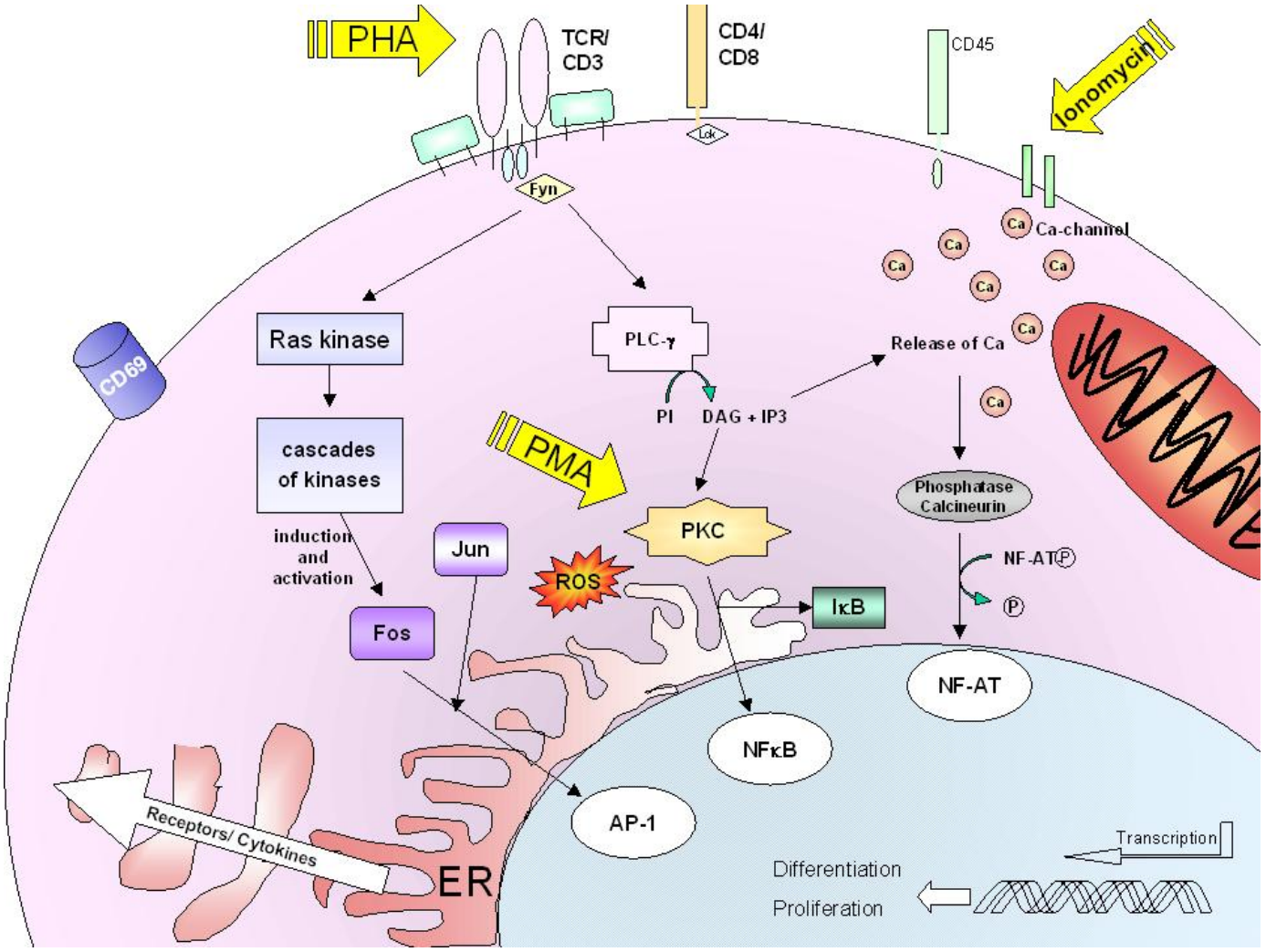


Figure 54. T cell activation pathways and mechanism of action of PHA-L and PMA/ Ionomycin that simulate antigenic stimulation *in vitro*.

The ratio of tyrosine phosphorylation of $CD4^+$ lymphocytes correlated significantly with the donor's age (***p<0.001; n = 30; open circles in). However, no statistically significant correlation was found for the amount of phosphorylated tyrosine residues with aging in the CD8 subset (p = 0.083; n = 30; open triangles in Figure 55). A comparison of the two age groups revealed a highly significant reduction of the phosphorylated tyrosine-ratio in T helper lymphocytes (CD4, young: 1.473 ± 0.065; old: 1.180 ± 0.283; ***p<0.001; n = 13-14; inset). In $CD8^+$ cells levels of phosphorylated tyrosine were reduced as well, though not statistically significant (CD8, young: 1.319 ± 0.063; old 1.209 ± 0.035; p = 0.074; n = 13-14; inset Figure 55).

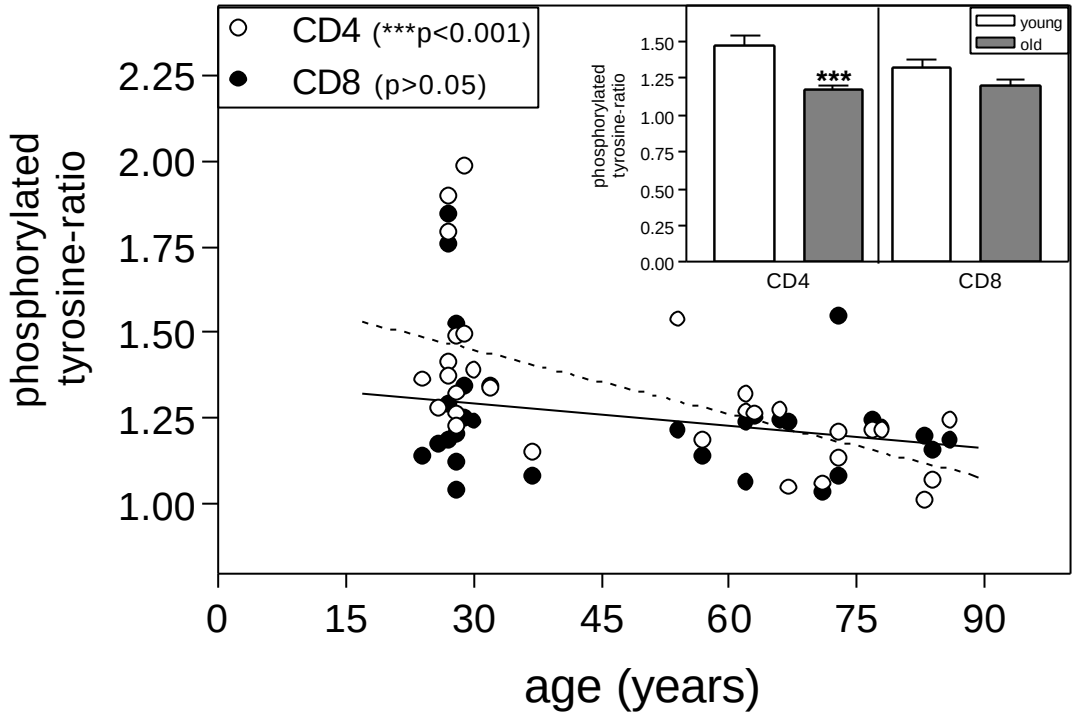


Figure 55. Phosphorylation of tyrosine residues upon PHA-L activation in aging. Whole blood cultures were stimulated with PHA-L for 3 minutes at 37°C and intracellulary stained with an antibody that detects phosphorylated tyrosine residues or an isotype-control. Data are expressed as ratio of PHA-stimulated and unstimulated cells. The ratio of phosphorylated tyrosines correlates significantly with the donor's age in $CD4^+$ cells. When the groups of young and aged donors were compared there was a significant decrease of phosphorylated tyrosine residues in the group of old volunteers (inset). In $CD8^+$ lymphocytes a tendency towards reduced levels could be observed with aging, however, those changes are not significant.

CD69 expression upon PHA-L and PMA/Ionomycin stimulation decreases with aging

Whole blood cultures were prepared as described above and stimulated with 10 µg/ ml PHA-L or 50 ng/ ml PMA and 200 ng/ ml Ionomycin. After 4 hours incubation leukocytes were stained with CD3-PerCp, CD8-PE and CD69-FITC and further processed as indicated. Figure 56 shows a representative set of density plots 4 hours without stimulation (Figure 56, upper panel) and with stimulation (Figure 56: PHA-L, middle panel and PMA + Ionomycin, lower panel) from a young and an old representative individual. Left quadrants represent $CD8^-$ cells, e.g. $CD4^+$ lymphocytes, right quadrants show $CD8^+$ T cells. The upper quadrants show $CD69^+$ lymphocytes, those in the left are $CD4^+CD69^+$, and the ones in the right upper quadrant are $CD8^+CD69^+$. Only CD3-positive cells were gated.

Basal levels of CD69 were quite low and not different between young and old humans (CD4, young: 1.16 ± 0.22%; old: 1.14 ± 0.17%; CD8, young: 1.91 ± 0.52%; old: 1.75 ± 0.38%; n = 19-20). A reduction of CD69 expression after T-cell activation with PHA-L was determined for both T-cell subsets in aging (CD4, young: 49.64 ± 2.64%; old: 39.59 ± 3.56%; *p<0.014; CD8, young: 53.09 ± 2.86%; old: 42.28 ± 4.30%; *p<0.02; n = 19-20; Figure 57A).

The phorbolester PMA and the calcium ionophore Ionomycin bypass the T-cell receptor by directly activating protein kinase C and by elevating the intracellular levels of Ca^{2+}. Levels of activated cells were significantly increased after PMA and Ionomycin treatment in both subsets and in both age groups when compared with PHA-L treatment (***p<0.0001; paired Student's t-test). CD8-cells derived from young donors show maximal CD69 expression after PMA and Ionomycin treatment. Interestingly, CD4-lymphocytes are not able to respond to this with the maximal capacity within 4 hours. CD69 expression decreases highly significantly on $CD8^+$ cells and significantly on $CD4^+$ lymphocytes in aging (CD4, young: 85.25 ± 2.40%; old: 75.84 ± 3.3%; *p<0.025; CD8, young: 96.94 ± 0.66%; old: 92.46 ± 0.91%; ***p<0.001; n = 19 in each group; Figure 57B).

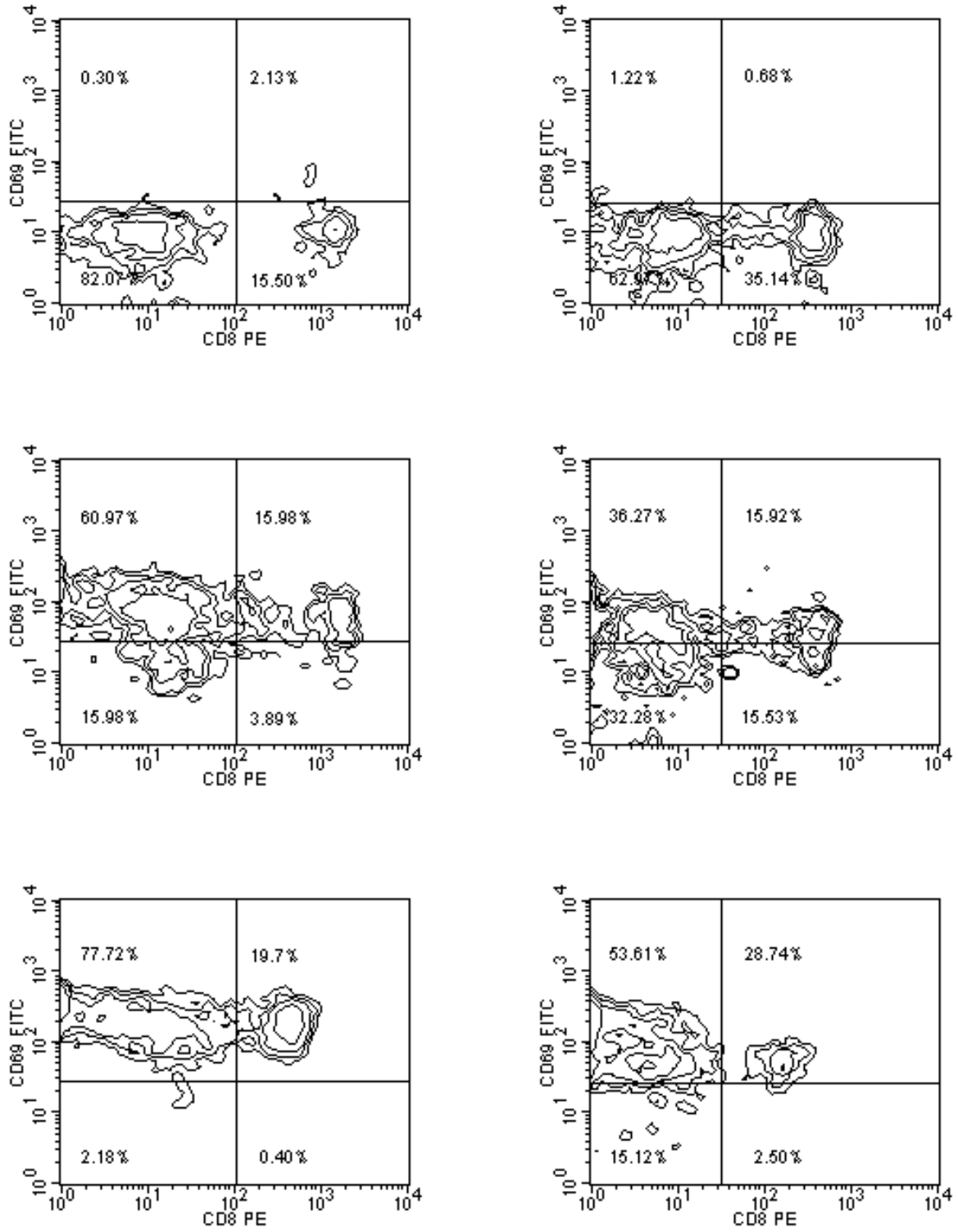


Figure 56. Density plots of CD69 expression at basal condition (upper panel) and after activation with PHA-L (middle panel) and PMA and Ionomycin (lower panel) for 4 hours of a young and an old representative donor. Only CD3-PerCp-positive gated leukocytes are displayed in the plot.

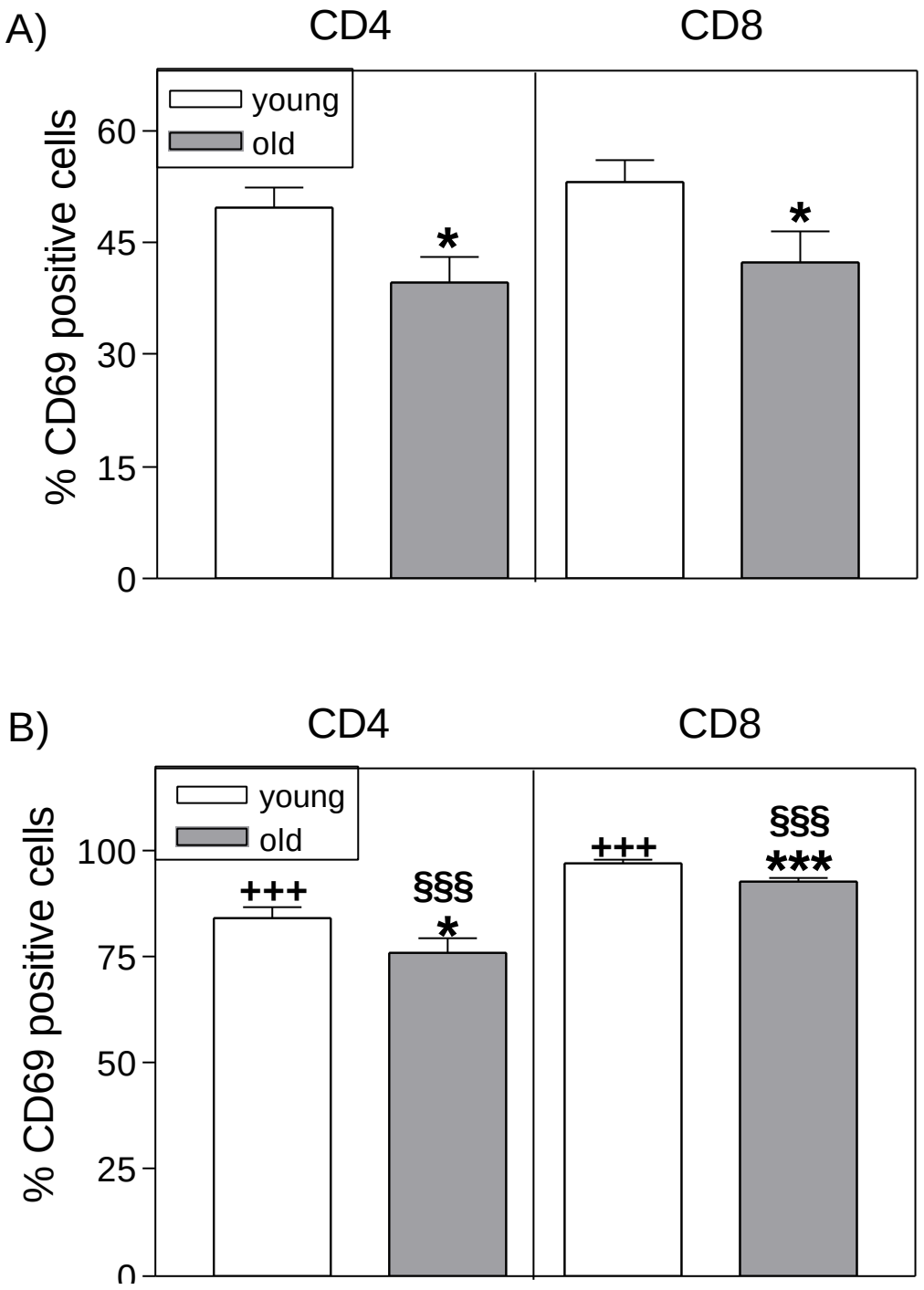


Figure 57. Expression of the early activation marker CD69 is reduced in aging. Whole blood cultures were activated with either PHA-L or PMA/ Ionomycin for 4 hours at 37°C. Subsequently, cells were surface stained for CD3, CD8 and CD69. Only CD3 positive gated cells were analyzed for CD69 expression. (A) TCR engagement triggered by PHA treatment revealed significantly reduced levels of CD69 expressing cells in aged donors in both T cell subsets. (B) This pattern remained when the TCR complex was bypassed with PMA/ Ionomycin, though the differences were not that large anymore.

Cytokine expression is reduced in lymphocytes derived from aged donors:

Whole blood cultures were incubated for 22 hours at 37°C in the absence (basal) or presence of mitogenic activation (PHA-L or PMA/ Ionomycin). 16 hours before the cells were harvested and stained, Brefeldin A was added to the cultures, in order to block the secretion of the cytokines. Thus, the cytokines were plugged in the Golgi and can, therefore, be stained with appropriate antibodies (Figure 58).

The percentages of cytokine expressing cells of both age groups are summarized in table 1. Lymphocytes derived from aged donors (n = 9) showed reduced levels of IL2 and Tumor Necrosis Factor-α (TNF-α) expression even basal but as well upon activation, when compared with young controls (n = 10). In contrast, production of Interferon-γ (IFN-γ) was significantly elevated in PHA-L activated cells from aged persons. Basal Interleukin-2 (IL2) expression was not altered in lymphocytes from aged volunteers. Subsequent to stimulation with PHA-L or PMA/ Ionomycin cells from old persons tend to display reduced levels of IL2 expressing cells. As a T_{H1} cytokine IL2 is mainly expressed in $CD4^+$ T lymphocytes. In addition, levels of IFN-γ, were determined. Here, the percentage of basal IFN-γ producing lymphocytes was not altered in lymphocytes from aged donors. Upon stimulation with PHA cells from old donors displayed significantly more IFN-γ positive cells in both T cells subsets when compared with old controls. Bypassing the TCR with PMA/ Ionomycin revealed the largest amounts of IFN-γ. However, changes between the age groups were not significant. Finally, the percentage of TNF-α positive cells was determined. The T helper subset showed age-related differences in the basal expression of TNF-α. In the $CD8^+$ population those changes were not that evident. Mitogenic activation of the cells tends to effect age-related changes more in the CD8 than in the CD4 subset.

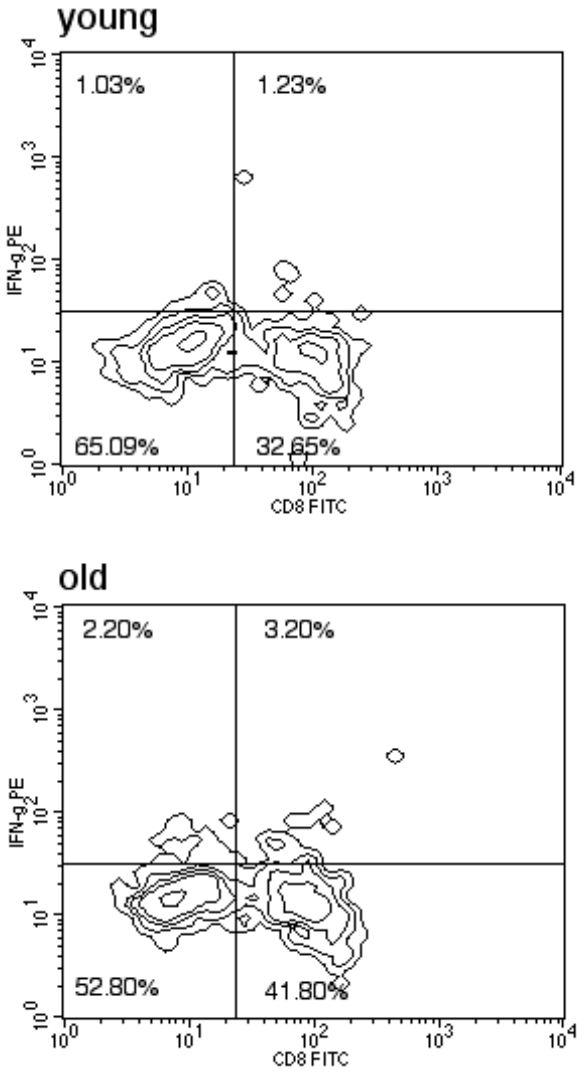


Figure 58. Plots of cells intracellulary stained for IFN-g after stimulation with PHA for 22 hours. Secretion of cytokines was blocked by brefeldin A treatment for 16 hours. Only CD3-PercP-positive gated leukocytes are displayed in the dot plot. Left quadrants represent $CD8^-$ cells, e.g. mainly $CD4^+$ lymphocytes, right quadrants show $CD8^+$ T cells. The upper quadrants show IFN-g^+ lymphocytes, those in the left are $CD8^-$ IFN-g^+, and the ones in the right upper quadrant are $CD8^+$.

		$CD4^+$			$CD8^+$		
		basal	PHA-L	PMA/ Ionomycin	basal	PHA-L	PMA/ Ionomycin
IL-2	young	1.43 ± 0.88	11.48 ± 5.95	30.80 ± 4.51	0.30 ± 0.20	4.95 ± 3.29	15.67 ± 7.21
	old	1.29 ± 0.64	5.17 ± 1.36	22.74 ± 4.33	0.41 ± 0.35	0.51 ± 0.90	10.13 ± 5.72
INF-γ	young	0.70 ± 0.34	0.89 ± 0.35	6.50 ± 1.35	1.40 ± 0.90	3.56 ± 1.24	16.38 ± 4.25
	old	0.25 ± 0.12	2.21 ± 0.35**	11.91 ± 5.31	0.47 ± 0.33	8.76 ± 1.30**	19.79 ± 3.04
TNF-α	young	3.22 ± 1.76	4.27 ± 1.68	31.91± 6.49	3.80 ± 1.91	8.05 ± 3.10	38.88 ± 12.20
	old	0.14 ± 0.07*	5.52 ± 3.01	25.50 ± 14.21	1.15 ± 0.71	4.17 ± 1.52	19.42 ± 7.86

Table 10. IL2, IFN-g, and TNF-a cytokine expression in lymphocytes from young and old donors. Data is expressed in % cytokine expressing cells (means ± S.E.M.). Whole blood cultures were stimulated either with PHA-L or PMA/ Ionomycin as described or remained untreated (basal) for 22 hours. Brefeldin A was added 16 hours prior harvesting the cells. Leukocytes were surface stained for CD3 and CD8, fixed and permeabilized. Staining with fluorochrome-coupled antibodies against IL2, IFN-g, TNF-a, or isotype-control identified cytokine expressing cells. **indicates p<0.01 compared to young controls; *indicates p<0.05 compared to young controls.

Lymphocyte proliferation is significantly reduced in aging

72 hr after preparation of the whole blood cultures and stimulation, leukocytes were stained as indicated above. Preliminary experiments showed that stimulation for 3 days and BrdU-pulse for 24 hours are optimal conditions for this purpose. Figure 59 shows representative density plots from a young and an old individual after PHA-L stimulation (10µg/ml).

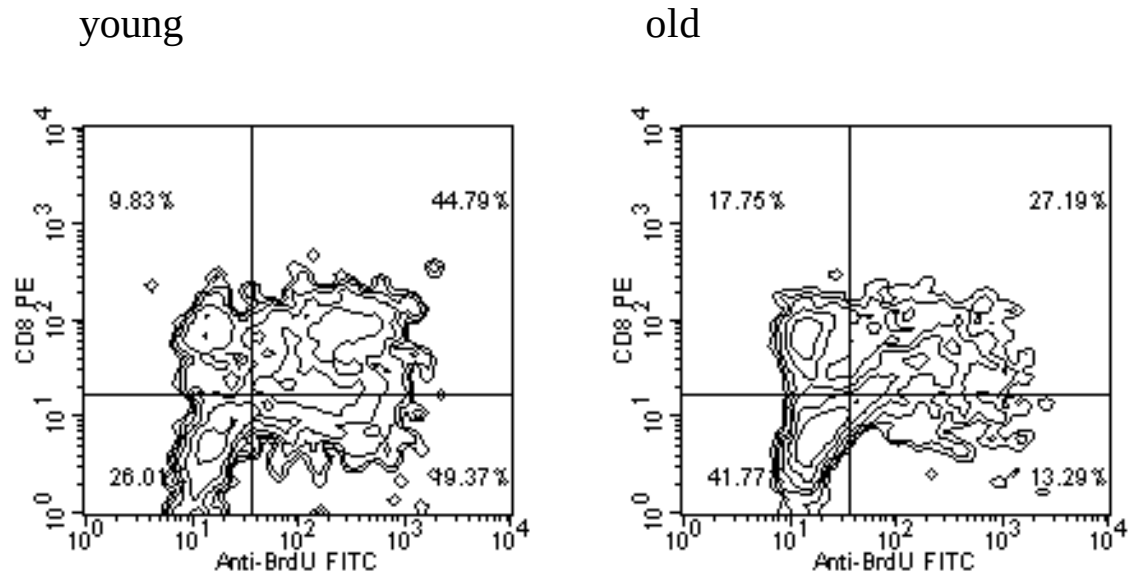


Figure 59. Dot plots of BrdU-incorporation upon stimulation with PHA-L taken from a representative young and old subject. Cells were stained with an isotype antibody as control or with an antibody raised against BrdU to explore the content of proliferating cells. Only CD3-PercP-positive gated leukocytes are displayed in the dot plot. Left quadrants represent $CD8^-$ cells, e.g. $CD4^+$ lymphocytes, right quadrants show $CD8^+$ T cells. The upper quadrants show $BrdU^+$ lymphocytes, those in the left are $CD4^+BrdU^+$, and the ones in the right upper quadrant are $CD8^+BrdU^+$. After 72 hr PHA-L activation, the T lymphocytes from the young individual clearly shows more cells positive for anti-BrdU staining than the T cells from the old person does.

When proliferation was induced by activation with PHA-L via TCR ligation, T cells derived from young donors incorporated significantly more BrdU within 24 hours than cells from elderly persons did, indicating a higher proliferation (CD4, young: 24.75 ± 1.98%; old: 16.07 ± 1.92%; **$p<0.005$; CD8, young: 42.21 ± 3.81%; old: 19.86 ± 1.76%; ***$p<0.0001$; n = 22-25; Figure 60A). By circumventing the TCR complex with PMA and Ionomycin the significant decrease of BrdU-positive cells in aging was still evident, though not that

obviously (CD4, young: 33.43 ± 2.84%; old: 24.35 ± 2.99%; *p<0.023; CD8, young: 50.70 ± 5.05%; old: 27.85 ± 2.86%; ***p<0.001; n = 22-25; Figure 60B). It is noteworthy that in both age groups the proliferative activity of the T lymphocytes was mainly triggered by the CD8 subsets.

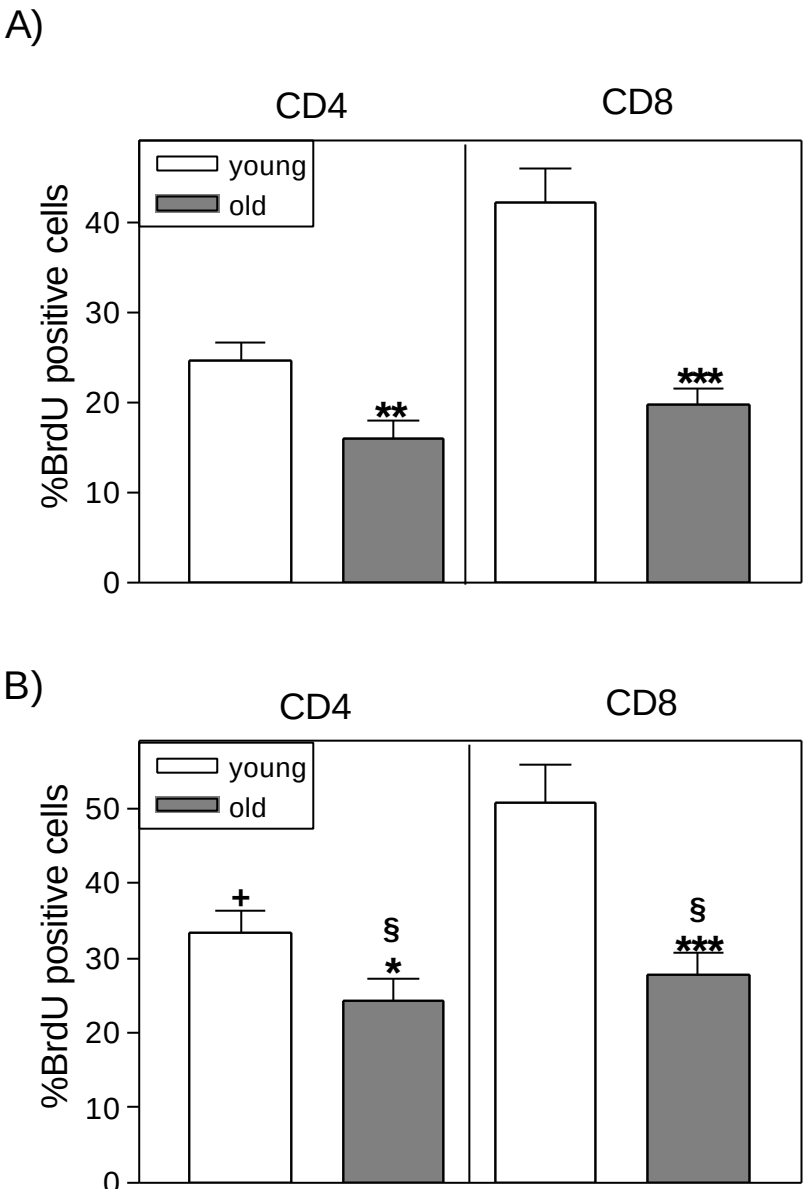


Figure 60. Proliferation according to activation decreases in lymphocytes of aged donors. (A) PHA-L stimulated cells show a markedly increased proliferation in the CD8 subset compared to CD4 positive lymphocytes. In aging the number of BrdU-incorporating cells is significantly reduced in both subsets. (B) Activation subsequent to PMA/ Ionomycin treatment showed as well a higher percentage of proliferating cells within the CD8 subset. Moreover, lymphocytes from young donors revealed significantly elevated levels of BrdU positive cells when compared with old controls.

Altered T cell activity in AD

To determine whether T cell activation of AD patients is comparable with that of elderly persons in the sense of immunosenescence, the established methods were used to analyze T cell reactivity in Alzheimer's disease.

Increased ratio of phosphorylated tyrosine residues in AD in both T cell subsets

Whole blood cultures of AD patients and elderly non-demented controls were stimulated with PHA-L or remained untreated for 3 minutes at 37°C as described above.

The ratio of tyrosine phosphorylation of $CD4^+$ and $CD8^+$ lymphocytes from patients with AD was significantly increased compared to controls (CD4, AD: 1.362 ± 0.0420, n = 6 vs. control 1.180 ± 0.0283, n = 13; **p<0.01; CD8, AD: 1.435 ± 0.1361, n = 6 vs. control: 1.209 ± 0.0349, n = 13; *p<0.05; Figure 61).

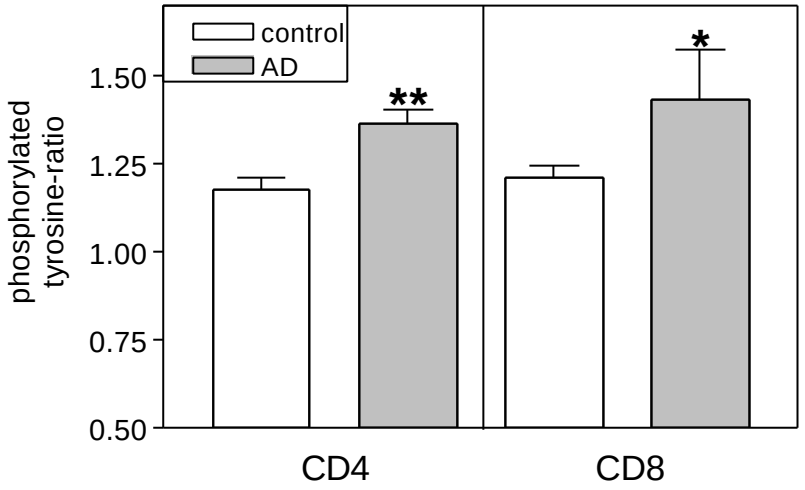


Figure 61. Ratio of phosphorylated tyrosine residues in AD-patients and elderly controls. In demented patients the ratio is significantly higher for both T cell subsets, indicating that mechanisms, like the uncoupling of the CD4-receptor that occurs in aging, do not seem to be evident here.

CD69 expression is enhanced in AD upon TCR ligation and even when bypassing the TCR

Whole blood cultures were prepared as described above and stimulated with 10 µg/ ml PHA-L or 50 ng/ ml PMA and 200 ng/ ml Ionomycin.

Basal levels of CD69 were unchanged in AD and elderly controls (CD4, AD: 0.87 ± 0.284%, n = 22 vs control: 1.196 ± 0.282%, n = 18; CD8, AD: 2.33 ± 0.537%, n = 22 vs. control : 4.64

± 2.810%, n = 18). A reduction of CD69 expression after T-cell activation with PHA-L was determined for both T-cell subsets in healthy aged volunteers when compared with AD patients (CD4, AD: 46.12 ± 3.382%, n = 18 vs. control: 39.59 ± 3.558%, n = 19; CD8, AD: 53.44 ± 4.971%, n = 18 vs. control: 42.28 ± 4.300%, n = 19; *p<0.05; Figure 62A).

When bypassing the T-cell receptor by directly activating protein kinase C with PMA and by elevating the intracellular levels of Ca^{2+} with ionomycin, levels of activated cells were significantly increased in AD after PMA and Ionomycin treatment in both subsets. (CD4, AD: 83.13 ± 2.764%, n = 19 vs. control: 75.84 ± 3.300%, n = 14; *p<0.05; CD8, AD: 95.64 ± 0.887%, n = 19 vs. control: 92.46 ± 0.913%, n = 14; *p<0.05; Figure 62B).

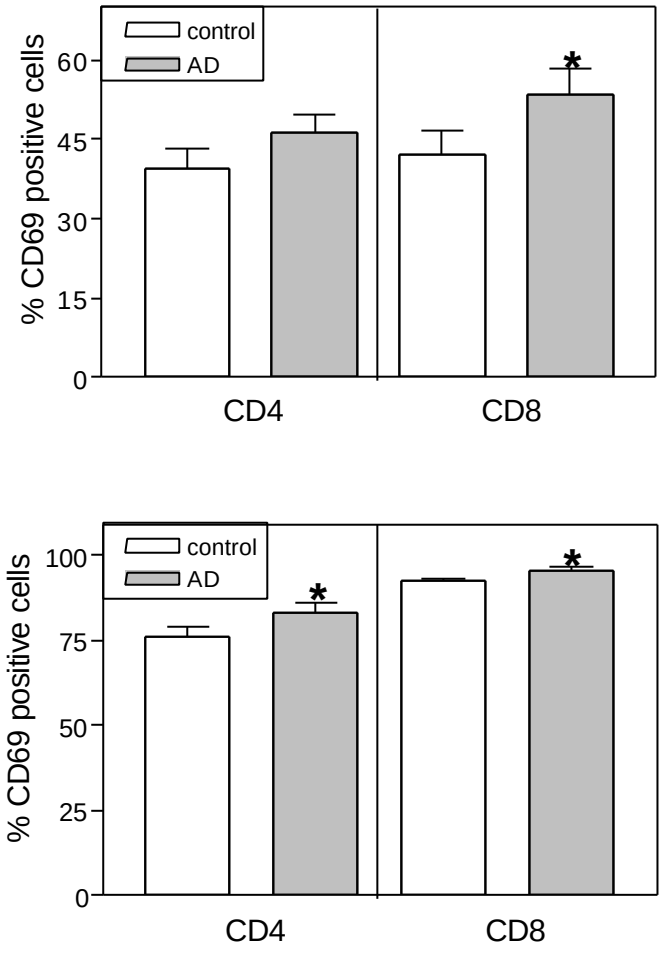


Figure 62. In concordance with the elevated phosphorylation-ratios observed above, the percentage of T cells, expressing the activation marker CD69 after 4 hours of stimulation, is significantly increased in AD-patients. The upper bar graph represents the wholeblood cultures after stimulation with PHA-L, the lower one after activation with PMA and Ionomycin.

Disproportionate lymphocyte proliferation in AD

When proliferation was induced by activation with PHA-L via TCR ligation, T cells derived from AD patients incorporated significantly more BrdU within 24 hours than cells from elderly healthy persons did, indicating a higher proliferation. In contrast, $CD8^+$ AD lymphocyte show significantly decreased proliferation (CD4, AD: 25.98 ± 2.288%, n = 18 vs. control: 16.070 ± 1.919%, n = 25; **p<0.01; CD8, AD: 16.66 ± 1.523%, n = 20 vs. control: 23.59 ± 3.066%, n = 25; *p<0.05; Figure 63A). By circumventing the TCR complex the significant changes of BrdU-incorporation were not evident anymore, only a slight tendency (CD4, AD: 30.94 ± 3.421%, n = 18 vs. control: 24.35 ± 2.991%, n = 22; CD8, AD: 26.21 ± 2.501%, n = 19 vs. control: 38.82 ± 5.375%, n = 22; Figure 63B).

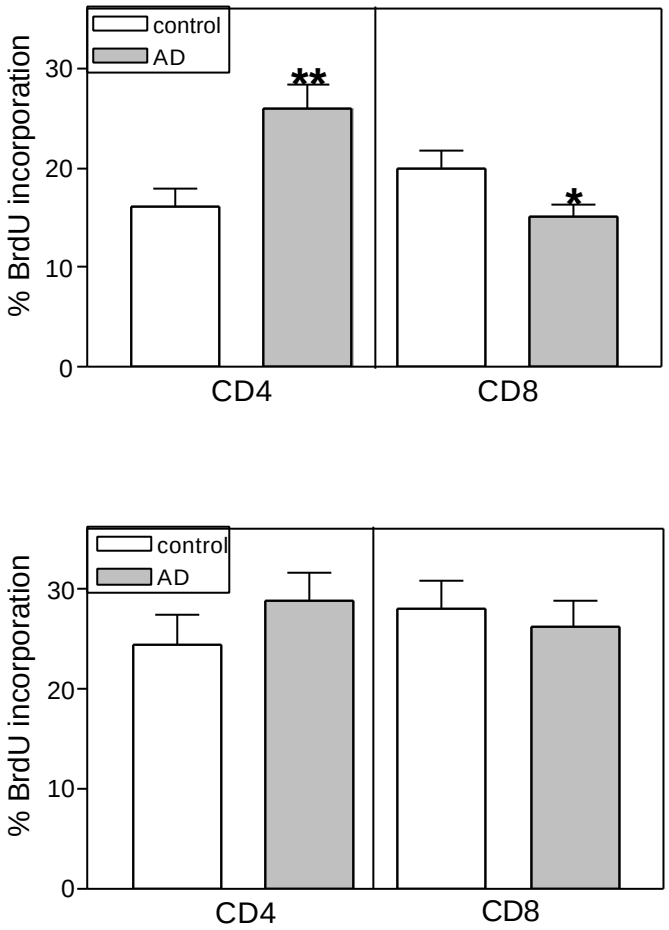


Figure 63. The percentage of proliferating cells within the T helper and T suppressor subsets after stimulation with PHA-L (A) and PMA and Ionomycin (B) in AD and elderly non-demented controls. Increased CD4 and decreased CD8 cell proliferation might be a possible explanation for the altered CD4/CD8-ratio in AD.

Suitability of murine lymphocytes to study age-related changes and effects of FAD-associated mutations on apoptosis

In this thesis, enhanced vulnerability to cell death in AD was found and, moreover, earlier findings revealed perturbed calcium homeostasis in peripheral lymphocytes from AD patients (Parshad *et al*., 1996; Eckert *et al*., 1997a, 1998, 2000a; Gasparini *et al*., 1998). In order to close the gap between studies using neuronal cell lines transfected with PS1 mutations, neurons from mutant PS1 transgenic animals and peripheral cells of AD patients, lymphocytes from mice transgenic for FAD-specific PS1 mutations were investigated.

Accordingly to demonstrate that enhanced apoptosis in aged humans is related to the aging process and not due to presymptomatic Alzheimer's disease, the basal and oxidative stress-induced apoptosis in lymphocytes from young and aged mice to exclude changes associated with AD were investigated. To monitor the protective efficacy of potential therapeutic drugs in preclinical studies, the effects of the *Ginkgo biloba* extract EGb761 on murine lymphocyte apoptosis were studied *in vitro* and *ex vivo* in young and aged mice. The rationale for the pharmacological intervention study is as follows: EGb761 is a potent antioxidant and has already been shown to inhibit apoptosis in tissue culture experiments (Ni et al., 1996; Ahlemeyer et al., 1999; Bastianetto et al., 2000a) and oxidative stress in general (Kose *et al*., 1997; Pietri *et al*., 1997). Its effectiveness in Alzheimer's disease has been demonstrated by recent trials (Kanowski *et al*., 1996;(Le Bars et al., 1997).

Age-related changes of apoptosis in murine lymphocytes: Similarities to human aging

Basal levels of apoptotic nuclei

T cells were isolated from the spleens of young (3 months) and old (24 months) mice. To reduce activation or cell death of T cells during the isolation procedure, B lymphocytes were removed by magnetic beads in order to leave the T cells untouched. Basal DNA fragmentation assessed immediately after isolation of the cells was analyzed by propidium iodide (PI) staining. Fluorescence intensity was plotted against number of counts. Significantly increased basal apoptotic levels could be determined in freshly isolated spleen cells from old mice (2.18 ± 0.23% apoptotic cells; n = 13) compared to young mice (1.77 ± 0.26%; n = 9; *$p<0.05$; Figure 65A).

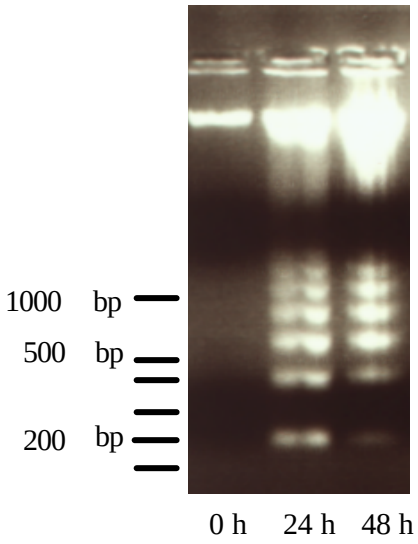


Figure 64. 2-deoxy-D-ribose (dRib) induces DNA-fragmentation demonstrated by DNA-ladder formation in murine spleen cells. Apoptotic cell death pattern in murine lymphocytes under basal conditions (immediately after isolation, 0 hours), levels of apoptotic cells are too low, to give a detectable DNA ladder formation. After 24 and 48 hours incubation with dRib, a massive apoptotic endonuclease activity occurs, which is evident by cleavage of chromosomal DNA between the nucleosomes producing oligomers of 180 basepairs (typical "DNA ladder").

Oxidative stress enhances the age-related differences in apoptotic cell death

2-deoxy-D-Ribose (dRib) depletes the intracellular pool of reduced glutathione thereby promoting states of enhanced oxidative stress inside the cell (Ceruti et al., 1997; Barbieri et al., 1994). Here it was demonstrated that dRib is able to induce apoptosis as well in murine lymphocytes. To further confirm the apoptotic nature of cell death, DNA laddering of nuclear fragmentation was assessed in murine T cells from individual mice. Figure 64 shows a representative experiment.

T cells of the spleen from young and old mice were incubated for 24 hours in the presence of 10 mM dRib. Spontaneous *in vitro* apoptosis (young: 25.15 ± 0.67% vs. old: 26.42 ± 1.65%) of cells incubated over the same period with medium alone was subtracted in order to reveal ROS/oxidative stress-induced apoptosis. Induction of oxidative stress revealed age-related changes in programmed cell death. A significant increase towards higher apoptotic levels was

found in spleen cells of aged animals (old: 7.90 ± 1.55%; young: 2.21 ± 0.45%; **p<0.01; n = 9-13 in each group; Figure 65B).

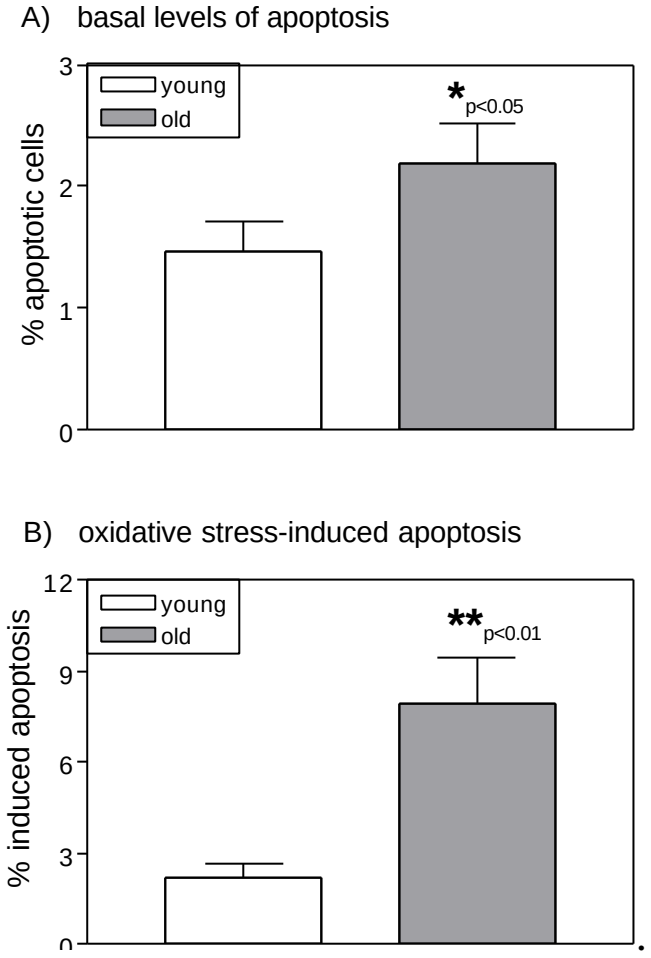


Figure 65. Basal (A) and dRib-induced (B) apoptosis in T cells of young and old mice. Percent sub-G_0 DNA content (termed as apoptotic cells) in freshly isolated lymphocytes derived from young (3 months of age, white bars) and old (24 months of age, gray bars) animals are shown. A) Apoptotic cell death was determined immediately after isolation, B) cells were incubated for 24 hours at 37°C in the presence of dRib (10 mM). Data are means ± S.E.M.; n = 9-13 per group, *p<0.05; **p<0.001.

Comparison from murine peripheral blood mononuclear cells (PBMC) with murine spleenocytes

To compare these findings with results in studies from human PBMC (see: Basal and spontaneous apoptosis are increased in lymphocytes from elderly subjects), murine PBMC were isolated according to standard procedures and basal, spontaneous and dRib-induced

apoptosis was determined. dRib-induced apoptosis was calculated by subtracting the spontaneous apoptosis. The results are summarized in the following table (Table 11).

	young	old	significance
basal apoptotic levels	0.79 ± 0.21%	0.99 ± 0.31%	n.s.
spontaneous *in vitro* apoptosis	9.37 ± 1.58%	15.63 ± 2.91%	*p<0.05
10 mM dRib-induced apoptosis	7.17 ± 0.5%	17.8 ± 0.74%	***p<0.001

Table 11. Basal, spontaneous and dRib-induced apoptosis in murine PBMC, n = 10 per group.

No differences in basal levels were found. In contrast, spontaneous and ROS-induced apoptosis after 24 hours *in vitro* was clearly enhanced in cells from aged mice. Compared with lymphocytes from the spleen, murine PBMC are less vulnerable to apoptotic cell death

In vitro effects of EGb761 on ROS-induced apoptosis in spleen cells from young and old mice

To determine the protective potential of *Ginkgo biloba* extract on ROS-induced apoptosis *in vitro*, freshly isolated spleen cells from young and old mice were co-incubated with various doses of EGb761 and 10 mM dRib for 24 hours. The level of ROS-induced apoptosis (dRib alone) occurring after 24 hours of any individual animal was taken as 100% (full apoptotic effect). The percentage of basal apoptosis from freshly isolated lymphocytes was set as 0% (Figure 66). *Ginkgo biloba* extract decreased dRib-induced apoptosis at concentrations above 10 µg/ml. Interestingly, EGb761 provides greater protective properties in cells from aged animals. 250 µg/ml and 500 µg/ml revealed a significant reduction of apoptosis in aged mice compared to young mice. Since absolute levels of dRib-induced apoptosis are higher in lymphocytes from old animals (Figure 65B), these findings demonstrate that cells from old mice specifically benefit from EGb761 treatment. In lymphocytes from young mice the reduction was less pronounced than in elderly animals. Two-way ANOVA revealed significant differences between mice groups and treatment with EGb761 (10-500 µg/ml; $p<0.05$).

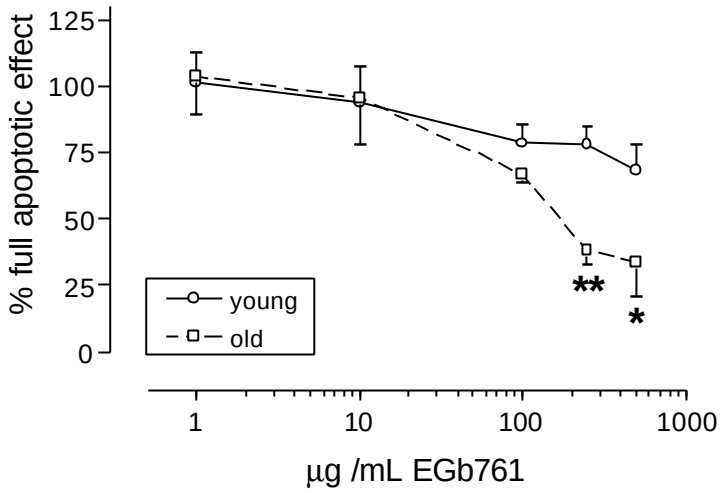


Figure 66. Prevention of ROS-induced apoptosis by increasing doses of EGb761. Percentage of apoptotic lymphocytes after 24 hours without EGb761 treatment were set 100%. Basal apoptosis of freshly isolated spleen cells was defined as 0%. The anti-apoptotic effects of EGb761 concentrations over 250 µg/ml differ significantly between the to age groups (n = 3 per group, **p<0.01; *p<0.05).

Ex vivo effects of EGb761 on ROS-induced apoptosis in young and old mice

In order to explore the effect of *Ginkgo biloba* extract on chronically treated cells and to determine its *ex vivo* anti-apoptotic function, we treated young and old mice with EGb761 in 0.2% agarose or with agarose alone as placebo over a period of 2 weeks. The dose used (100 mg/kg) has been previously shown to improve learning deficits in aged mice (Stoll et al., 1996). After 14 days of treatment, mice were killed by cervical dislocation and spleens were prepared as described above. No effect of EGb761 on basal apoptosis could be seen in both groups (young: placebo; 1.77 ± 0.26%; EGb761, 1.76 ± 0.28%, n = 9 per group; old: placebo, 2.18 ± 0.23%; EGb761, 2.19 ± 0.31%, n = 13 per group), indicating no direct *in vivo* effect of EGb761 on physiological apoptosis. In contrast, *ex vivo* ROS-induced apoptosis triggered by treatment with dRib was significantly reduced in the placebo group of young animals (placebo: 100.0 ± 3.10%; EGb761: 79.80 ± 3.58%; n = 9 in each group; ***p<0.001; Figure 67A). Interestingly, as already shown by our *in vitro* experiments, lymphocytes from old animals revealed a significant (EGb761old: 55.55 ± 10.65% vs. EGb761young: 79.80 ± 3.58%; *p<0.05) greater protection by the plant extract than cells from younger mice did (old,

placebo: 100.0 ± 23.51%; EGb761group 55.55 ± 10.65%; n = 13 per group; $^{+}p<0.05$; Figure 67B).

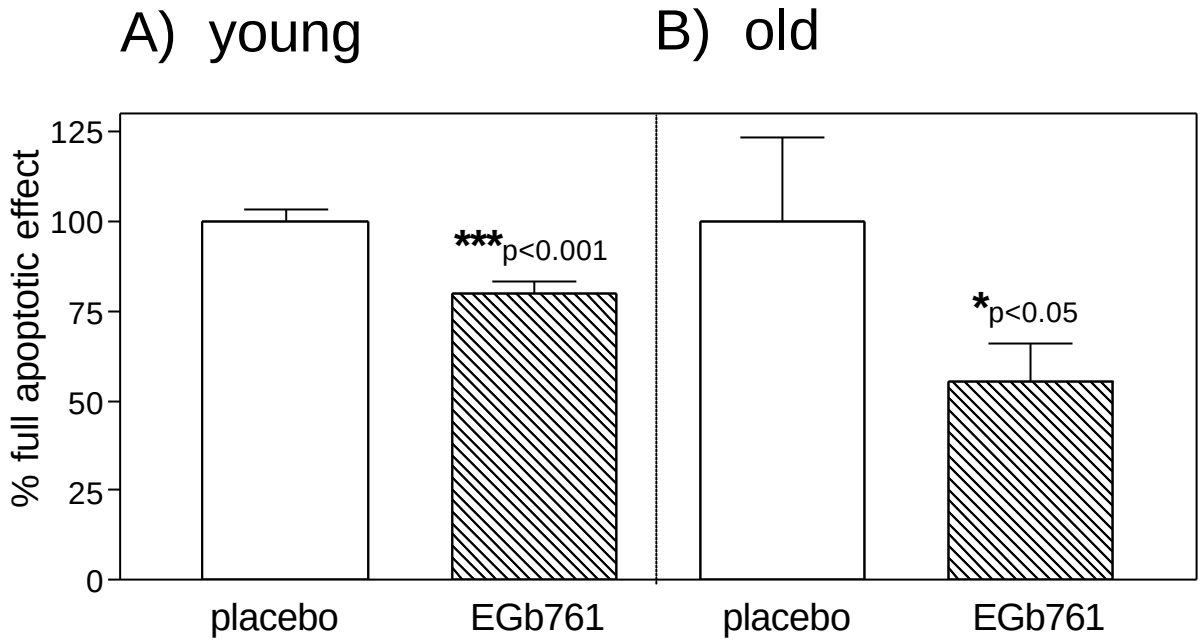


Figure 67. Effect of oral EGb761-treatment on ROS-induced apoptosis in spleenic T cells from young and old mice. Young A) and old B) female NMRI mice were treated with 100 mg/kg EGb761 or vehicle (placebo) orally for 2 weeks. T cells were isolated from the spleens and incubated with 10 mM dRib for 24 hours. Mean levels of dRib-induced apoptosis of the placebo group were set 100% (100% full apoptotic effect). Basal apoptosis of freshly isolated spleen cells was defined as 0%. A) In spleen cells from young animals, ROS-induced apoptosis was diminished by EGb761 by about 20% when compared with placebo treated mice (n = 9 per group, ***p<0.001). B) In cells derived from old mice this effect was even more amplified. Apoptosis was reduced by about 45% in the EGb761-treated group, though levels fluctuated more in aged animals, that results in a lower significance (n = 13 per group, *p<0.05). However, cells from aged mice revealed a significant greater benefit from EGb761-treatment than lymphocytes of young animals did (placebo old (B) vs. placebo young (A): $^{+}p<0.05$).

Transgenic animal models for AD

Impact of mutant PS1 to apoptosis of peripheral lymphocytes

Three different types of trangsgenic mice were generated by N. Touchet and C. Czech (Rhone Poullenc/ Aventis Pharma, Vitry-Sur-Seine, France) and kindly donated to our labaratory. Genetic engineered mice were either expressing a human wildtype Presenilin-1 (PS1 wt) transgene or a PS1 transgene expressing either a single pathogenic mutation at codon M146 (PS1 M146L) or multiple FAD-specific mutations M146L + H163R + A246E + L286V + C410Y (PS1 M5), since the combination of different PS1 mutations shows additive effects on Aβ1-42 secretion (Citron *et al*., 1998). Transgene expression is under the control of the human HMG-CR-promotor which represents a housekeeping-type promotor that shows a strong and ubiquotious expression pattern with high expression in neurons (Czech *et al*., 1997; 1998). Transgenic and non-transgenic animals were littermates or subsequent generation siblings and are therefore strain matched. PS1 transgenic mice showed normal growth and development, and revealed no apperenet behavioural changes compared to littermate controls. As shown by other studies, transgenic mice overexpressing mutant PS1 show an elevation in Aβ42, however they do not develop amyloid deposits by 24 months of age (Chui *et al*., 1999; Czech *et al*., 1998). In order to assess whether human PS1 is expressed in peripheral cells of transgenic mice, a Western Blot analysis of spleenocytes homogenates from transgenic and nontransgenic animals was performed. The blot was probed with a monoclonal antibody against the N-terminal part of human PS1. The proteolytic expression against of transgenic PS1 is different between the constructs. The characteristic N-terminal fragment of PS1 is detectable in cells of mice expressing PS1 wt and PS1 M146L with the appearance of small amounts of full-length PS1 M146L most likely due to saturation of processing events as described previously in transgenic mice and rats expressing high amounts of PS1 (Czech *et al*., 1998; Thinakaran *et al*., 1996). However, multiple mutations in PS1 M5 resulted in a clear reduction in the N-terminal fragment and accumulation of full-length PS1. This is most likely due to saturation of processing since the levels of PS1 M5 expression are lower than the levels of PS1 wt or PS1 M146L. Instead, the accumulation of full-length protein suggests that PS1 M5 has severe structural alterations which make it no longer a substrate for the PS-cleaving protesases.

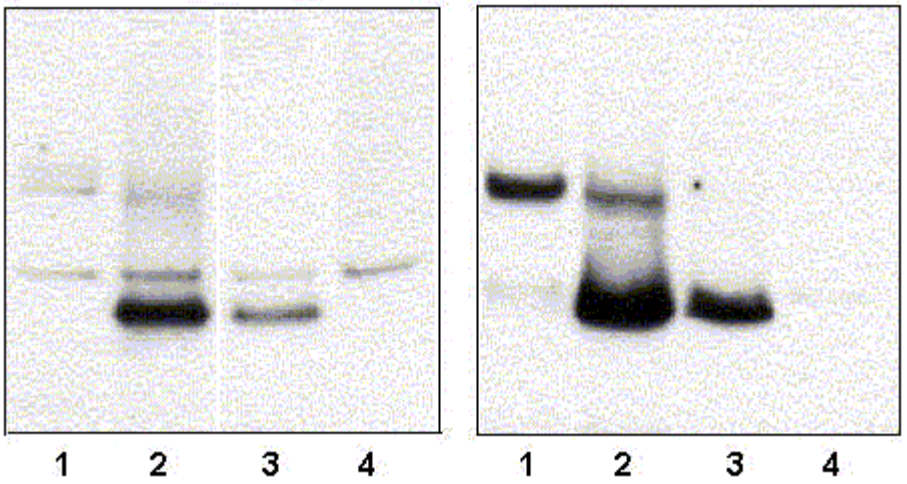


Figure 68. Western Blot analysis of PS1 expression in spleenocytes (left blot) and homogenated brains (right blot) from transgenic mice (lane 1, PS1 M5; 2, PS1 M146L; 3, PS1 wt) and non-transgenic littermate controls (lane 4).

	control	PS1 wt	PS1 M146L	PS1 M5
female	10	4	2	2
male	8	7	8	6
Σ	18	11	10	8

Table 12. Number and sex of transgenic and control mice included in the study.

Basal, spontaneous and agent-induced apoptosis was determined in enriched T lymphocytes from the spleen ($CD3^+$ cells approximately 85%) from transgenic mice and littermate controls.

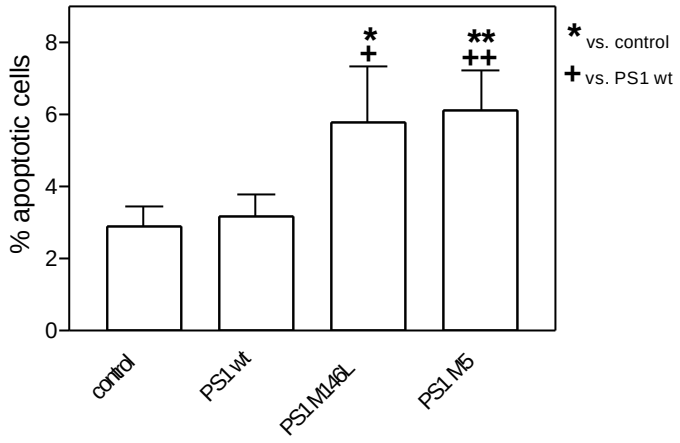


Figure 69. Basal apoptotic cells of spleenic T cells from different PS1 transgenes. Cells derived from mice with single and multiple mutations of PS1 show an elevated percentage of apoptotic cells compared with PS1 wt and littermate control.

Freshly isolated lymphocytes from mice expressing mutant PS1 (PS1 M146L and PS1 M5) stained with PI showed significantly enhanced basal levels of apoptotic cells compared to T cells from PS1 wt transgenic (tg) and non-transgenic (non-tg) littermate controls (Figure 69). Mutant tg mice exhibited a twofold increase in basal apoptosis. These findings could be confirmed by our laboratory in an additional assay detecting cytosolic nucleosomes built during apoptotic cell death (Eckert *et al*., 2001b).

Next, *in vitro* apoptosis after 2.5 and 24 hours incubation was determined. Lymphocytes from PS1 M5 tg mice showed significantly enhanced vulnerability to spontaneous *in vitro* apoptosis when compared with that of T cells from PS1 wt tg and non-tg control mice, whereas the vulnerability of the single mutation PS1 M146L was not markedly increased. (Figure 70). The difference in vulnerability of T cells from PS1 M5 tg mice to apoptotic cell death was not salient anymore 24 h after ex vivo conditions (data not shown; Eckert *et al*., 2001b). Again, multiple mutations in PS1 showed much more apoptotic cells than cells from PS1 wt tg and control mice did.

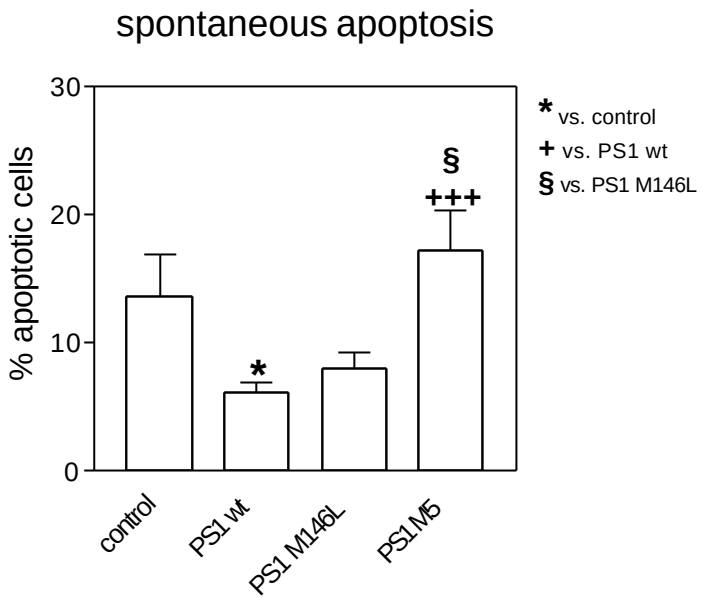


Figure 70. Spontaneous apoptosis after 2.5 hours *in vitro*. PS1 M146L displays elevated levels of apoptosis, though not significantly compared with PS1 wt. Cells with PS1 M5 transgene are significantly elevated compared with wildtype protein. Interestingly, cells derived from non-tg control mice display significantly increased levels compared with PS1 wt, indicating a possible protective effect of overexpressed wildtype PS1.

APP/PS1 double transgenic mice

Mice bearing a triple mutation in the human APP gene were kindly donated by C. Czech and mated in our breeding facility with PS1 M146L tg mice, in order to generate mice that show AD like brain pathology at an age of 6 months (Moussaoui *et al*., 2000). The offspring consisted of tg mice with human mutated PS1 (PS1 M146L), human APP_{695}SDL alone, double transgenes (PS1 M146L x APP_{695}SDL) or non-tg littermates, as confirmed by genomic PCR (see methods, Figure 23). Those four different constructs were compared with PS1 wt mice. Apoptosis was determined by 7-AAD-staining, to distinguish between apoptosis in CD4 and CD8 cells.

	controls	PS1 wt	PS1 M146L	$APP_{695}SDL$	PS1 M146L x $APP_{695}SDL$
female	0	0	3	3	0
male	6	6	3	3	6

Table 13. Number and sex of transgenic animals and non-transgenic littermates included in the study. All mice were at an age of 3 to 4 months.

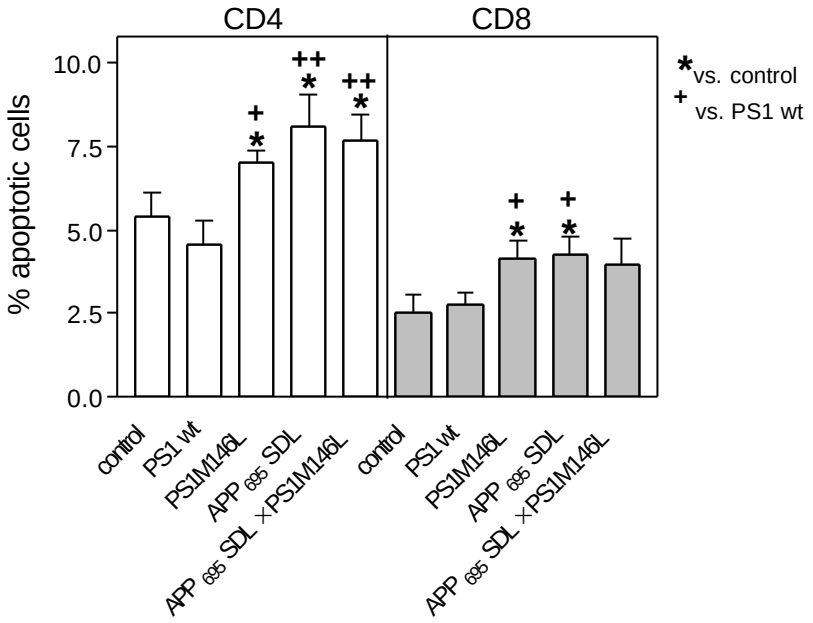


Figure 71. Basal apoptosis in T cell-enriched spleenocytes derived from transgenic mice with either a mutated APP or PS1 transgene, or both, PS1 wt and non-transgenic controls. White bars on the left represent $CD4^+$ cells, dotted bars on the right are $CD8^+$ cells. In concordance with the data from human T lymphocytes derived AD patients, the FAD-associated pathogenic mutation display significantly elevated levels of basal apoptotic cells. The increase of apoptosis is more evident in T helper cells (compare with Figure 50 and Table 9).

Moreover, spontaneous, ROS- and dexamethasone induced apoptosis was assessed by 7-AAD-staining after 24 hours *in vitro*.

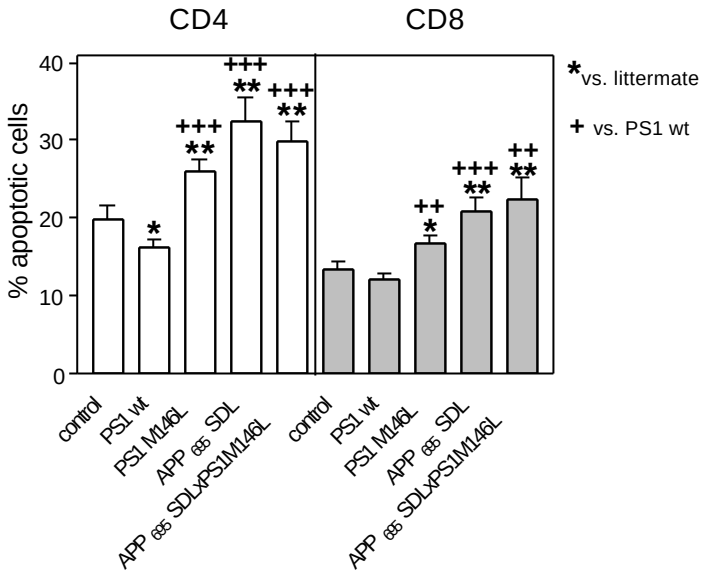


Figure 72. Spontaneous apoptosis of transgenic mice after 24 hours culture in vitro. White bars on the left hand represent CD4 cells, dotted on the right are CD8.

Pathogenic FAD-associated mutations display dramatically increased levels of apoptotic cells, especially in $CD4^+$ cells, in concordance with findings from PBMC of AD-patients (see Table 9).

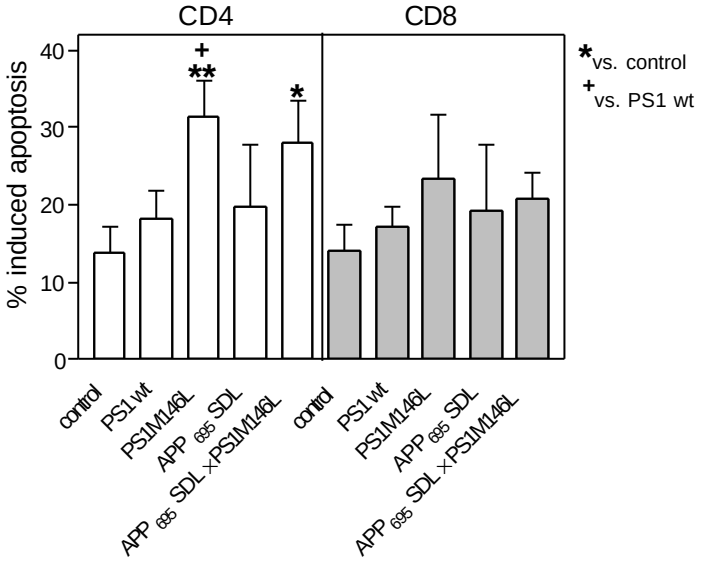


Figure 73. ROS-induced apoptosis in spleenocytes from transgenic mice. Only T hepler lymphocytes (white bars) display significantly elevated levels compared with controls. $CD8^+$ cells from FAD-associated mutations (dotted bars) transgenes are increased,

though changes are not significantly altered. These data are in concordance with findings in PBMC from AD-patients (see Table 9).

Enhanced vulnerability to ROS-induced apoptosis seems to be mainly restricted to mutant PS1. Mice carrying the mutant APP do not show increased apoptotic values upon dRib treatment.

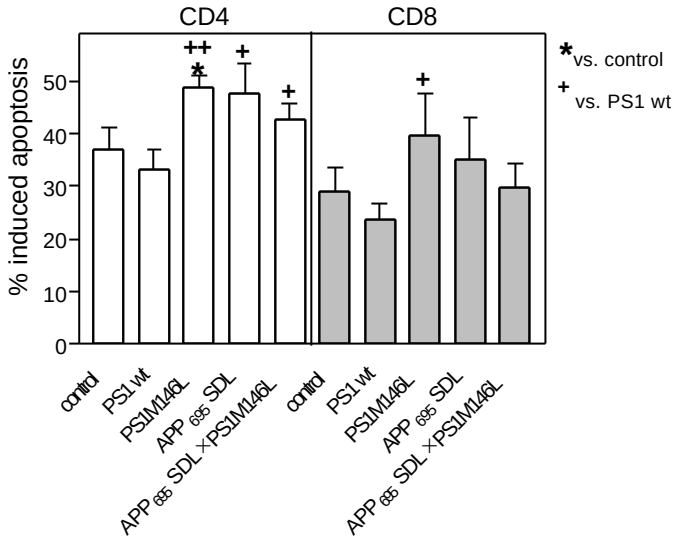


Figure 74. Dexamethason-induced apoptosis in spleenocytes from transgenic mice. Pathogenic FAD-mutation are more vulnerable against cell death induced by glucocorticoids. T helper cells (white bars) are more involved than $CD8^+$ lymphocytes (dotted bars).

It is noteworthy that PS1 wt tg mice show decreased apoptotic features compared to littermate controls (see Figure 71- Figure 74), indicating a probably anti-apoptotic role of overexpressed PS1. This finding will be further explored in the subsequent experiments (see next chapter).

Antiapoptotic action of PS1 C-terminal fragments

Familial Alzheimer's disease is predominantly connected with mutations in the Presenilin family. Presenilin (PS) 1 and 2 have been shown to be involved in apoptosis. Recently, it was found that ALG-3, a truncated form of PS2 rescued T-cells from Fas-induced cell death (Vito *et al*., 1996), whereas overexpression of the full-length not proteolytic processed PS1 and PS2 enhances apoptosis. Presenilins are substrates of caspases. Caspase mediated cleavage of PS1 occurs between aspartate 345 and serine 346 (Grünberg *et al*., 1998).

In previous experiments (see APP/PS1 double transgenic mice) reduced apoptosis of spleen T lymphocytes derived from PS1-transgenic mice compared to their non-transgenic littermate controls was found. The transgenes carry their endogenous murine PS1 and additionally under the control of the HMG promotor the human PS1. Therefore, tg PS1 wt mice express higher amounts of total PS1 compared with non-tg controls. However, Western blot analysis revealed that PS1 is processed normally in tg mice (Figure 76), whereas overexpression under a strong promotor in cell lines often limits proteolytic capacity thereby leading to enhanced apoptosis due to full-length PS1 (Kim *et al.*, 2001). To further analysis the molecular basis of this observed anti-apoptotic behavior in T cells from PS1 wt tg mice, a construct that encodes a 531 bp fragment of PS1 and is analogously to ALG-3, named PS1short (PS1s, Met^{292} - Ile^{467}) and a construct, that encodes the C-terminal fragment processed by caspase cleavage, named PS1Cas (Figure 78), were generated and transfected into Jurkat T cells and compared with cells transfected with PS1 wt, PS2 wt and ALG-3 . Apoptosis was induced by Fas-ligand engagement.

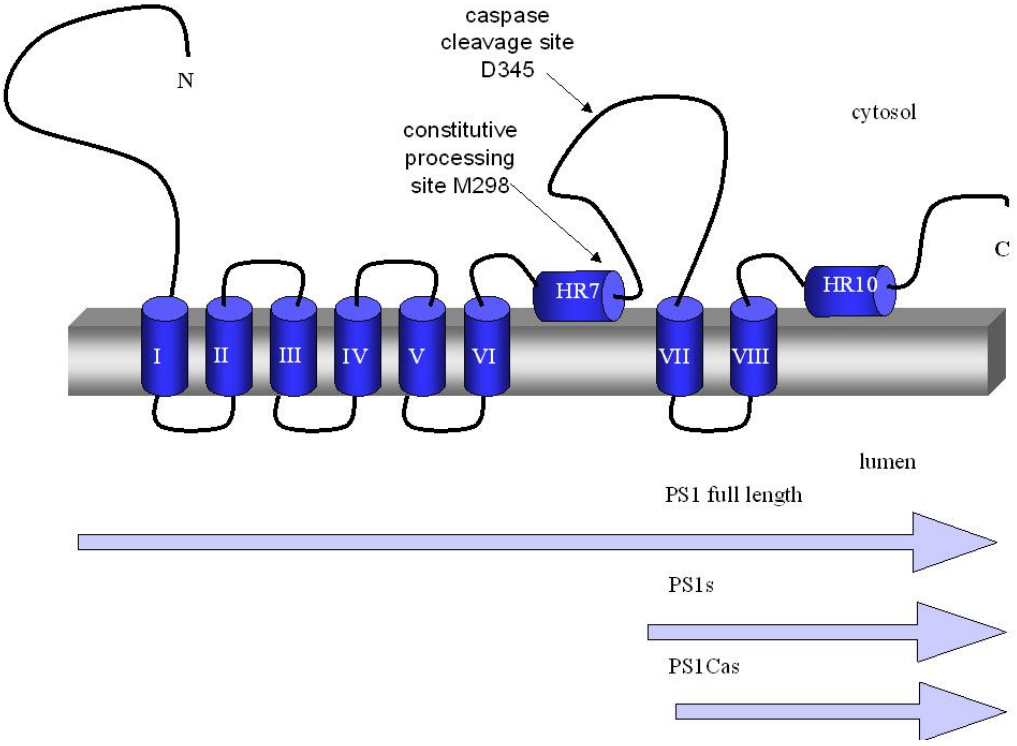


Figure 75. Membrane topology of PS1. Arrows indicate constructs of PS1 and C-terminal fragments.

In lymphocytes from PS1-transgenic mice, it was observed that that spontaneous apoptosis is significantly reduced in $CD4^+$ T-cells from PS1-transgenic mice compared to non-transgenic littermate controls (Figure 77), the basal apoptotic levels showed clear tendency, but were not significantly altered (data not shown).

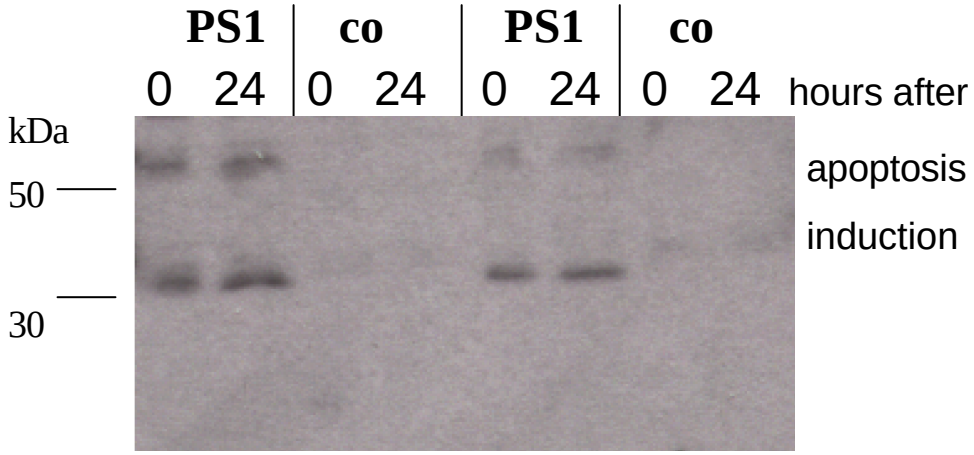


Figure 76. Western blot analysis of spleenocytes derived from PS1-transgenic (PS1) mice and littermates (lt) controls. By blotting with an N-terminal-specific antibody it is obvious, that cells from PS1-tg express much more PS1 than lt, which only express their endogenous PS1.

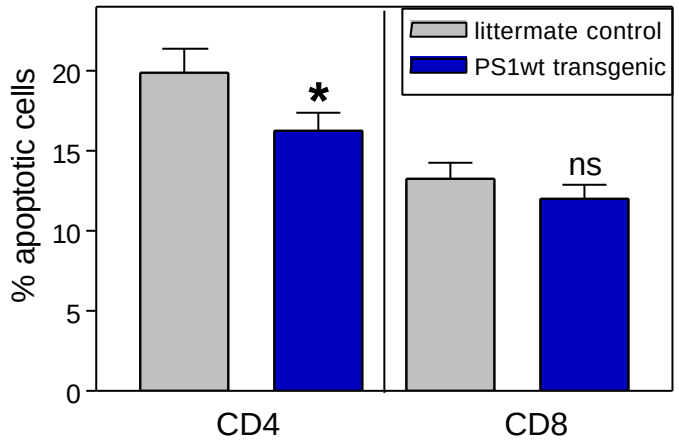


Figure 77. Spontaneous apoptosis after 24 hr ex vivo is significantly decreased in $CD4^+$-spleenocytes of PS1-transgenic mice when compared with littermates controls, indicating a protective effect of additional PS1 during apoptosis.

Protein analysis by western blotting revealed the existence of short PS1 fragments (~13 kDa) when cells undergo apoptotic cell death (Figure 78). We hypothesized that this fragment derived from casapse cleavage of PS1 during apoptosis. This fragment, termed PS1Cas, is much more prominent in PS1 wt-transgenic animals than in littermate controls, though it is produced in detectable amounts.

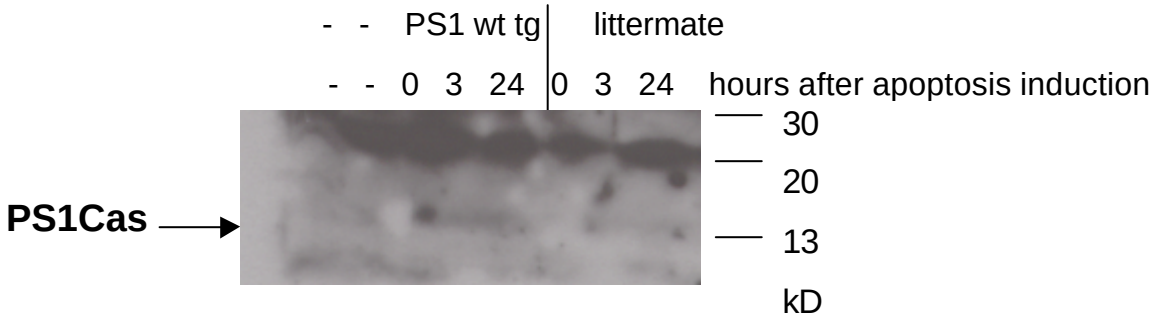


Figure 78. Western blot analysis of spleenocytes derived from PS1-transgenic (PS1) mice and littermates (lt) controls. Blotting with a C-terminal-specific antibody small fragment can be detected after induction of apoptosis. In wt-tg more fragments can be detected.

In order to investigate this fragment and its effects on apoptosis, several constructs encoding full-length PS2, ALG-3, full-length PS1, and PS1s (a PS1-analoug to ALG-3; see Figure 75) were generated and transfected into Jurkat T-cells. Apoptosis was induced with Fas-ligand for 4 and 24 hours. An additional construct was designed encoding the C-terminal fragment of PS1 up to the considered caspase-cleavage site, named PS1Cas. Transfection and induction of apoptosis in Jurkat cells showed that this fragment provides the same antiapoptotic action like PS1s and ALG-3 (Figure 79). Fas-L induced apoptosis can be clearly diminished by C-terminal fragments of PS2 (ALG-3) and PS1 (PS1s and PS1Cas) by about 40%.

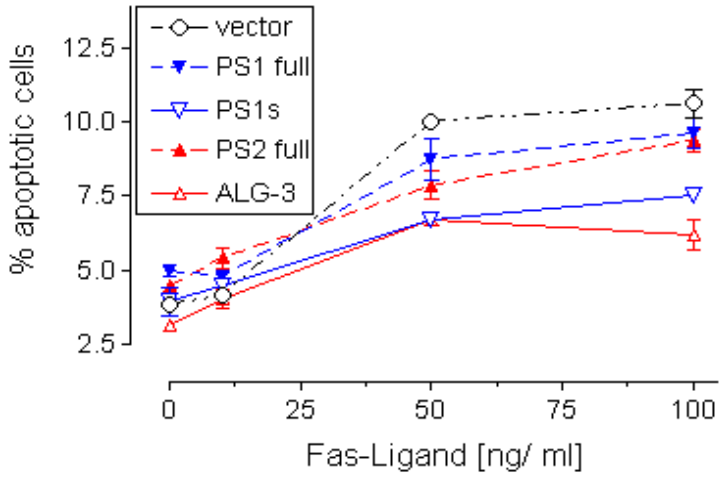


Figure 79. Apoptosis 4 hours after Fas-ligand treatment in Jurkat cells transfected with PS1, PS2, PS1s, and ALG-3. PS1 and PS2 show slight protective effects against apoptosis while the C-terminal fragments diminish significantly apoptosis by 40% when compared with vector transfected cells. Basal levels are not significantly changed. After 24 hours Fas-ligand treatment, changes between the different transfected constructs are not significant (data not shown).

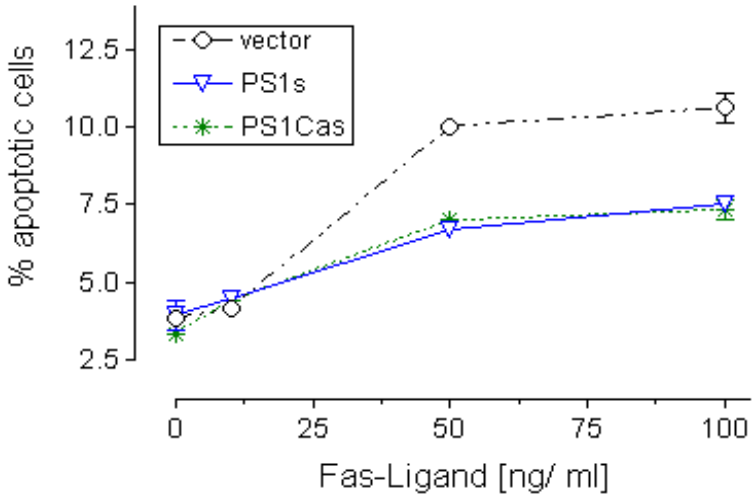


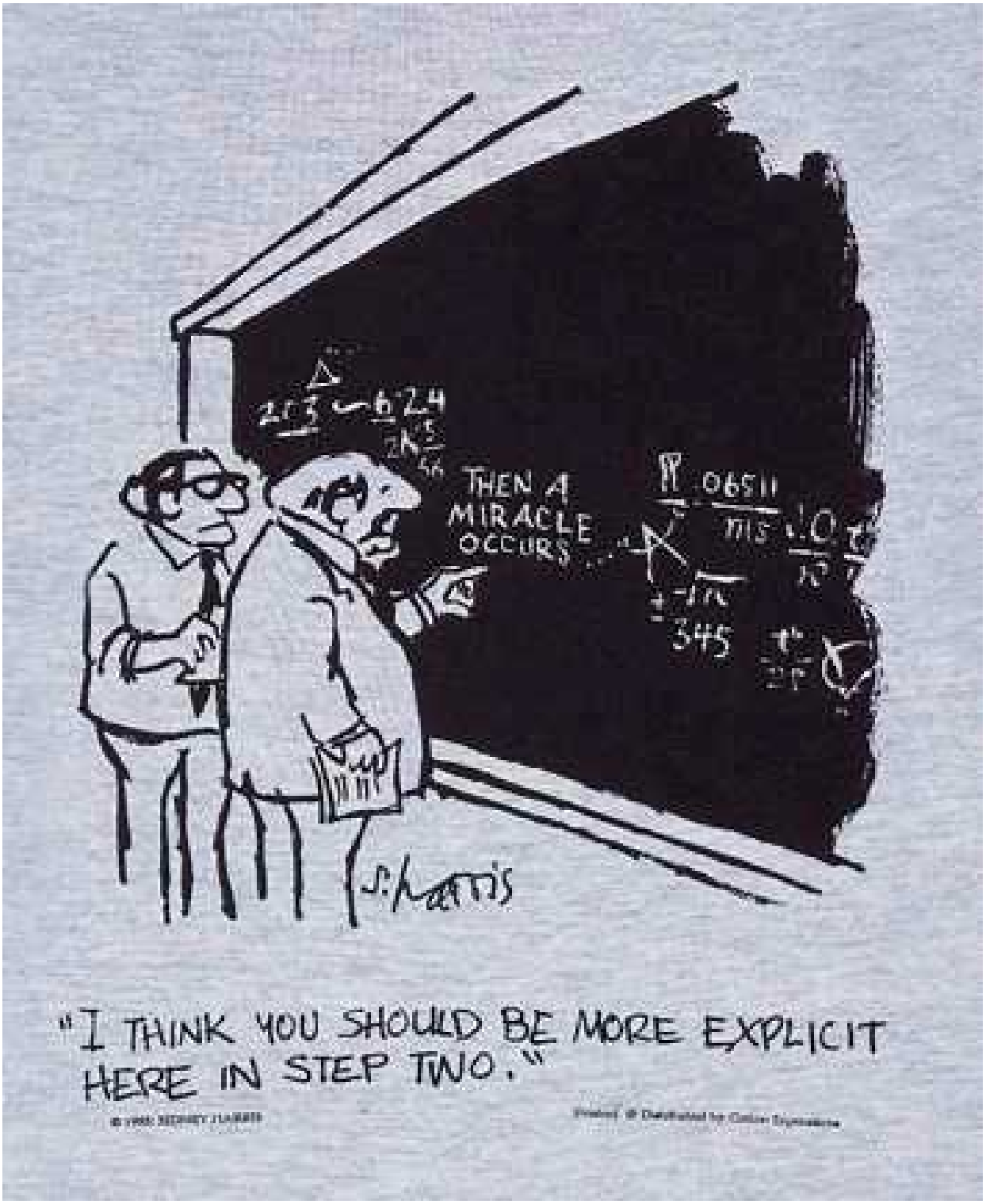
Figure 80. Transfection of Jurkat cells with PS1Cas and induction of apoptosis by Fas-ligand. Apoptosis was measured after 4 hours by PI-staining and FACS analysis. PS1Cas provides the same anti-apoptotic properties as PS1s.

The proteolytic cleavage of the presenilins and the generation of the C-terminal fragment seems to play an important anti-apoptotic feedback mechanism in programmed cell death. Several mutants interacting with the proteolytic processing are known to be involved in familiar to Alzheimer's disease.

Analysis of Presenilin-1 carrying a mutation at site D345A (D = aspartate was mutated to A = alanine by site-directed-mutagenesis) are currently under investigation. Aspartate is needed as recognition motif for most caspases. If cleavage of aspartate 345 by caspases is essential to form this anti-apoptotic fragment (PS1Cas), cells carrying that mutation should not show any decrease in apoptotic level.

Presenilins are involved in the Notch-cascade, playing an important role in fetal development. However, the biological relevance of this antiapoptotic feedback for developmental processes and in AD remains to be elucidated.

Discussion


THEN A MIRACLE OCCURS...
"I THINK YOU SHOULD BE MORE EXPLICIT HERE IN STEP TWO."

The present thesis demonstrates that Alzheimer's disease and familiar Alzheimer's disease-related mutations have a significant impact on apoptosis of peripheral lymphocytes in patients and transonic mice. To determine these changes from those present in 'healthy' aging, programmed cell death was assessed in PBMC from young and elderly volunteers.

Previous results of our and other group reported an increased content of basal and dRib-induced apoptotic cells in PBMC from patients with Alzheimer's disease relative to age-matched controls (Eckert *et al*., 1998; 2001a; Lombardi *et al*., 1999). This effect is probably not related to the physiological process of immunosenescence. In addition, apoptosis is a hallmark in brains derived from Alzheimer's patients, possibly associated with states of increased oxidative stress (Cotman *et al*., 1995; Simonian and Coyle, 1996; Tritschler *et al*., 1994; Yamatsuji *et al*., 1996). Similar alterations could also be detected in peripheral cells of AD patients (Mecocci *et al*., 1997; Parshad *et al*., 1996). In order to understand the specific contribution of AD to the enhanced susceptibility of lymphocytes to apoptosis it is of particular importance to know first the role of normal aging without dementia. Therefore, in this thesis the age-related apoptotic behavior of peripheral human lymphocytes and the role of oxidative stress were explored.

Altered apoptosis of peripheral lymphocytes in aging

Age-related differences in the vulnerability to cell death were clearly shown. Basal levels of apoptotic nuclei in freshly isolated PBMC are significantly elevated in the group of aged donors compared to younger ones. In parallel, basal levels of reactive oxygen species are similarly increased in aging (Leutner, 2001). PBMC and activated $CD3^+$ lymphoblasts from elderly subjects have an enhanced susceptibility to spontaneous and activation-induced *in vitro* apoptosis. This enhanced vulnerability is probably not related to a decline in IL2 production (Phelouzat *et al*., 1997) but must be correlated with an increased basal expression of activation markers (e.g. CD95, CD25, CD69, HLA-DR) and higher levels of ROS. Usually, apoptotic cells are eliminated quite fast after generation. A higher percentage of basal apoptotic levels in aged human PBMC could indicate an impaired phagocytosis of these cells in the aging organism. Thus even basal apoptotic levels might reflect a mild stimulation of the cells due to the isolation procedure. The content of Bcl-2 slightly raises in peripheral cells with aging. Moreover, other investigators and our laboratory could show that resting peripheral blood cells do not express large amounts of Bcl-2 at all, but the content of Bcl-2

increases significantly after activation with PHA-L and IL-2 (Reed *et al.*, 1992). Basal amounts of Bcl-2 correlate with the content of basal apoptotic cells in aging. Since Bcl-2 is a physiological apoptosis inhibitor, it could be possible that cells prone to undergo apoptosis express higher amounts of Bcl-2 as a defending mechanism. Therefore, it appears not to be surprising that apoptotic cells display more Bcl-2 at basal levels. Activated lymphoblasts displayed the more Bcl-2 the younger the donor was. These data implicate a strong correlation between lymphocyte's functionality concerning activation and the donor age. It seems that the decreased amount of Bcl-2 in activated lymphoblasts in aging might be one factor contributing to immunosenescence.

This higher susceptibility to apoptosis in aging might be due to an enhanced production and/ or not satisfactionally elimination of reactive oxygen species in aging, thereby leading to apoptosis. Mitochondria are the major site, where free oxygen radicals are produced. In aging, mutations accumulate in mtDNA, due to its near vicinity to radical formation (Mecocci *et al.*, 1994). The mutant mtDNA leads to mitochondria with impaired respiratory function (Anderson *et al.*, 1998; de Grey, 1997). In addition, ROS act as intracellular messenger and mediate lymphocytes activation (Dalton *et al.*, 1999) as well as apoptosis (Buttke and Sandstrom, 1994) by interacting with redox-sensitive transcription factors like NFκB.

Reduced glutathione (GSH) is an important antioxidant factor in the cell. Depletion of intracellular GSH inhibits T-cell function (Droge *et al.*, 1994). Recently, it could be shown that the levels of GSH decrease in aging in human lymphocytes (Wieshout and Peters, 1998). The sugar 2-deoxy-D-ribose provokes oxidative stress in cells probably by depleting the intracellular levels of reduced glutathione (Kletsas *et al.*, 1998). It is involved in the formation of free radicals (Ceruti *et al.*, 1997), as it significantly elevates the level of ROS in lymphocytes compared to untreated controls (Leutner, 2001). The apoptotic process initiated with dRib can be blocked with N-acetyl-L-cysteine (NAC) a potent radical scavenger (Schindowski *et al.*, 1999). Therefore dRib is a useful tool to simulate oxidative stress in human lymphocytes as shown by (Eckert *et al.*, 1998). Here, it was found that with increasing donor age, peripheral blood cells could cope worse with oxidative stress induced by incubation with 10 mM dRib. Compared to spontaneous apoptosis, dRib induced apoptosis increases more with age. A possible cause for this finding could be the decreased levels of GSH found in aging lymphocytes (van Lieshout and Peters, 1998). It can be concluded that the less GSH a cell contains the higher is the susceptibility to apoptosis induced by 10 mM dRib. Moreover, dRib induces significantly higher levels of ROS in PBMC derived from aged

donor compared to younger ones. However, higher concentrations of dRib induce rather more necrosis, but still apoptosis but the content of apoptotic cells induced by this stimulus did not correlate with age. Interestingly, the percentage of 50 mM dRib induced apoptotic cells correlates significantly with the content of lymphocytes basal expressing the activation markers CD25, CD95 and HLA-DR. Apoptotic cells induced with lower concentration of dRib (10 mM) did correlate as well with the expression of CD25. Correlation of 10 mM dRib with CD95 and HLA-DR could not be done as their expression is age-related as well and is therefore linearly related. It seems that dRib leads activated cells more in apoptosis than resting cells, maybe by a different mechanism, providing a powerful tool to explore lymphocyte apoptosis.

It is important to note that the age-related increased susceptibility of PBMC to dRib was not found for staurosporine. Staurosporine-induced elevation of ROS (Leutner, 2001) and staurosporine-induced apoptosis were not different in young or aged lymphocytes. Staurosporine produced comparable levels of ROS as dRib did (Leutner, 2001). However, due to the involvement of caspases in staurosporine-induced death signaling upstream of ROS (Krohn *et al*., 1998), higher levels of apoptotic cells were reached in young as well as in old subjects when compared to dRib-induced apoptosis. Staurosporine-mediated ROS serve rather as a second messenger (Buttke and Sandstrom, 1994) and GSH is primarily not affected. Obviously, only certain specific become mechanisms disturbed in aged lymphocytes.

In concordance to other groups it was found, that the basal expression of functional CD95 increases with age (Lechner *et al*., 1996; Phelouzat *et al*., 1997). We determined expression of CD95 surface molecule as well as its function with an agonistic antibody leading to apoptosis-specific DNA fragmentation. T lymphocytes from young donors expressed CD95 by about 12% less compared to elderly individuals, while only 0.3% of PBMC of young subjects displayed apoptotic morphology after 24 hours of incubation with anti-CD95. In the group of aged genders apoptosis could be induced in about 2.3% of the PBMC. These data implicate that most of the expressed CD95-molecules on $CD3^+$ cells are not fully functional, maybe due to a lack the FADD-protein (Fas-associated death domain). In aging, the number of functional CD95 expression raises. This data concerning expression of CD95 on native $CD3^+$ lymphocytes are consistent with the findings of Phelouzat *et al.* (1997), who reported that enhanced susceptibility to apoptosis of T lymphocytes from elderly is associated with increased expression of CD95-receptor. The frequency of $CD95^+$ cells may rise through adulthood, but go on to decrease in certain but not all subsets in old age (Aspinall *et al*.,

1998). The amount of soluble Fas in the blood of elderly donors was also reported to be significantly increased compared to young donors (Seishima *et al*., 1996). Moreover, several studies have shown increased CD3- or PHA-mediated AICD in the elderly (Phelouzat *et al*., 1996; Lechner *et al*., 1996, Potestio *et al*., 1998). This was found not to be due to IL 2 deprivation, nor was it associated with decreased Bcl-2 expression (Phelouzat *et al*., 1996).

Apoptosis in human subesets in aging

When analyzing the PBMC's subsets, apoptosis is increased at basal levels in T-, B- and NK-lymphocytes. In addition, in this small cohort investigated, only T cells show significant changes concerning spontaneous apoptosis in aging. Depletion of GSH by dRib amplified the age-relaed effects nicely in T and NK cells, however, in B cells treatment with dRib induces as well necrosis, therefore levels of apoptotic cell death seemed to be apparently lower in elderly than in young controls. Thereof, it can be concluded that all lymphocyte subsets are affected by aging, however, the T cell subset seems to be particularly involved, concerning apoptosis.

The aging process does not significantly alter the amount of Bcl-2 in the subsets. Nevertheless, a positive correlation was observed when plotting Bcl-2 content versus the percentage of apoptosis for each subset.

Basal, spontaneous and ROS-induecd apoptosis of the T cell subsets CD4 and CD8 correlated significant with the donor's age, indicating the close association of T cell apoptosis with age-related changes. Moreover, both subsets seem to be effected in the same manner, indicating that healthy aging influences T cells in general and no subset is preferred by these changes, in contrast to AD. Therefore, it is interesting that the amount of anti-apoptotic Bcl-2 decreases significantly only in $CD8^+$ cells with aging. In addition, a similar correlation as observered for B cells was detected for CD4 T lymphocytes when plotting Bcl-2 content versus percentage of apoptotic cells. It can be, therefore, speculated that B and $CD8^+$ T cells are responsible for the same correlation seen in whole PBMC using ELISA technique (see Figure 32). The mechanisms and biological relevance for this finding remains to be elucidated.

Another recent study examining CD95 expression in the elderly found increased levels of expression of both Fas and Fas-ligand on CD4 and CD8 cells, and these did not seem to decrease at more advanced age (Agarwal and Gupta, 1998). In the Aggarwal study but not in the Aspinall study the CD95 changes were noted on $CD45RO^+$ (e.g. memory) cells as well,

together with decreased levels of Bcl-2 and correspondingly increased levels of CD95 antibody-triggered apoptosis in all subsets with increased CD95 expression (Agarwal and Gupta, 1998). In a different system, in which T cells were stimulated by PHA and cultured with IL-2 for up to 6 days, apoptosis (not induced by CD95 mAb) was reported to be increased in $CD45RO^-$ but not RO^+ cells in the elderly compared to the young (Herndorn *et al*., 1997). It was therefore suggested that differential susceptibility to apoptosis late after T cell stimulation might contribute to explaining the preponderance of $CD45RO^+$ cells in the elderly, although the differences actually measured in these studies were minimal.

Consistent with the findings of increased susceptibility to AICD *ex vivo*, $CD4^+$ T cell clones aged in culture also become increasingly susceptible to AICD (Pawalec *et al*., 1996). Moreover, although T cell lines derived from old donors upregulated CD95 more slowly than those derived from young donors, their loss of ability to downregulate CD95 occurred faster, resulting in more rapidly increased susceptibility to Fas-mediated apoptosis (Lechner *et al*., 1996). However, the idea that increased levels of AICD are detrimental to functioning of the immune system must be reconciled with data from several sources suggesting an age-associated increase in resistance to apoptosis on the part of cells from various tissues including lymphocytes. It was suggested that some of the manifestations of aging on the immune system were related to downregulated apoptosis (Zhou *et al*., 1995). Some further supporting data for the concept of decreased apoptosis in aged cells may be found in the report of Lechner *et al*. (1996) who found decreased inducibility of CD95 after CD3-stimulation of old persons´ T cells compared to young. However, susceptibility to AICD of T cell lines established from old donors was greater than those from young donors (Lechner *et al*., 1996). Culture-aged CD4+ T cell clones show enhanced AICD compared to young cells from the same clone, although they do not express higher levels of CD95 (Pawelec *et al*., 1996).

In conclusion, aging leads to quite specific changes of the susceptibility of human lymphocytes for programmed cell death. This knowledge will allow getting a more detailed information about the additional changes taking place in AD.

Altered apoptosis in lymphocytes of aged mice and impact of antioxidative EGb761 treatment

In the present thesis, it was confirmed that lymphocytes derived from old mice are also more vulnerable to dRib-induced apoptosis than lymphocytes from young mice. Proposing that dRib exhausts the glutathione pool, cells that generate more ROS should be more prone to apoptosis. Since apoptosis is significantly elevated in dRib treated cells from elderly mice, one can conclude that lymphocytes derived from old animals as compared with young, either generate more ROS during metabolism or are more sensitive to ROS. However, higher sensitivity of old animals to dRib can not excluded.

Apoptosis is an important aspect in the aging processes in many tissues including brain and immune system. Enhanced oxidative stress seems to be one major causal factor. Enhanced vulnerability to oxidative stress-induced cell death has been found in this thesis for human lymphocytes in aging as well as in Alzheimer's disease (Eckert *et al*., 1998; 2001a). In order to finally confirm the role of the aging process *per se*, we determined additionally basal, spontaneous and ROS-induced apoptosis in lymphocytes of young and aged NMRI mice. In addition, the influence of the radical scavenger Ginkgo biloba extract EGb761 on apoptosis was studied *in vitro* and *ex vivo* after treatment over 14 days.

Basal levels of apoptotic lymphocytes increase only slightly with aging, most likely because apoptotic cells are eliminated quite fast by phagocytotic cells. In concordance, it was shown in this thesis that basal apoptosis of human PBMC revealed also only small changes in aging, indicating either an increased incidence of basal apoptosis or decreased elimination of apoptotic cells in aging. Moreover, it could be clearly demonstrated that spleen lymphocytes are much more susceptible to cell death than PBMC.

By contrast, spontaneous apoptosis was more clearly elevated in PBL derived from aged mice, suggesting that indeed enhanced vulnerability of the lymphocytes to apoptosis is involved.

ROS occur as physiological intermediates in aerobic metabolism and are usually eliminated inside cells by antioxidant pathways that require reduced glutathione (GSH) for regeneration. dRib provokes oxidative stress in cells. There are growing evidences that the underlying mechansim is diminishing the intracellular levels of GSH (Kletsas *et al*., 1998) and it seems to be therefore involved in the formation of free radicals (Ceruti *et al*., 1997). Depletion of GSH makes the cell defenseless against elevated levels of ROS, which can trigger the cell to undergo apoptosis by DNA damage or directly act as second messengers in the apoptotic

pathway. Buttke and Sandstrom (1994) found that apoptosis in lymphocytes is often mediated by ROS, considering that already low concentrations of peroxides can initiate apoptosis. Therefore, dRib represents a useful tool to generate intracellular oxidative stress in lymphocytes (Barbieri *et al*., 1994; Eckert *et al*., 1998). In the present thesis, it was found that lymphocytes derived from old mice are more vulnerable to dRib-induced apoptosis than lymphocytes from young mice. Proposing that dRib exhausts the glutathione pool, cells that generate more ROS should be more prone to apoptosis. Since apoptosis is significantly elevated in dRib treated cells from elderly mice, one can conclude that lymphocytes derived from old animals as compared with young, either generate more ROS during metabolism or are more sensitive to ROS. However, a higher sensitivity of old animals to dRib can not be excluded.

Several *in vitro* studies using primary neurons in tissue culture have already shown that EGb761 can protect from oxidative stress-induced cell death (Bastianetto *et al*., 2000b; Chen *et al*., 1999; Ahlemeyer *et al*., 1999; Ni *et al*., 1996). The presented results confirm and extend these findings by demonstrating that the protective effect of EGb761 is (1) also seen in fully differentiated lymphocytes *in vitro* and (2) can also be observed after *ex vivo* treatment where it seems to take place at lower concentrations. Moreover, these experiments reveal that EGb761 treatment has a higher protective effect in cells derived from elderly animals and that the degree of protection is similar *in vitro* and *in vivo.* In paralell, our laboratory demonstrated that dRib enhances significantly the levels of ROS in peripheral lymphocytes isolated from aged humans (findings of S. Leutner published in: Schindowski *et al*., 2000). Therefore, one might speculate that the percentage of prevention by EGb761 is greater, as more radicals generated by dRib can be eliminated. Even more important, apoptotic mechanisms needed for the physiological elimination of cells (basal apoptosis) were not affected by the EGb761 treatment. This fact may be relevant to the physiological role of programmed cell death in carcinogenesis, development, and homeostasis of immune system. However, “pathological apoptosis” e.g. apoptosis induced by an additional stimulus, which seems to mimic enhanced oxidative stress in pathological conditions like Alzheimer disease, can be significantly prevented by Ginkgo treatment. Thus, the findings not only demonstrate that enhanced vulnerability to oxidative stress-induced apoptosis is a common feature of aged lymphocytes, but also that EGb761 might specifically interfere with this pathological mechanism, leaving physiological aspects of programmed cell death rather unaffected.

Based on these findings, lymphocytes seem to represent a suitable model system for monitoring drug effects on cell death mechanisms in further preclinical and clinical studies using EGb761 or other antioxidant drugs.

Immunogerontology

In order to explore the rationale for incraesed apoptosis and several other altered features in PBMC of AD patients, T cell functionality should be assesed in physiological aging first to compare these findings with impaired changes evident in AD.

The incidence and severity of infectious disease is increased in the elderly. The relative mortality rates of many infectious diseases in the elderly are more than twice those of the young (Yoshikawa, 1997). Autopsy data on the very old suggest that the accepted prime age-associated causes of death in the "younger old" (ie. cardiovascular, cancer) do not necessarily apply to the very old. Studies from Leiden, Geneva and Tokyo have found the prime cause of death to be infectious disease in the over 80´s (however, whether these were really opportunistic infections is still not clear). An extensive study on major causes of death in Japan suggests that unlike those causes showing deceleration or neutrality with advancing age, those showing acceleration in old age (ie. pneumonia, influenza, gastroenteritis, bronchitis) mostly involve infectious agents (Horiuchi and Wilmoth, 1997).

The decline of immune function with aging has been a subject in various studies, reviews, and comments. However, unfortunatly most studies focus on only one event after mitogenic activation, so that a novel whole blood activation assay was established in this thesis.

T lymphocyte-mediated immunity includes the primary immune response, effector functions and immunological memory. *In vivo* the primary immune respons begins usually in the peripheral lymphoid organs when naive T cells are stimulated by professional antigen-presenting cells (APCs). This step provides cell-cell contact, antigen recognition and costimulatory signals and results in activation into effector cells and in generation of memory cells, those cells that are not eliminated to teminate immune response. Reexposure (re-call) of memory T cells to same or similar (cross-reactive) antigen causes a rapid effector response.

The T cell receptor complex (TCR) on T lymphocytes is involved in antigen recognition is composed of a number of glycoproteins. The TCR is a heterodimer, usually (on 95% of all T cells) consisting of an α and a β chain, linked via a disulphide bond. These molecules are structurally related to immunoglobulins, having a variable (V) and constant (C) region. The V

region forms a domain that contains the antigen-binding site of the T cell receptor (Weir DM, 1999). The TCR complex is responsible for the recognition of specific mjor histocompatibility complex (MHC)/antigen complexes and will be different for every T cell clone. However, other membrane molecules are needed to form the functional TCR. On of these is the CD3 complex, that is present on all T cells and is involved in proper signal transduction through TCR. Cytoplasmic residues are susceptible to phosphorylation. CD4 and CD8 molecules are necessary for functional signaling and restrict binding of T cells to MHC I- (CD8) or MHC II-positive cells (CD4).

TCR engagement, that was mimicked here by the lectin PHA-L that is believed to be quite similar to that taking place in the organism, results in phosphorylation of tyrosine-residues mediated by Src and Zap-70-family members (Weiss and Littman, 1994), followed by activation of Ras and Raf/MAP kinase cascade and PLC-γ (phospholipase-γ) that interacts with PKC (protein kinase C). Gene expression is induced by the rapid inactivation of IκB-α and subsequent nuclear migration of active NF-κB dimers (Kuo and Leiden, 1999). Ras/Raf/MAP kinase activation leads to oligomerization of Fos and Jun that form the transcription factor AP-1. In addition, IP_3 concentration raises due to PLC activity and proceeds to release of Ca^{2+} from intracellular stores. Elevated Ca^{2+} levels initiate the phosphatase calcineurin, which dephosphorylates the transcription factor NF-AT, thereby becoming active (Janeway and Travers, 1995). Treatment of lymphocytes with phorbol ester (PMA) plus Ionomycin bypasses the TCR engagement by direct interaction of the PMA with PKC and by increasing the intracellular Ca^{2+} levels due to ionophore Ionomycin. Altered T cell functionality due to impaired TCR function and/ or decreased TCR-complex expression should be avoided by PMA plus Ionomycin regimen (see summary in Figure 54).

A significantly decreased number of total T lymphocytes in CD3 and in the CD4 and CD8 subsets in aged humans was observed. However, the CD4/CD8-ratio remained unchanged with aging. The enhanced incidence of apoptosis seen in physiological aging might be associated with the decreasing number of $CD3^+$ cells with advanced age. A lack of T cells may be one of the numerous causes of immunosenescence, since fewer cells providing immune function are available to maintain immune homeostasis. In agreement with our findings, a dramatical decrease of lymphocyte count has been observed during the last 3 years before death in a prospective study (Bender *et al.*, 1986).

Soluble CD8 receptor is a marker for T suppressor cell activation (Lenkei *et al*., 1998). Reduced density of CD8 receptor on T suppressor cells from elderly could be related with increased levels of soluble CD8. Interestingly, in neurological disorders like DAT increased levels of soluble CD8 are involved as well (Singh, 1994).

Due to thymic involution, however, the number of untouched CD4 and CD8-cells declines with increasing age and with the number of infections during life. Unprimed cells carry the CD45RA isoform of the CD45-receptor, while memory cells express the CD45R0 epitope (Vitetta *et al*., 1991). However, no significant changes of the CD4/ CD8 ratio with aging were found (O'Leary *et al*., 1988; Yen *et al*., 2000). This is an interesting fact as some neurological disorders (DAT and MS, for instance) show increased CD4/CD8-ratios (Shalit *et al*., 1995; Crucian *et al*., 1995).

Mitogenic activation of T cells leads to the phosphorylation and activation of $p56^{lck}$ kinase. $p56^{lck}$ associates with CD4 and CD8 costimulatory receptor molecules (Abraham, 1991). Here, it was shown that tyrosine phosphorylation was age-related decreased in $CD4^+$ cells. $CD8^+$ lymphocytes display insignificantly reduced phosphorylation ratios. Recently, an altered association of $p56^{lck}$ with coreceptors such as CD4 was found in the elderly (Tinkle *et al*., 1998), suggesting that alterations in $p56^{lck}$ tyrosine kinase and its association with CD4 may underlie lowered T cell function during aging.

Moreover, in earlier studies of our laboratory lowered Ca^{2+} mobilization after mitogenic stimulation with PHA-L in lymphocytes derived from aged individuals (Eckert *et al*., 1997a; 1997b; 1998b) was determined.

CD69 was formerly known as activation inducer molecule (AIM) and is a 60-kDa glycosylated costimulatory molecule for T lymphocyte proliferation, which is expressed after ligation of CD3/T cell receptor complex. Although the physiological role of CD69 is still unknown, crosslinking of CD69 induces proliferation. Expression of CD69 in T cells is dependent on appropriate activation of several intracellular signaling pathways including IP_3 production, increases in intracellular Ca^{2+} and the activation of Ras/Raf-1/MAP kinase pathway (Perfetto *et al*., 1997). In concordance with Rutella et al. (Rutella *et al*., 1999), this thesis revealed that $CD8^+$ cells display more CD69-expression after PHA-L activation for 4 hours than $CD4^+$. $CD4^+$ and $CD8^+$ lymphocytes derived from aged persons had reduced CD69 surface expression compared to young ones. After treatment with phorbol ester and Ca^{2+} ionophore, the expressed amount of CD69 was significantly higher than after TCR engagement in both age groups. Moreover, a significant age-related difference in expression

of CD69 was evident when bypassing the TCR complex with PMA and Ionomycin. These data indicate that impaired expression of the activation marker CD69 in aging by engagement and even by bypassing the TCR. However, reduced activation of lymphocytes from elderly humans seems therefore not to be only due to lower expression of CD3 and/ or followed by reduced signal transduction (Fulop *et al*., 1995) after TCR ligation, but also age-related diminished transcription might be involved in our findings (Whisler *et al*., 1996).

Many earlier reports using different techniques to determine serum levels of cytokines indicate that the lymphocyte's response to mitogens is impaired in aged subjects (Bruunsgaard *et al*., 2000a; Nagel *et al*., 1988; Murasko *et al*., 1986). In this study, we could only detect a tendency of lymphocytes from young donors to produce more IL-2. However, due to large standard derivation we could not show significant age-related differences with this method. The relevance of decreased production of IL2 (Barcellini *et al*., 1988; Rabinowich *et al*., 1985) in aging, the major growth factor for T cells, remains unclear. However, this thesis revealed no changes in CD25 (α-chain of the IL2 receptor) expression with aging. The role of interferons in aging is not clear yet. Decreased levels of interferons (IFN-α and –γ (Miller, 1980; Lio *et al*., 2000)) were found by some laboratories while other investigators determined increased levels (Fahey *et al*., 2000; Sakata-Kaneko *et al*., 2000; Yen *et al*., 2000). In addition, our data are in concordance with the latter showing that activated T cells of both subsets from elderly individuals produced more IFN-γ than from young controls. The serum concentration of the inflammatory cytokine TNF-α increases with aging (Bruunsgaard *et al*., 2000b). However, it remains to be elucidated which cells secrete more TNF-α in the elderly. An increased activity of monocytes and macrophages (Sadeghi *et al*., 1999; Han *et al*., 1995) or NK cells (Solana and Mariani, 2000), observed in aged individuals, could be responsible for elevated levels of TNF-α as well. Moreover, in neurological disorders that are related to neuroinflammation and increased activity of glial cells, elevated levels of TNF-α and IL6 are detected as in DAT (Singh, 1994) and in parkinsonism (Dobbs *et al*., 1999).

A decreased amount of transcription factors (AP-1, NFκB and NFAT) have been observed in aged individuals (Trebilcock and Ponnappan, 1996), resulting in fewer transcription of genes after mitogenic activation. Since not only protein levels but as well mRNA levels of IL2 are decreased in aging (Barcellini *et al*., 1988; Nagel *et al*., 1988), reduced activation of transcription factors surely is another aspect in immunosenescence.

Decline of immune function with increasing age is not restricted to T lymphocytes: several groups reported of impaired B cells function (Burns *et al*., 1993; Paganelli *et al*., 1992; Antonaci *et al*., 1985; Hijamns *et al*., 1984). However, we could show that aging effects $CD4^+$ cells that interact with B lymphocytes. A defective T helper activity might contribute to an altered B cell functionality and could account as one aspect for the altered humoral activity associated with aging (Guidi *et al*., 1998). Natural Killer lymphocytes are supposed to be involved in immunosurveillance against tumors and in viral defense. Their age-related changes in activity are still controversly discussed through literature (Murasko *et al*., 1986b), but most studies show increased activation of NK cells with advancing age. In the present thesis a significant correlation with donor's age of the percentage of NK cells within the lymphocyte population was determined.

However, despite the findings discussed above, it remains the case that the precise clinical relevance of T cell immunosenescence is hard to define. Indeed, there are studies suggesting that the NK status of subjects may also be important or more important than T cells. Ogata *et al*. reported that not the numbers but the functional activity of NK cells was a parameter correlating with death (due to infection) in the follow-up period for 44 elderly subjects (1997). Moreover, inclusion of T cell functional parameters has been shown to predict mortality in a Swedish prospective study (Ferguson *et al*., 1995).

In most aging studies in human, individuals > 60 years are commonly considered "old". Longitudinal studies are required to establish the critical changes within the immune system and what may be associated with "healthy aging". There may be surprises, as in the recent report that in the over-85´s, high cholesterol levels were associated with greater survival over a ten-year follow-up (Weverling-Rijnsburger *et al*., 1997). Studies of the very old (i.e. the survivors) may also be informative. Centenarians may be considered to be that very small proportion of successfully aged individuals. Healthy centenarians are indeed found to have well-preserved immune functions, much more similar to the "young" immune system than average for less extremely old donors. Thus, as summarized by Franceschi (Franceschi *et al*., 1995), T cell proliferative responses are well-maintained (albeit taking place more slowly), the T cell repertoire still contains all Vß families, hematopoiesis is maintained, autoantibodies are absent, and interestingly, there is a high level of lymphocyte genomic stability (low spontaneous breaks etc., which otherwise are thought to increase with age in average, non-centenarian, donors (King *et al*., 1994)).

The composition of the T cell compartment changes during aging as a result of antigen exposure, clonal expansion and contraction, regulatory T cell interactions and memory cell formation, and changed thymic output. Thus, many studies have addressed the question of whether the numbers and proportions of T cells and other lymphocytes are altered during aging. In general, alterations in numbers of T cells are relatively small and may be influenced by underlying disease (Remarque and Pawalec, 1998), although not all studies agree on this (Huppert *et al*., 1998). It is probably true to say that all in all, the consensus is that age-associated alterations in T cell numbers are minimal (Pawelec, 1999). However, age-associated changes in the proportions of T cell subsets have been repeatedly documented in rodents and humans. Mice seem to show a relative loss of CD4 cells in the blood with age compared to CD8 cells (Miller, 1997), and the same tendency may be true in humans, although the small reduction in CD4 cells is also associated with poor nutritional status (Masari and Lesourd, 1998). Accumulating evidence suggests that even where the overall numbers of T cells are not obviously changed, and even within a particular T cell subset as characteriazed above, there may be further age-associated alterations in terms of the specificities of the TCR expressed. For example, whereas the TCR2 repertoire of CD4 cells in mice was initially reported not to be obviously changed compared to young cells, the CD8 repertoire was found to be markedly altered in early reports, suggesting expansion of a small number of CD8 cells during aging (Callahan *et al*., 1993). In human, early reports also indicated that it was the CD8 cells rather than the CD4 cells which were primarily affected in this way. Thus, in $CD8^+$ but not $CD4^+$ T cells, up to 30% of the entire population may consist of oligo- or even monoclonal cells expressing the same TCR-Vß markers (Possnett *et al*., 1994). Within the CD8+ cells, these oligo- or monoclonal populations are prevalent in the CD28-negative subset (Possnett *et al*., 1994) and the CD57-positive subset, which essentially overlaps with the CD28-negatives (Battliwalla *et al*., 1996). It is interesting to note that it is this $CD28^-CD8^+$ subpopulation which was identified many years ago as containing so-called "suppressor" cells (Damle *et al*., 1983). These data may explain the observation that alterations in proportions of different T cell subsets may also be more marked in CD8+ than in CD4+ cells of aged humans (Hoshino *et al*., 1993). However, this phenomenon seems not be absolutely limited to CD8 cells (Crisi *et al*., 1996).

Depressive illness and affective disorders are associated with immune and cytokine alterations (Zaharia *et al*., 1993; Irwin *et al*., 1987). Reductions of lymphocyte response to PHA and *in*

vitro IL2 production are effects of repeated stress in rats (Batuman *et al*., 1990). The neurodegenerative disorder itself has proven effects on central and peripheral immune competent cells as well (Eckert *et al*., 2001a; Garibaldi and Zhang, 1999). In this thesis it was shown that the CD4/CD8-ratio of Alzheimer's disease patients significantly correlates with disease's severity (measured as cognitive decline). Since there is no change of the ratio of T helper to T suppressor cells associated with aging, the impact of the pathological increase of $CD4^+$ cells and the decrease of $CD8^+$ cells seen in DAT should be further explored.

In conclusion, the age-related modifications of human peripheral lymphocytes are important in order to elucidate additional changes taking place in neurological diseases of geronto-psychiatric patients.

Increased apoptosis of peripheral lymphocytes from AD-patients

Earlier findings of our group indicate enhanced apoptosis in PBMC from AD patients compared to elederly non-demented controls (Eckert *et al*., 1998; 2001a). One aim of this thesis was to verify these results with different methods and to investigate the underlying mechanisms. In order to understand the specific contribution of AD to the enhanced susceptibility of lymphocytes to apoptosis it is of particular importance to determine the cellular origin of the increased cell death. Apoptosis is regulated by genes that are expressed in neuronal and non-neuronal cells. Accordingly, the enhanced vulnerability of lymphocytes to apoptosis in AD patients might be due to a dysregulation of apoptosis-relevant genes or related mechanisms, lowering the sensitivity threshold for apoptosis.

Basal, spontaneous and Fas-L induecd levels of apoptotic nuclei, analyzed with PI, were significantly elevated in PBMC in AD. So, the findings by Eckert *et al*. (1998; 2001a) were confirmed with a different cohort of AD patients and controls and with another method (flow cytometry) to determine apoptosis, indiacting that the older findings were not limited to this patient cohort or nucleosome ELISA.

Moreover, there was a significant correlation with the severity of the dementia: the lower the cognitive performance of the patient was, the higher were the percentage of apoptotic PBMC. This defect itself appears not to be sufficient to lead to a pathological leukopenia in AD patients (see Table 4), probably since lymphocyte can be rapidly restored.

These data raises the questions why do PBMC of AD display more basal apoptotic cells and, moreover, why are the cells more vulnerable to spontaneous and Fas-L induced cell death, as well. What evoiremental conditions or genetical factors make the cells going into apoptosis?

First, it was essential to find out, if all cells of the PBMC (T-, B-, and NK-lymphocytes and monocytes) were effected or only some subsets show this impaired behaviour.

Monocytes show *in vitro* an adherent form to plastic vessels, indicating that they are hardly detectable by flow cytometry, using polypropylene (PP) tubes. Staining with CD14 mAb, as a marker for myelotic cells, revealed that monocytes can be found in PBMC by flow cytometry only in traces, though monocytes/ macrophages are obvious visible in the microscope, before subjecting to the PP tubes (data not shown). Therefore, the flow cytometric-analyzed PBMC consist mainly of lymphocytes, namely T-, B-, and NK-lymphocytes. For these reasons, samples of AD patients, young and elderly controls were analyzed for apoptotic cell death for each lymphocytes subset. All subsets showed typical age-related changes, however, those changes were most prominent in T cells. In AD, elevated levels of basal apoptosis were evident in T and NK lymphocytes, B cell apoptosis was not altered in demented persons. Moreover, the sum of apoptotic cells in all subsets covers the amount detected with PI taking into account the percentage content in the PBMC (apoptosis in T cells of elderly controls: 1.08%; apoptosis in B cells of elderly controls: 5.03%; apoptosis of NK cells in elderly controls: 1.47%; apoptosis of T cells in AD patients: 1.54%; apoptosis of B cells in AD patients: 4.89%; apoptosis of NK cells in AD patients: 2.78%. → controls: (1.08% x 0.6596) + (5.032% x 0.1088) + (1.47% x 0.2436) = 1.618% apoptotic cells in PBMC by 7-AAD staining in elderly controls. AD: (1.54% x 0.6979) + (4.89% x 0.1034) + (2.78% x 0.2332) = 2.229% apoptotic cells in PBMC by 7-AAD staining in AD). The following table shows the determined apoptosis of PBMC with PI and the calculated data for PBMC determined with 7-AAD. It is evident that there variations between both methods. It should be noted that only a few identical individuals (patients and controls) were included in both studies. Most subjects were different, a propable reason for the deviation. However, AD-patients show elevated levels by both flow cytometric methods and by nucleosome ELISA (Eckert *et al*., 1998; 2001a).

elderly control		AD patients	
PI (PBMC)	7-AAD (Σ of subsets)	PI (PBMC)	7-AAD (Σ of subsets)
1.35%	1.62%	2.37%	2.23%

Table 14. Basal apoptosis in PBMC from AD patients and elderly controls.

Altered apoptosis in subsets of AD patients

It can be assumed that T cells provide the major impact on the apoptotic properties of PBMC from AD patients, since they showed elevated levels in basal, spontaneous and Fas-L induced apoptosis (due to small number of included volunteers failed to be statitstically significant). Moreover, the percentage of basal apoptotic cells correlated significantly with the cognitive decline of the patients. NK lymphocytes display elevated levels of apoptotic cells under basal conditions, nevertheless, spontaneous and agent-induced apoptosis was not altered and there was no correlation with the severity of the cognitive decline. Moreover, looking at the subset distribution between AD patients and controls, there was an increase in the CD4/CD8-ratio. For these reseons, the T cells were the subject of the following studies, though there are mechanisms evident that affect NK lymphocytes, as well. As another fact, there is much more literature and knowledege about apoptosis and its mechansims of T cells than of NK lymphocytes.

The altered CD4/CD8-ratio gave the rationale to further investigate the apoptosis of the T cell subsets, CD4 and CD8 T lymphocytes, by three color flow cytometry with 7-AAD. This determination showed that in fact the cell death function of the T cells is impaited in AD. CD4 lymphocytes display elevated levels of apoptosis compared to elderly controls. In contrast, in the investigated cohort of patients, no significant changes compared to controls were evident in CD8 cells, but interestingly, correlation with the cognitive performance of the patients was detected, indicating that CD8 T cells became more vulnerable to apoptosis as the disease proceeds. Earlier findings indicate that all $CD4^+$ lymphocytes are positive for Aβ whereas only the majority of $CD8^+$ lymphocytes show Aβ-immunoreactivity (Suh *et al.*, 1996). Assuming that Aβ induces oxidative stress and apoptosis in cells, this could explain some aspects of our results. The overall amount of APP transcript in PBMC is not altered in AD (Ledoux *et al.*, 1995; Ebstein *et al.*, 1996). However, APP is upregulated upon mitogenic stimulation (Bullido *et al.*, 1996; Fukuyama *et al.*, 1994; Ledoux *et al.*, 1993; Monning *et al.*, 1990).

The data concerning apoptosis of peripheral lymnphocytes in AD can be summarized as follows: (1) Apoptotic levels are elevated in PBMC from AD patients and correlate with the MMSS. (2) T- and NK- lymphocytes are the targets that show enhanced apoptosis in AD, B cells are not altered. Only T cells show a relation to the MMSS. (3) The CD4/CD8-ratio is increased in AD patients and there is a correlation with the MMSS. (4) CD4 lymphocytes

dispaly elevated apoptotic levels without correlation to the MMSS while CD8 lymphocytes do not show significant altered levels, but correlate significantly with the MMSS.

Influence of ApoE genotype

30 patients were *apo*ε-genotyped. 2 were ε2/ε3, 9 were ε3/ε3, 16 were ε3/ε4, and 3 were ε4/ε4, indicating that in our cohort of demented patients AD was associated in 63% with ε4. In this study, patients that carry *apo*ε4 displayed elevated levels of apoptotic cells compared to non-*apo*ε4 patients. Unfortionally, the number of patients is too small to determine a proper statistical analysis for this finding. However, enhanced numbers of apoptotic nuclei were found in whole PBMC as in CD4 and CD8 positive cells.

It was shown that ApoE suppresses glial activation, ApoE4 has a lower activity compared ApoE3 and ApoE2 (Laskowitz *et al.*, 1997). Activated glia in AD is known to secrete inflammatory molecules that mostly can pass the blood brain barrier. In addition, sera from AD-patients with ApoE4 genotype induced microglial activation in vitro, indicating the presence of factors (e.g. cytokines) that mediate immune activity (Lombardi *et al*., 1998). Perhaps, these factors are responsible as well for the enhanced apoptosis observed in this thesis.

The above mentioned findings raised the question for the objective of T cells to undergo apoptosis in AD. Apoptosis of immune competent cells is a comon feature to maintain leukocyte homeostasis. For instance, an immune response like an acute infection stimulates antigen-specific T and B lymphocytes to get activated and proliferate. In order to teminate the immune response, lymphocytes undergo apoptosis in absence of appropiate growth factors (e.g. IL-2 for T cells) to eliminate the accumulated lymphocyte clones. Very littele of these cells rescue for not yet fully eluciadated reasons as memory cells. This kind of physiological apoptotic cell death was termed AICD (activation induced cell death).

Unfortunatley, it is not as easy to determine why the cell underwent apoptosis as it is do differntiate between apoptosis and necrosis. It can be easily deteced wheter a cell dispalys some apoptotic features like DNA-fragmenations, caspae-activity, surface presence of phosphatidylserine (detectable by Annexin V-staining) etc. But it is still very complex to determine the rationale for cell death, if it was not induced under *in vitro* conditions. Rather

comparable to someone commiting sucide without a farewell letter: only the fact of commiting sucide is apparent, the reasons remain unknown.

If the found apoptosis were due to incraesed activation of the T cells, a possible result of the chronic neuroinflammation ongoing in AD, subsequent activation of T cells should be altered. Therefore, T cells from AD patients were activated via their T cell receptors (TCR) using PHA-L and TCR were additionally bypassed using PMA and Ionomycin.

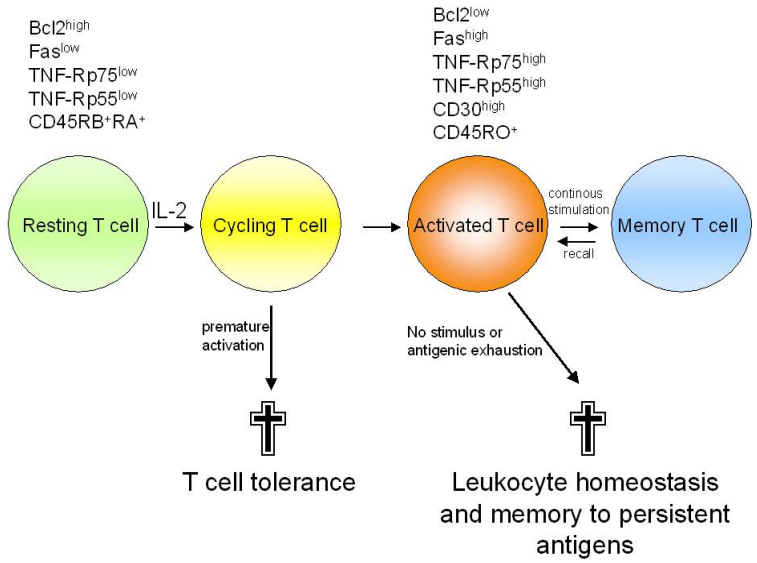
To determine changes specific for the subsets, all analyszes were done by three color flow cytometry to distinguish between CD4 and CD8 lymphocytes.

Altered inducibility of T lymphocytes from AD patients

Compared with elderly non-demented controls, T cells of AD patients showed increased phosphorylation of tyrosine residues and enhanced expression of early activation marker CD69 as response to PHA-L traetment. Thsese effects are not related to changes in the TCR density in contrast to physiological aging as bypassing the TCR shows the same result in CD69 expression. Moreover, both T cell subsets (CD4 and CD8) reveal this incraesed inducibility, indicating a general involvment of T cells in AD. In addition, proliferation of T lymphocytes showed a disproportionate profile from AD patients. In healthy young and aged control, $CD8^+$ T cells mainly trigger proliferative capacity. In AD, the T helper population incorporated significantly more BrdU, indicating a higher percentage of proliferating cells, while $CD8^+$ T cells revealed significantly decreased amount of cells with DNA synthesis subsequent to PHA treatment. Those changes were only evident as a tendency when PMA and Ionomycin were used for activation. The study in this thesis examining immunogerontology revealed that 'healthy' $CD8^+$ T cells provide the major impact for T cell proliferation. The lower content of proliferating $CD8^+$ lymphocytes in AD results in decreased proliferative capacity when the whole PBMC are inspected. This could explain the decreased proiliferative acticity found in PBMC from AD patients in several earlier studies (Shalit *et al.*, 1995; Lombardi *et al.*, 1999).

What are the rationale for the increased inducibility of AD T cells and the decreased proliferation of CD8 T lymphocytes from AD patients? Earlier finding in this thesis showed that T cell function decreases with aging, why do T lymphocytes derived from AD patients show apparently better function? A valuable explanation would be the generation of peripheral memory T cells in AD: Memory cells display incraesed 're-call' activity, indicating

a faster and more efficient inducibility upon TCR ligation. In the above mentioned experiments we stimulated T cells with PHA in order to induce TCR mediated activation. If in AD there is a higher percentage of memory cells in peripheral leukocyes, more T lymphocytes should show the measured signs of activation. Interestingly, $CD8^+$ memory T lymphocytes display increased Ca^{2+} mobilization but decraesed proliferation (Schlunck *et al*., 1990). Earlier result (Adunsky *et al*., 1991; 1995; Ibarreta *et al*., 1997) could show enhanced Ca^{2+} influx im PBMC from AD patients but decreased Ca^{2+} increase in $CD4^+$ T cells (Grossmann *et al*., 1993) and here we demonstrated decraesed proliferation of T cytotoxic/supressor cells in AD. As mentioned in the introduction, chronic neuroinflammation in the AD brain is a major feature in AD pathology. In this thesis it could be shown first in detail that peripheral lymphocytes from AD patients seem to react with altered function to this chronic stimulus. There are some hints that this encanced inducibility subsequently to mitogenic stimulation might reflect a generation of memory T lymphocytes in AD patients. These data do not support the hypothesis of Antonaci *et al*. (1990) that claims AD to be a distinct entity of immunosenescence with decreased T helper function.



The data found in this thesis support the assumption that the activation of T cells and a possible generation of memory cells (summarizied in the scheme on the left) is a process that seems to occur long before the clinical onset of AD. In addition, it seems to be mostly finished in present AD patients since we could not found any relation with the cognitive decline in those experiments. Apoptosis of $CD8^+$ T cells, however, did show significant correlation with the MMSS in patients suffering from AD, indicating that here are processes evident that seem to be still ongoing, even after the clinical manifestation of AD.

With our findings we can postulate that AD involves the peripheral immune system, but is definitively not an advanced type of immunosenescence.

Is the observed enhanced apoptosis in AD a consequence of chronic activation of the T cells, the so-called AICD (activation-induced cell death)? Generation of activated T lymphocytes (possibly memory T cells) in AD as a result of chronic neuroinflammation.

Here, we could demonstrate much data that supports the hypothesis of changes in the peripheral immune system in AD. Those chanegs could be responsible for increased apoptosis of T lymphocytes in the disease. Several other groups have found similar results indicating altered lymphocyte function. However, most of the studies are lacking a meaningful explanation for these observations.

Earlier results concerning impaired funtion of AD lymphocytes

Increased apoptosis

Enhanced levels of apoptotic PBMC from AD patients were found after 72 hours *in vitro* culture in the presence as well as in the absence of α-CD3-stimulus or hyperthermia. Interestingly, the number of Fas-R (CD95) increased on CD4 and decreased on CD8 cells following this treatment in AD, but not in vascular dementia or Parkinson's disease (Lombardi *et al*., 1999). The presence of an apoptotic factor in Alzheimer plasma was proposed (Maesaka *et al*., 1999) due to observations, that AD plasma increased apoptosis fourfold in a cultured cell line.

Altered Calcium homeostasis

According to the calcium hypothesis of brain aging, disturbances of free intracellular calcium homeostasis $[Ca^{2+}]_i$ play a key role in pathology of Alzheimer's disease (Pascale and Etcheberrigigaray, 1999). Earlier data from neuronal tissue culture support the contribution of Aβ to neurodegeneration in AD, probably by disruption of the intracellular Ca^{2+} regulation (Hartmann *et al*., 1994a; 1994b; 1994c, Müller *et al*., 1996b).

Elevated basal levels of free intracellular Ca^{2+} in PBMC from AD patients were found (Adunsky *et al.*, 1991; 1995; Ibarreta *et al*., 1997) when compared to elderly controls, patients with multi-infarct dementia or patients suffering from depressive illness. However, only some investigated patients showed levated basal levels, but no one out of the control or multi-infarct dementia group. In addition, B-lymphoblasts from AD patients revealed elevated basal levels, as well (Ibarreta *et al*., 1997). Increased concentrations of intracellular Ca^{2+} could indicate

that the lymphocytes are in an activated state. Moreover, treatment with super-optimal doeses of PHA (100 µg/ml) revealed increased Ca^{2+} influx in PBMC in AD (Adunsky *et al*., 1991; 1995). In concordance with our results concerning mitogenic activation, the authors could not detect any correlation between the cognitive grade and the amount of Ca^{2+}.

The PHA-induced Ca^{2+} response in circulating human lymphocytes of healthy volunteers is affected by Aβ and its fragment $A\beta_{25-35}$ in a fashion similar to its effects on central neurons, whereas no effect of Aβ on receptor-activated Ca^{2+} response in neutrophils were found. The amplifying effect of Aβ on Ca^{2+} signalling was significantly reduced in lymphocytes from AD patients (Eckert *et al*., 1995). Moreover, Ca^{2+} responses to $A\beta_{25-35}$ were not different between early- and late- onset AD patients. These findings indicate that the sensitivity of the lymphocyte for the effects of Aβ is reduced in a high percentage of patients with probable or possible AD. As possible explanation a similar reduction of the sensitivity of the lymphocyte membrane for the fluidity-decreasing properties of Aβ was observed. Finally, the inhibition of the PHA-induced Ca^{2+} response by tetraethylammonium (TEA) was lower in the AD group compared to aged controls. This could suggest the presence of a K^+ channel dysfunction on AD lymphocytes, as it has been shown on skin fibroblasts of AD patients (Etcheberrigaray *et al*, 1994). Interestingly, in AD B-lymphoblasts, the Ca^{2+} influx was increased after addition of $A\beta_{25-35}$ or αIgM (Ibarreta *et al*., 1997).

A specific stimulation with a monoclonal α-CD3 antibody has been reported to elict an increased (Adunsky *et al*., 1991) or a decreased (Grossmann *et al*., 1993) intracellular Ca^{2+} concentration in AD lymphocytes in comparsion with control cells. It should be taken into account that the study from Grossmann and co-workers has been performed in $CD4^+$ lymphocytes thus non-CD4 population (e.g. CD8 T cells) might be responsible for the incraesed Ca^{2+} response shown in the previous studies.

Impaired proliferation

A marked reduction in the proliferative ability of AD PBMC upon mitogenic stimulation was observed (Shalit *et al*., 1995; Lombardi *et al*., 1999). No data exsits throuhout literature concerning proliferation of CD4 or CD8 cells in AD.

Remarkedly, Aβ stimulates lymphocytes to express IL-2 receptor and to proliferate. However, lymphocytes from AD patients do not show incraesed proliferation upon Aβ exposure (Trieb *et al*., 1996). The authors speculate that this might reflect T cell anergy in AD, due to chronic contact to Aβ and suggest that autoreactive lymphocytes with specificity for metabolic

products of APP occur in healthy individuals. These cells may be of relevance for the elimination of potentially amyloidogenic substances. This mechanism might be impaired in AD patients.

Altered cytokine secretion

Basal expression of CD25, a part of the IL-2-receptor, was increased on CD4 and CD8 T cell in patients suffering from AD (Ikeda *et al*., 1991b). Following stimulation with PHA, the expression of IL-2-R was markedly reduced in AD (Shalit *et al*., 1995). In addition, the same study showed a significant elevation of secreted IL-2 subsequent to PHA treatment. Soluble serum levels of IL-2 and TNF-α receptors were increased and there was a negative coorelation between sTNF-α-R and MMSS (Leblhuber *et al*., 1998).

When stimulated with lipopolysaccharide (LPS) that activates monocytes and macrophages, levels of IL-1β, IL-10, TNF-α and IL-6 were significantly increased in whole blood cultures of AD patients but not of vascular demented patients (Lombardi *et al*., 1999). Moreover, when the patient group was divided according to the disease's stage, the average level of IL-1β, TNF-α, IL-6 and IL-10 increased in severe dementia (MMSS: 10 ± 3).

The binding of IFN-γ and IL-6 to T lymphocytes was reduced in AD (Bongioanni *et al*., 1997a; 1998). The number of functional TNF-α-receptors was increased on T cell of Alzheimer's disease patients (Bongioanni *et al*., 1997b).

Altered expression of activation markers

An increase in HLA-DR expresssion was observed in AD and was more obvious in later stages of the disaese (Shalit *et al*., 1995). Ikeda and co-workers (1991a; 1991b) found out that HLA-DR was increased on CD4 and on CD8 T lymphocytes. However, other studies (Lombardi *et al*., 1999) and in this thesis only a tendency of increased levels was observed.

The number of $CD8^+$ cells expressing CD28, a TCR-related co-stimulatory molecule, or CD11b (Mac-1, Integrin on NK lymphocytes) was significantly decraesed in AD (Lombardi *et al*., 1999), indicating imapired suppresor/cytotoxic T cell and NK lymphocyte function. The activation markers CD26 and CD38 were incraesed on CD8 lymphocytes (Lomabrdi *et al*., 1999). In $CD4^+$ cells, the percentage of CD25 and CD28 were significantly increased (Lombardi *et al*., 1999). CD28 mediates activation of naive T lymphocytes. The finding of Lombardi and co-workers supports the disproportionante proliferation measured in CD4 and CD8 T cells, possibly leading to incraesed CD4/CD8-ratio in the present thesis in AD. All this

observations were specific for AD patients and could not be found in vascular dementia or Parkinson's disease (Lombardi *et al*., 1999).

The number of peripheral benzodiazepine (BDZ) receptors is decreased on lymphocytes from patients suffering of AD (Bonngioanni et al., 1997c). The physiological role of the peripheral BDZ receptors is still unclear, however, a strong relation to immunemodulating properties, such as stimulation of human monocyte chemotaxis (Ruff *et al*., 1985), modulation of cytokine release (Bessler *et al*, 1992), enhancement of T cell-dependent antibody response (Zavala *et al*., 1987) and energy metabolism (Anholt *et al*., 1986) is speculated.

Distribution of lymphocyte's subpopulation

An increase of CD4 and a reduction of CD8 lymphocytes in relation to the stage of the disease was found by Shalit *et al*. (1995). A decreased CD4/CD8-ratio was additionaly found by other groups (Singh, 1990; Pirrtilä *et al*., 1992; Lombardi *et al*., 1999). They showed that this alteration was mainly due to a decrease of CD8 cells in AD. No chnages concerning CD4 and CD8 levels were found in vascular dementia or patients with Parkinson's disease (Pirrtilä *et al*., 1992; Lombardi *et al*., 1999). Moreover, the percentage of $CD7^+$ cells, an antigen expressed throughout lymphocyte differentiation, was reduced in AD (Lombardi *et al*., 1999).

In addition, it was recently demonstrated that Down's Syndrome (DS) is associated with impaired T cell function as well. PHA-stimulated proliferation of T lymphocytes is reduced and cytokine produktion is altered (Park *et al*., 2000). CD4/CD8-ratio seems not to be altered in DS while the levels of HLA-DR positive T cells are increased, as well (Rabinowe *et al*., 1989).

Changes in other lymphocyte subsets

The percentage of B lymphocytes remains unchanged in AD (Pirrtilä *et al*., 1992). Moreover, B lymphoblasts of sparadic and familar AD have reduced GSH levels (Cecchi *et al*., 1999). Levels of anti-microglia (Lemke *et al*., 1999) and anti Aβ (Xu and Gaskin, 1997) antibodies are incraesed in sera of AD patients.

Interstingly, Lombardi *et al*. (1999) found decraesed levels of CD56, a marker for NK lymphocytes. In this thesis, no changes were discovered concerning pecentage of NK lymphocytes were observed. However, here NK cells were detected with a comercially available antibody mixture raised against CD16 and CD56, so that a loss of of $CD56^+$ cells only might be not observed. In addition, enhanced cytotoxic response of NK cells from AD

patients to IL-2 was found (Solerte *et al*., 1996). In addition, IFN-γ and TNF-α production is enhanced in NK cells from AD patients in correlation with their Mini Mental State Score (Solerte *et al*., 2000). There are hints that defects in protein kinase C may be involved in altered NK cell activity in AD (Solerte *et al*., 1998).

T lymphocytes in the AD brain

Activated T cells (CD25 and HLA-DR positive) were found in cerebrospinal fluid of AD patients (de los Angeles Robison-Agramonte *et al*., 1998), indicating that they do cross the blood brain barrier in AD and might contribute to damage the barrier.

Earlier reports show that detectable amounts of invaded lymphocytes were found in AD brains while remaining undetectable in normal aged control brains. Both, CD4 and CD8 antigens were identified in hippocampus and temporal cortex of AD patients (McGeer *et al*., 1989; Fiala M, 2000), however T cytotoxic/suppressor lymphocytes (determined as CD8 immunoreactivity) are more dominant in AD brains compared to CD4/T helper inducer cells (Itagaki *et al*., 1988; Rogers and Mufson, 1990). Highest staining activity was found in sites with increased signs of gliosis (enhanced GFAP and HLA-DR expression).

In MS and EAE brains a disproportionate recruitment of $CD8^+$ T cells into the CNS by professional antigen-presenting cells was found (Carson *et al*., 1999). Data exsist that activated astroglial cells selectively recruits CD8+ T lymphocytes while microglia seems to interact more likely with CD4+ T cells (Sedgwick *et al*., 1991). One can speculate that peripheral changes in T cells might reflect ongoing changes in glial cells. However, the relevance of this findings and their possible use a diagnostic marker should evaluted in further studies.

Taking together all these findings, it becomes evident that T lymphocytes of AD patients are in an activated state, a possible explanation for the incraesed apoptosis found in peripheral cells of AD patients. Most of our and other data could indicate that AD patients provide a higher percentage of memory T cells compared with non-demented aged controls. Those memory cells could be possibly the result of the chronic neuroinflammation. This hypothesis can explain most of the various findings summarized above concerning impaired activity of peripheral lamphocytes in AD.

The following figure summarizes the data of AD T cells found in this thesis and by others.

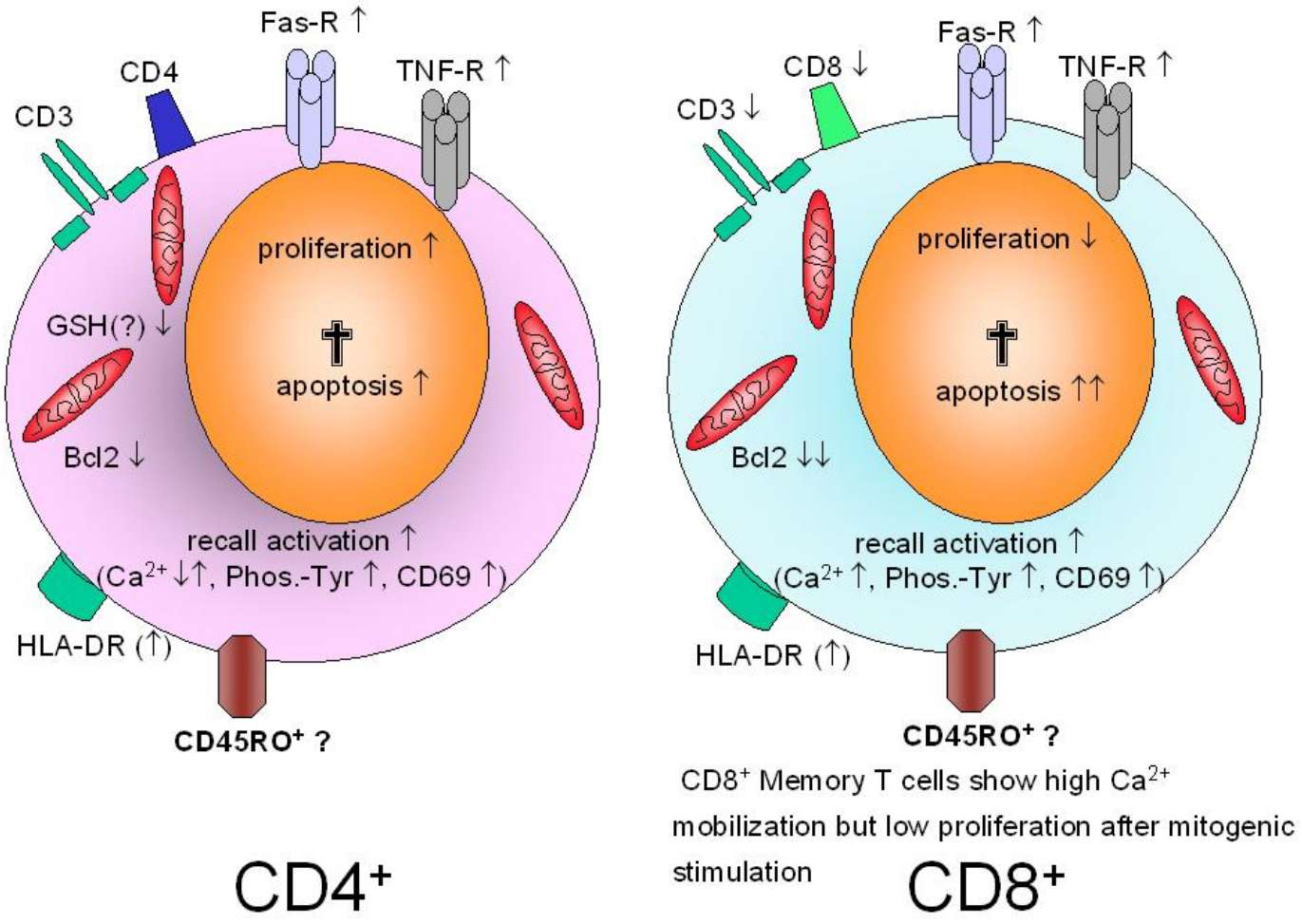


Figure 81. Summary of data observed in the present thesis and by other groups of changes of T lymphocytes in AD.

There is some evidence that memory cells (in humans: $CD45RA^-$ and $CD45RO^+$) are not quiescent long-lived cells, but represent T cell clones in a constant state of activation. It may be that unlike naive cells, memory cells do not require antigen to survive and proliferate, but only the self MHC molecule (i.e. have a lower functional activation threshold; Tanchot *et al*., 1997). Memory cells show incraesed activation and cytokine secretion upon stimulation and display incrased levels of activation markers. Interestingly and in concordance with our findings, do $CD8^+$ memory T cells show decraesed proliferation compared to $CD4^+$ memory T lymphocytes (Schlunck *et al*., 1990).
To verify this hypothesis expression of $CD45RA^-$ and $CD45RO^+$ should be determined in AD lymphocytes in further studies. Moreover, if the presence of memory T cells can be confirmed in AD, it would be rather interesting whether there were specific to plaques and their contents.

In addition, the incidence of increased levels of anti-brain antibodies in sera of AD patients supports the hypothesis that the amount of memory B cells (plasma cells) is increased in AD, as well. However, since there are not many data available on the physiological role of NK bisides their important function in natural cancer prevention, it is rather difficult to speculate on their mechanisms in neuroinflammatory diseases. Further resaerch is necessary to elucidate the roles of the B and NK lymphocytes and their impact on neuroinflammation in AD.

The changes in immune cell function in AD patients and animal models described above suggest the existence of an immunological component to the pathogenesis and progression of the disease. How might this occur? One possibility is that the immune system normally plays an important role in removing potentially toxic aggregating forms of Aβ from the brain, thereby preventing plaque formation. Evidence supporting such a mechanism comes from recent studies showing that APP mutant mice and APP/PS1 double mutant mice can be ‚immunized' by challenging their immune system with aggregated forms of Aβ (Schenk *et al*., 1999). Mice so challenged may generate antibodies against aggregated Aβ, resulting in suppression of Aβ deposition in the brain, as well as clearance of previously deposited Aβ.

Transgenic mouse models for AD

Oxidative stress, mitochondrial function, and intracellular calcium regulation are early and important contributors to either apoptotic or necrotic cell death. In the present thesis apoptosis and oxidative stress-induced cell death in lymphocytes of transgenic mice carrying either a single or multiple human Alzheimer-linked PS1 and APP mutations were investigated. Consistent with the rationale that different PS1 mutations within the same molecule cause more dysfunction and higher $A\beta_{1-42}$ levels than one alone (Citron *et al*., 1998), we found a higher accumulation of apoptotic cells in lymphocytes from mice carrying multiple PS1 or APP mutations (PS1 M5 Tg, APP-SDL Tg, respectively).

Our results concerning apoptosis in Tg animals bearing FAD related mutations additionally indicate that comparable disturbances in apoptosis regulation due to PS1 and APP mutations can be detected not only in brain but also in peripheral cells such as lymphocytes. Furthermore, lymphocytes from mutant APP or PS1 Tg mice showed an increased susceptibility to cell death compared to T cells from PS1 wt Tg mice or non-Tg littermate controls. Already basal levels of apoptotic cells were elevated in APP and PS1 mutant mice. However, spontaneous or ROS-induced in vitro apoptosis was extremely increased in lymphocytes from APP-SDL Tg mice, but not in PS1 M146L Tg mice. In contrast, necrotic-type cell death was elevated in both PS1 M146L and PS1 M5 Tg mice (Eckert *et al*., 2001b). These findings suggest that PS1 may enhance vulnerability to both types of cell death. Mainly the severity of insult, cellular homeostasis, and preexisting damage may determine the pattern of cell death to be either more apoptotic or more necrotic in nature. Additional experiments, e.g., assessing caspase-3 activation, might be needed to clarify whether cell death as observed in lymphocytes from PS1 and APP-SDL transgenic animals belongs to the apoptotic or necrotic pathway. PS1 mutations on the one hand sensitizes PC12 cells, neuroblastoma cells, or primary neurons from PS1 M146KI mice to apoptosis (Guo *et al*., 1997, 1999b; Tanii *et al*., 2000) and on the other hand make hippocampal cells from PS1 M146KI mice more vulnerable to necrotic insults (Guo *et al*., 1999a).

Very interestingly, some evidence is provided that a similarly increased vulnerability to oxidative stress-induced DNA damage is present in lymphocytes from FAD patients bearing PS1 mutations (Parshad *et al*., 1996). Whether this is solely due to an increased production of neurotoxic $A\beta_{1-42}$ by mutant PS1 or whether additional mechanisms are involved, is not known yet. Apoptosis is often mediated by oxidative stress and ROS (Buttke and Sandstrom, 1994). Our results are therefore in accordance with findings demonstrating reduced activities

of cytosolic superoxide dismutase and glutathione reductase in the brain of the same transgenic animals (Leutner *et al*., 2000) and with data from lymphoblasts of FAD patients with mutant PS1 indicating altered levels of reduced glutathione (Cecchi *et al*., 1999). They are further consistent with findings demonstrating increased vulnerability of PS1 M146KI hippocampal neurons to lipid peroxidation after exposure to Aβ (Guo *et al*., 1999).

An association between PS1 and Bcl-2 (Alberici *et al*., 1999) or Bcl-XL, another antiapoptotic member of the Bcl-2 family (Passer *et al*., 1999), has been found. Therefore, redistribution of cytochrome c, one of the best known downstream activators of the apoptotic cascade, could be one of the crucial events influenced by PS1/Bcl-2 interaction.

However, the molecular mechanisms by which FAD PS or APP mutations enhance apoptotic activity in this context are not known. One can speculate that mutant PS1 may generate molecules with proapoptotic activity or may negatively regulate the antiapoptotic effects of Bcl-2 family members, possibly by indirectly regulating the activity of Bax, finally leading to enhanced cytochrome c release in response to an apoptotic signal (Guo *et al*., 1997; Passer *et al*., 1999). One potential mechanism by which APP mutations enhance the apoptotic process could be the increased production of Aβ, which is able to induce apoptotic cell death. It has been shown that differentiated PC12 cells expressing mutant APP show an increased basal apoptotic activity (Zhao *et al*., 1997). Recently, our group could show that, in PC12 cells bearing mutant APPsw, caspase-3 activity, indicative for ongoing apoptosis, was significantly enhanced (Eckert *et al*., 2001c). Elevated caspase-3 activation has also been found in the brains of AD patients (Stadelmann *et al*., 1999; Gervais *et al*., 1999) as well as in cell lines transfected with mutant PS-1 (Kovacs *et al*., 1999; Guo *et al*., 1999). At present, it is not clear whether these effects are mediated either by Aβ-induced caspase activation, in that all FAD mutations lead to increased Aβ levels, or by more complex proapoptotic mechanisms.

Very importantly, our present findings in lymphocytes from PS1 and APP mutant transgenic mice mirror very well comparable findings in lymphocytes from AD patients shown here in this thesis.

Our mouse model shows AD-like pathology in APP- and APP-PS1-double transgenic mice. At an age of 18, 6 months, respectively, plaque formation is observed in brains of APP_{695}SDL or APP_{695}SDLxPS1 M146L transgenic mice (Moussaoui *et al*., 1999).

The transgenic mice used here are similar to the APP23 mice that were first reported to show AD-like pathology. APP23 transgenic mice used in a study by Sturchler-Pierrat and

Staufenbiehl overexpress the human APP with the Swedish double mutation only under the control of a neuron-specific promoter. Those mice show Aβ plaque formation starting at an age of six months. Peripheral amyloid deposits are not detectable, indicating that all Aβ originates from neural tissue. In addition, gliosis can be detected as early as the first small deposits become discernible. While microglia activation is largely restricted to the plaques, hypertrophic astroctyes are found also at a distance, but only in plaque containing brain regions. Degenerative processes are obvious around compact amyloid plaques. It seems that the APP23 mice develop plaque-associated early neurofibrillary pathology. Very interestingly, this mouse models shows a loss of cholinergic fibers and neuron loss in the CA1 region of the hippocampus, hallmarks quite selectively for AD pathology. Moreover, when crossed with PS1 transgenic mice carrying AD-relevant mutations, the double transgenic offspring show enhanced plaque formation (Sturchler-Pierrat and Staufenbiehl, 2000).

Since all of these specific alterations can be picked up in lymphocytes from sporadic AD patients or from FAD patients as well as from transgenic mice carrying FAD-specific mutations, it seems possible that enhanced cellular vulnerability to cell death, represent a pathological pathway for sporadic and familial AD.

Antiapoptotic action of PS1 C-terminal fragment

Moreover, PS1 wt Tg mice seem to be protected of apoptosis and basal and spontaneous conditions. Jurkat leucemic cells transfected with a C-terminal construct of PS1 showed increased resistance to apoptosis as well (Vezina *et al*., 1999). In addition, similar findings have been described for PS2 and its C-terminal fragment ALG-3 (Vito *et al*., 1996). Here, we detected this phenomenon even under in vivo condition in PS1 wt Tg mice. As transgenic mice express more PS1, the human and their endogenous murine PS1, it seems that those effects are due to a gene-dose effect. PS1 interacts with anti-apoptotic Bcl2 family members that differentially regulate sequential stages of progenitor cell survival (Mehler and Gokhan, 2001). It is further known that PS protein interact with Ca^{2+} binding proteins (Buxbaum *et al*., 1998) and PS1 mutants sensitize to apoptosis as demonstrated above. If maintenance of intracellular Ca^{2+} concentration is a physiological function shared by PS and Bcl2, one can speculate that PS overexpression may stabilize their reciprocal association. The enhanced apoptosis of cells bearing PS1 mutations might reflect the loss of this anti-apoptotic mechanism. Moreover, recently we could show that antioxidative system in brains derived

PS1-transgenic mice display elevated activity when compared with littermate controls (Leutner *et al*, 2000). Possibly here the same mechanism is evident, as oxidative radicals are mediators of apoptosis. Further experiments to explore the nature of this finding are currently under investigation.

In this study it was clearly shown that C-terminal fragments of Alzheimer's disease related PS1 generated during apoptosis provide an antiapoptotic action, similar to that previously reported for PS2 and its C-terminal fragment ALG-3.

To our knowledge, we are the first to demonstrate this antiapoptotic effect of C-terminal fragments of the Presenilin family *ex vivo* in cells derived from PS1-transgenic animals. As transgenic mice express more PS1, the human and their endogenous murine PS1, it seems that those effects are due to a gene-dose effect.

PS1 interacts with anti-apoptotic Bcl2 family members that differentially regulate sequential stages of progenitor cell survival (Mehler and Gokhan, 2001). It is further known that PS protein interact with Ca^{2+} binding proteins (Buxbaum *et al*., 1998b) and PS1 mutants sensitize to apoptosis as demonstrated above. If maintainace of intracellular Ca^{2+} concentration is a physiological function shared by PS and Bcl2, one can speculate that PS overexpression may stabilize their reciprocal association.

The rationale of the antiapoptotic action of PS1Cas during caspase activation remains to be elucidated. It could be possible that the PS1/ Bcl2/ BclXL interaction represents one of the yet still rare negative feedback mechansim in apoptosis regulation.

It should be expermintally explored whether PS1 mutants are able to bind to Bcl2 family members or not. The enhanced apoptosis of cells bearing PS1 mutations might reflect the loss of this anti-apoptotic mechansim.

Mover, recently we could show that antioxidative system in brains derived PS1-transgenic mice display elevated activity when compared with littermate controls (Leutner *et al*., 2000). Possibly here the same mechanism is evident, as oxidative radicals are mediators of apoptosis.

Peripheral cells as model in AD research

Recent findings suggest that genetic and environmental factors may facilitate the neurodegenerative process of AD, by acting at different points in a feed-forward cyclical neurodegenerative cascade that shares features with other neurodegenerative disorders (Eckert *et al*., 1995b). Primary abnormality in cellular calcium signaling in the pathogenesis of some

inherited forms of AD, and the implications of this defect for all cases of AD might be a general defect in neural tissue in this disorder. The possibility that dysregulation of cellular calcium homeostasis is central to the pathogenesis of AD fits nicely within the well-established role of calcium in regulating synaptic changes that underlie learning and memory, and the involvement of calcium overload in the pathogenesis of a range of neurodegenerative conditions (Mattson *et al*., 2001). Moreover, emerging evidence for alterations in calcium signaling in cells of the immune system suggests to us a pivotal role for altered calcium signaling in immune dysfunction that occurs in AD patients. During the past decade considerable evidence has accumulated suggesting that perturbations in the regulation of intracellular Ca^{2+} levels contributes to the pathogenesis of AD (Mattson *et al*., 1993). For example, overactivation of glutamate receptors, and other conditions that result in a sustained elevation of intracellular Ca^{2+} levels, can induce alterations in the neuronal cytoskeleton similar to those seen in neurofibrillary tangles. By increasing Aβ production and decreasing sAPP production, alterations in APP processing that result from presenilin mutations can disrupt cellular calcium homeostasis and render neurons vulnerable to degeneration and death (Mattson *et al*., 2001).

Studies of fibroblasts and lymphocytes from patients with late-onset sporadic forms of AD have also revealed defects in calcium signaling (Eckert *et al*., 1993a).

While histological studies in postmortem brain tissue indicate enhanced features of apoptotic cell death such as DNA fragmentation (Stadelmann *et al*., 1999), evidences for an enhanced cellular vulnerability to apoptosis mainly originates from studies using lymphocytes from sporadic AD patients (Parshad *et al*., 1996; Eckert *et al*.,1998; 2001a; Mecocci *et al*., 1998). Very importantly, the present findings in lymphocytes from FAD associated transgenic mice reflect comparably findings in lymphocytes from AD patients found in this thesis and by others (Parshad *et al*., 1996; Eckert *et al*., 1998; Lombardi *et al*., 1999). Since these specific alterations can be picked up in lymphocytes from sporadic AD patients or from FAD patients as well as from transgenic mice carrying FAD-specific mutations, it seems possible that enhanced cellular vulnerability to cell death represent a common pathological pathway for sporadic and familial AD caused by mutations in PS1. The present thesis demonstrates that we could bridge the gap between research using transgenic animal models or transfected cells and studies using tissue from AD patients.

Future prospectives

How does the information get from the CNS to the periphery?

Infiltration across the blood brain barrier

Activation and loose-tethering of blood-borne leukocytes to cerebrovascular endothelial cells involves intracellular adhesion molecules-1 (ICAM-1) on endothelial cells and its integrin receptor on leukocytes, the lymphocyte function-associated antigen-1 (LFA-1). Crosslinking of ICAM-1 activates T cells and macrophages, resulting in the production of pro-inflammatory molecules (e.g. INF-γ, IL-1 and NO). Induction of NO and cytokines alters the permeability of the blood brain barrier, increases ICAM-1 and LFA-1, and induces another adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), on endothelial cells and glia (Merrill and Murphy, 1997). VLA-1 was identified to play a crucial role for the entry of $CD4^+$ T cells (Baron *et al*., 1993). Once cells have crossed the blood brain barrier, they are directed to migrate toward gradients created by chemoattractant chemokines. The small cytokines can be quite specific in determining the immigration patterns of lymphocytes and macrophages to certain inflammatory regions and are capable of enhancing activation by integrins through redistribution of adhesion molecules, thereby modulating leukocyte-endothelial cell interaction (Merrill and Murphy, 1997).

However, the fate of brain-infiltrated lymphocytes remains to be elucidated. Older theories describing immune privileged zones like the CNS claim that activated T lymphocytes are subjected to apoptotic cell death via the Apo/Fas-pathway (Janeway and Travers, 1995). But latest research validates that the brain and neural cells definitively provide immune activity (see Figure 82). Probably some infiltrated lymphocytes can re-enter the blood brain barrier and mediate their information in the periphery. Moreover, invaded activated lymphocytes secrete cytokine and inflammatory molecules that cross the blood brain barrier. In the periphery those molecules are able to stimulate other leukocytes.

Possible future therapies to treat AD

There are several new ideas for treating and preventing AD. Apoptosis of peripheral blood lymphocytes and mitogenic activation of T lymphocytes could represent a good tool to monitor these new therapeutic strategies from periphery with minor invasive methods. The most promising novel therapies are as follows:

Inhibitors of secretases

The identification of β-secretase in 1999 (Yan *et al*., 1999) and the progress in the characterization of the still unknown g-secretase over the last two years has provided new opportunities to develop therapies to inhibit secretase activity. To many researchers, inhibition of secretase activity appears to be one of the most promising strategy for preventing AD (Citron, 2000). At this point there is no evidence of additional functions of Aβ, so that are no serious concerns about reduction of this metabolite. However, there are possible remaining pitfalls for this therapy: (1) Secretase are present in many body cells and APP may not be the only and most relevant substrate and (2) amyloid production is the top of the cascade and dementia is at the bottom. Therefore, secretase inhibition might be not useful to treat AD but to prevent AD in earlier years.

Immunization with Aβ

Immunization of transgenic mice carrying the London APP mutation (APP-V717F, PDAPP-mouse) with $A\beta_{1-42}$ prevents amyloid deposition and astrocystosis in young PDAPP mice (Schenk *et al*., 1999). In elderly mice, immunization appears to arrest the progression of amyloidosis. Immunized mice developed and maintained high serum antibody titer against Aβ One possible mechanism of action is that anti-Aβ antibodies facilitate clearance of Aβ either before deposition, or after plaque formation, by triggering monocytic/microglial cells to clear Aβ using signals mediated by F_c receptors. It has been suggested that anti-inflammatory regimens might be of therapeutic value. The findings of Schenk and coworkers argue that an alternative approach, one that augments a highly specific immune response involving the peripheral adaptive immune system, can markedly reduce pathology in an animal model of the disease. Aβ immunization appears to increase the clearance of amyloid plaques, and may therefore be novel and effective approach for the treatment of AD. However, patients in later stage of the disease are unlikely to benefit from this therapy.

Stemcells

A most exciting field of discovery, from the standpoint of therapeutic intervention in neurodegenerative disease, is that even the adult CNS posses populations of neuronal stem cells that are capable of proliferating and then differentiating into neurons and glial cells (Scheffler *et al*., 1999).

All earlier mentioned therapies (e.g. immunization, NSAID use) show their beneficial effects only, if administered early, years to decades before the onset of AD. Conventional therapies (anticholinergic treatment or use of antioxidant like EGb761) that focus on demented persons do only slower the progression of AD about a year or two but are not capable for healing it. The only therapy for healing AD would be to substitute the lost neurons thereby restoring synaptic transmission. There are some hints that neurons generated from ecotopic stem cells can integrate into neuronal circuits in the adult brain (Snyder *et al*., 1997). A neuronal stem cell-based approach would most likely be applied to patients in the early stages of AD with the hope of replacing damaged neuronal synapses and restoring learning and memory abilities (Mattson, 2000).

The findings of this thesis represent a basis to generate a suitable peripheral testmodel to monitor and evaluate new therapeutic approach.

Summary

Aging and age-related diseases are becoming more and more important for our society and our health care system. Alzheimer's disease (AD) is a disorder that destroys some parts of the brain and is characterized by global cognitive decline including a progressive irreversible loss of memory, orientation, and reasoning. “Healthy aging”, therefore, is one of the major aims for modern medicine. Apoptosis, or programmed cell death, plays an important role for example in fetal development, as well as for learning processes. T-lymphocytes usually undergo apoptosis in order to terminate an acute inflammation.

The aim of this thesis was to explore the changes in the apoptotic mechanism of peripheral lymphocytes from Alzheimer’s disease (AD) patients in contrast to physiological aging. The experiments were conducted with lymphocytes of healthy volunteers of different ages, AD patients and young and aged mice. Moreover, transgenic mice carrying familiar AD-related mutations were examined.

The aging study of peripheral cells of ‘healthy’-aged volunteers revealed an age-related increase of basal apoptosis. In addition, spontaneous apoptosis as well as apoptosis induced by oxidative stress (ROS) or by Fas engagement were enhanced in aging. A closer look at the subcellular basis of the lymphocytes (e.g. B-, NK-, $CD4^+$-, and $CD8^+$-T cells) determined that all lymphocyte subsets were affected by aging. Therefore, it could be concluded that the regulation of apoptosis is generally impaired in lymphocytes of aged persons. The increased susceptibility to oxidative stress supports the ‘Free radical theory of aging’ that claims the radicals to be the cause for the aging-process.

In mice an increase of basal, spontaneous and ROS-induced apoptosis was detected in T cells from the spleen, as well. An oral treatment over two weeks with the *Ginkgo biloba* extract EGb761 showed a clear reduction of ROS-induced apoptosis in the treated group. Interestingly, basal and spontaneous apoptosis, e.g. physiological apoptosis, were not effected by the plant extract. This is an important benefit for therapy since physiological apoptosis has a great relevance in the elimination of cancer-cells for example. In conclusion, the anti-dementive drug EGb761 reduces specifically ROS-induced apoptosis that a plays an important role in aging as shown in this thesis.

Based on the data found in healthy aging, lymphocytes from AD patients were assessed for apoptosis. The cells show enhanced levels of basal, spontaneous, and Fas-induced apoptosis. In subsequent experiments it was demonstrated that mainly the T cells were responsible for

the findings. However, the NK-cells provided an important impact as well. In concordance with AD-affected neurons, peripheral lymphocytes of AD patients show clear signs of apoptotic cell death. In addition, basal apoptosis of T cells and the CD4/CD8-ratio showed a correlation with the severity of the dementia. Therefore, it could be speculated that apoptosis is due to activation-induced cell death (AICD) that occurs in acute and chronic activation of adaptive immunity. In AD there is a chronic neuroinflammation in the CNS triggering degeneration of neural tissue. In order to explore this, the experimental model of lymphocyte's activation was established in healthy aging first.

The study included the detection of various events of lymphocyte's activation on the basis of the T cell subsets ($CD4^+$ and $CD8^+$). The inducibility to mitogenic stimulation clearly decreased in both subsets in aging. In contrast, T lymphocytes from AD patients showed an enhanced activation subsequent to mitogenic stimulation compared with age-matched non-demented persons. Only proliferation of $CD8^+$ T cells was clearly reduced in AD. This data could be clues that an increased generation of memory T cells due to chronic neuroinflammation might be evident in AD. Memory T lymphocytes show increased inducibility upon mitogenic activation. Interestingly, $CD8^+$ memory T cells display decreased prolifertive capacity. Due to activation, cells die by apoptosis later on. It could be concluded that AD patients display an increased amount of memory T cells compared to controls. The data implicate that there could be a cross talk between inflammatory within the brain and inflammatory cells of the periphery. This is an interesting point since the brain used to be assumed as immune-privileged zone. According to the experiment, the information of the diseased brain is transferred to white blood cells. The connection of those two compartments might raise the opportunity to observe and probably to influence easily not-accessible regions like the brain.

Transgenic mice carrying mutations in familiar AD-relevant genes (Amyloid-Precursor-Protein, Presenilin-1, respectively) displayed enhanced levels of apoptotic T cells from the spleen, as well. It seems that those mutated proteins influence the regulation of apoptosis. Probably, they are involved in the increased cell death of T- and NK-cells, as well. Animals overexpressing Presenilin-1 showed reduced levels of apoptotic cell death. It was demonstrated with molecuar biology tools that Presenilin-1, processed during apoptosis, has an anti-apoptotic effect.

Zusammenfassung

Altern und damit verbundene Alters-bedingte Erkrankungen stellen ein enormes Problem in der westlichen industrialisierten Welt dar. Apoptose, der programmierte Zelltod, spielt bei vielen pathologischen, aber auch bei physiologischen Prozessen eine wichtige Rolle, so zum Beispiel in der Embryonalentwicklung sowie bei Lernprozessen. Lymphozyten beispielsweise, die während einer Immunreaktion stark proliferieren, werden anschließend apoptotisch eliminiert.

Ziel der vorliegenden Arbeit war die Erfassung von Veränderungen des apoptotischen Verhaltens an peripheren Lymphozyten in der Alzheimer Demenz und zum Vergleich im physiologischen Alterungsprozess. Die Untersuchungen wurden mit Lymphozyten von gesunden Probanden unterschiedlichen Alters, Alzheimer-Patienten und Mäusen verschiedener Altersgruppen durchgeführt. Ausserdem wurden transgene Mäuse untersucht, die mutierte Gene tragen, die für die familiäre Form der Alzheimer Demenz verantwortlich sind.

Der Alt-Jung Vergleich von peripheren Zellen von "gesund"-gealterten Menschen zeigte einen Alters-bedingten Anstieg der basalen Apoptoserate. Ausserdem waren die spontane, die durch oxidativen Stress (ROS)- sowie Fas-induzierte Apoptose deutlich mit zunehmenden Lebensalter erhöht. Eine genauere Untersuchung der einzelnen Zellsubtypen zeigte, dass dieser Anstieg bei allen Lymphozyten (B-, NK-, $CD4^+$- und $CD8^+$- T-Lymphozyten) zu messen ist. Man kann daher vermuten, dass die Regulation der Apoptose im Alter allgemein in peripheren Lymphozyten verändert ist. Ausserdem legte sich eine verstärkte Vulnerabilität gegenüber oxidativen Stress dar, eine weiterer Befund zur Stützung der "Free radical theory of aging", die den Radikalen eine ursächliche Bedeutung im Alterungsprozess beilegt.

In der Jung-Alt-Studie am Mausmodell zeigte sich sowohl an peripheren Blutlymphozyten als auch an T-Lymphozyten aus der Milz, ein deutlicher Anstieg der basalen, spontanen und ROS-induzierten Apoptose. Eine zweiwöchige perorale Behandlung mit dem *Ginkgo biloba*-Extrakt EGb761 zeigte eine deutliche Reduktion der ROS-induzierten Apoptose, aber keinerlei Beeinflussung der physiologischen basalen und spontanen Apoptose. Dies hat einen besonderen Vorteil für die Therapie, da Apoptose physiologisch eine wichtige Rolle zur Elimination bestimmter Zellen, wie beispielsweise Tumorzellen, hat. Das Antidementivum EGb761 hat daher nur spezifisch Einfluss auf den oxidativen Stress-induzierten Zelltod, der, wie hier gezeigt werden konnte, im Alterungsprozess eine grosse Rolle spielt.

Basierend auf den Befunden zur Apoptose im physiologischen Alterungsprozess, wurden Untersuchungen mit peripheren Blutzellen von Alzheimer-Patienten durchgeführt. Die Zellen der Patienten zeigten eine Erhöhung der basalen, spontanen und Fas-induzierten Apoptose, die bei Untersuchungen der Subtypen (B-, T- und NK-Lymphozyten) auf die T-Lymphozyten zurück geführt werden konnte, die NK-Zellen aber auch deutlich betroffen waren. Wie die Neuronen im ZNS, so zeigen auch die peripheren Lymphozyten eindeutige Veränderungen, die die Apoptose kennzeichnen. Ausserdem erwies sowohl die basale Apoptose der T-Zellen, als auch das Verhältnis der CD4- zu CD8-positiven T-Zellen eine Abhängigkeit zum Schweregrad der Erkrankung. Dies lies die Vermutung aufkommen, dass es sich bei diesem verstärkten Zelltod um den Aktivierungs-induzierten Zelltod (AICD) handeln könnte, der bei einer akuten aber auch chronischen Aktivierung der adaptiven Immunität auftritt. In der Alzheimer Demenz kommt es zu einer jahrelangen chronischen Neuroinflammation, die mit starker Degeneration einher geht. Um diesen Aspekt näher zu beleuchten, war es notwendig, die Lymphozyten in vitro zu stimmulieren.

Untersuchungen, zur Aktivierbarkeit der T-Lymphozyten wurden zunächst wieder im Alterungsprozess etabliert. Hierbei konnte deutlich in einer Studie, die mehrere Zeitpunkte sowie die genauere Betrachtung der T-Zellsubpopulationen ($CD4^+$ und $CD8^+$) mit einschloss, gezeigt werden, in wieweit das physiologische Altern die Immunfunktion beeinflusst. So konnte insgesamt eine verminderte Aktivierbarkeit beider T-Zellsubsets von alten Probanden festgestellt werden. Im Kontrast dazu zeigten sowohl $CD4^+$ als auch $CD8^+$ T-Zellen von Alzheimer-Patienten eine verstärkte Aktivierbarkeit verglichen mit nicht-dementen Alters-gemittelten Kontrollpersonen. Nur bei der Proliferation nach mitogenen Stimulieren zeigten $CD8^+$ T-Lymphozyten eine geringeres Ansprechen. Diese Befunde könnten Hinweise darauf geben, dass bei dieser Demenzerkrankung verstärkt Gedächtnis-T-Zellen, wahrscheinlich bedingt durch die chronische Inflammation im ZNS, generiert werden. Gedächtnis-T-Lymphozyten zeigen eine verstärkte Aktivierbarkeit nach mitogener Stimulation. Interessanterweise, zeigen $CD8^+$ Gedächtniszellen eine geringere Proliferation. Aufgrund der Aktivierung sterben die Zellen anschliessend apoptotisch ab. Daher liegt die Vermutung nahe, dass bei Alzheimer-Patienten eine erhöhte Anzahl an Gedächtnis-T-Zellen im vorliegt. Diese Befunde stützen die These, daß der im Zentralnervensystem stattfindende Zelltod über die aktivierten Glia auch in das periphere Immunsystem überstrahlt. Über die Verknüpfung dieser beiden Kompartimente des Abwehrsystems ist es möglich, Veränderungen in einer nicht

zugänglichen Region wie dem Gehirn zu beobachten und möglicherweise auch zu beeinflussen.

Transgene Mäuse, die Mutationen (im Amyloid-Precursor-Protein- bzw. im Presenilin-1 Gen) tragen, die für die familiäre Form der Alzheimer-Demenz verantwortlich sind, zeigen auch eine erhöhte Apoptoserate an T-Lymphozyten aus der Milz, was darauf hindeutet, dass diese Genprodukte einen signifikanten Einfluss auf die Regulation der Apoptose haben. Möglicherweise sind diese Mechanismen auch am erhöhten Zelltod der T- und NK-Zellen beteiligt. Bei Tieren, die das Wildtyp Presenilin-1 Protein überexpremieren, konnte, mittels molekularbiologischer Methoden, gezeigt werden, dass dieses während der Apoptoseinduktion prozessiert wird und einen antiapoptotischen Effekt ausübt.

References

ABRAHAM, N., MICELI, M.C., PARNES, J.R., VEILLETTE, A., 1991. Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase p56lck. Nature 350, 62-66.

ADUNSKY A, DIVER-HABER A, BECKER D, HERSHKOWITZ M, 1995. Basal and activated intracellular calcium ion concentrations in mononuclear cells of Alzheimer's disease and unipolar depression. J Gerontol A Biol Sci Med Sci 50: B201-4.

ADUNSKY A, BARAM D, HERSHKOWITZ M, MEKORI YA, 1991. Increased cytosolic free calcium in lymphocytes of Alzheimer patients. J Neuroimmunol 33: 167-72.

AGGARWAL S, GOLLAPUDI S, GUPTA S, 1999. Increased TNF-alpha-induced apo-ptosis in lymphocytes from aged humans: changes in TNF-alpha receptor expression and activation of caspases. J Immunol 162: 2154 –61.

AGGARWAL S, GUPTA S, 1998. Increased apoptosis of T cell subsets in aging humans: Altered expression of Fas (CD95), Fas ligand, Bcl-2, and Bax. J Immunol 160: 1627-37.

AHLEMEYER B, MOWES A, KRIEGLSTEIN J, 1999. Inhibition of serum deprivation- and staurosporine-induced neuronal apoptosis by Ginkgo biloba extract and some of its constituents. Eur J Pharmacol 367:423-430.

AISEN PS, DAVIS KL, 1994. Inflammatory mechanisms in Alzheimer's disease: implications for therapy. Am J Psychiatry 151: 1105-13.

AKIYAMA H, KAWAMATA T, YAMADA T, TOOYAMA I, ISHII T, MCGEER PL, 1993. Expression of intercellular adhesion molecule (ICAM)-1 by a subset of astrocytes in Alzheimer disease and some other degenerative neurological disorders. Acta Neuropathol (Berl) 85: 628-34.

ALBERICI A, MORATTO D, BENUSSI L, GASPARINI L, GHIDONI R, GATTA LB, FINAZZI D, FRISONI GB, TRABUCCHI M, GROWDON JH, NITSCH RM, BINETTI G, 1999. Presenilin 1 protein directly interacts with Bcl-2. J Biol Chem 274: 30764-9.

ALLSOPP RC, VAZIRI H, PATTERSON C, GOLDSTEIN S, YOUNGLAI EV, FUTCHER AB, GREIDER CW, HARLEY CB, 1992. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A 89: 10114-8.

ALZHEIMER A, 1907. Über eine eigenartige Erkrankung der Hirnrinde. Allg Z Psychiatr Psych-Gerichtl Med 64, 146-8; 1997.

ANDERSEN K, LAUNER LJ, OTT A, HOES AW, BRETELER MM, HOFMAN A, 1995. Do nonsteroidal anti-inflammatory drugs decrease the risk for Alzheimer's disease? The Rotterdam Study. Neurology 45: 1441-5.

ANDERSON R, BARNES JC, BLISS TV, ET AL, 1998. Behavioural, physiological and morphological analysis of a line of apolipoprotein E knockout mouse. Neuroscience 85: 93–110.

ANHOLT RR, PEDERSEN PL, DE SOUZA EB, SNYDER SH, 1986. The peripheral-type benzodiazepine receptor. Localization to the mitochondrial outer membrane. J Biol Chem 261: 576-83.

ANTONACI S, GAROFALO AR, CHICCO C, POLIGNANO AV, PUGLIESE P, MISEFARI A, JIRILLO E, 1990. Senile dementia, Alzheimer type: a distinct entity in the immunosenescence? J Clin Lab Anal 4: 16-21.

ANTONACI, S., JIRILLO, E., GALLITELLI, M., GAROFALO, A.R., AND BONOMO, L., 1985. Impairment of T immunoregulatory activities in the induction of antibody specific response in aged humans. Mech.Ageing Dev. 30, 251-259.

ARAGA S, KAGIMOTO H, FUNAMOTO K, TAKAHASHI K. 1991. Reduced natural killer cell activity in patients with dementia of the Alzheimer type. *Acta Neurol Scand* 84: 259-63.

ARENDT, T. 1999. Pathologische Anatomie der Alzheimer Krankheit. In: Alzheimer Demenz: Grundlagen, Klinik und Therapie. edited by Förstl, H., Bickel, H. and Kurz, A. Springer Verlag, Berlin.

ARRASATE M, PEREZ M, VALPUESTA JM, AVILA J, 1997. Role of glycosaminoglycans in determining the helicity of paired helical filaments. Am J Pathol 151: 1115-22.

ASPINALL R, CARROLL J, JIANG SS, 1998. Age-related changes in the absolute number of CD95 positive cells in T cell subsets in the blood. Exp Gerontol 33: 581-91.

BALES KR, DU Y, HOLTZMAN D, CORDELL B, PAUL SM, 2000. Neuroinflammation and Alzheimer's disease: critical roles for cytokine/Abeta-induced glial activation, NF-kappaB, and apolipoprotein E. Neurobiol Aging 21: 427-32, discussion 451-3

BANATI RB, GEHRMANN J, SCHUBERT P, KREUTZBERG GW, 1993. Cytotoxicity of microglia. Glia 7: 111-8.

BARBIERI D, GRASSILLI E, MONTI D, SALVIOLI S, FRANCESCHINI MG, FRANCHINI A, BELLESIA E, SALOMONI P, NEGRO P, CAPRI M, 1994. D-ribose and deoxy-D-ribose induce apoptosis in human quiescent peripheral blood mononuclear cells. Biochem Biophys Res Comm 201:1109-1116.

BARCELLINI, W., BORGHI, M.O., SGUOTTI, C., PALMIERI, R., FRASCA, D., MERONI, P.L., DORIA, G., AND ZANUSSI, C., 1988. Heterogeneity of immune responsiveness in healthy elderly subjects. Clin.Immunol.Immunopathol. 47, 142-151.

BARGER SW AND HARMON AD, 1997. Microglial activation by Alzheimer amyloid precursor protein and modulation by apolipoprotein E. Nature 388: 878-81.

BARNUM SR, JONES JL, 1994. Transforming growth factor-beta 1 inhibits inflammatory cytokine-induced C3 gene expression in astrocytes. J Immunol 152: 765-73.

BARON JL, MADRI JA, RUDDLE NH, HASHIM G, JANEWAY CA JR, 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. J Exp Med 177: 57-68.

BASTIANETTO S, RAMASSAMY C, DORE S, CHRISTEN Y, POIRIER J, QUIRION R 2000a The Ginkgo biloba extract (EGb 761) protects hippocampal neurons against cell death induced by beta-amyloid. Eur J Neurosci 12:1882-1890.

BATLIWALLA F, MONTEIRO J, SERRANO D, GREGERSEN PK, 1996. Oligoclonality of CD8+ T cells in health and disease: Aging, infection, or immune regulation? Hum Immunol 48: 68-76.

BATUMAN, O.A., SAJEWSKI, D., OTTENWELLER, J.E., PITMAN, D.L., AND NATELSON, B.H., 1990. Effects of repeated stress on T cell numbers and function in rats. Brain Behav.Immun. 4, 105-117.

BAUER J, GANTER U, ABEL J, STRAUSS S, JONAS U, WEISS R, GEBICKE-HAERTER P, VOLK B, BERGER M, 1993. Effects of interleukin-1 and interleukin-6 on metallothionein and amyloid precursor protein expression in human neuroblastoma cells. Evidence that interleukin-6 possibly acts via a receptor different from the 80-kDa interleukin-6 receptor. J Neuroimmunol 45: 163-73.

BAUER J, KONIG G, STRAUSS S, JONAS U, GANTER U, WEIDEMANN A, MONNING U, MASTERS CL, VOLK B, BERGER M, et al 1991. In-vitro matured human macrophages express Alzheimer's beta A4-amyloid precursor protein indicating synthesis in microglial cells. FEBS Lett 282: 335-40.

BAUER MK, LIEB K, SCHULZE-OSTHOFF K, BERGER M, GEBICKE-HAERTER PJ, BAUER J, FIEBICH BL, 1997. Expression and regulation of cyclooxygenase-2 in rat microglia. Eur J Biochem 243: 726-31.

BEARD CM, KOKMAN E, KURLAND LT, 1991. Rheumatoid arthritis and susceptibility to Alzheimer's disease. Lancet 337: 1426.

BEHL C, SCHUBERT D, 1993. Heat shock partially protects rat pheochromocytoma PC12 cells from amyloid beta peptide toxicity. Neurosci Lett 154: 1-4.

BENDER BS, NAGEL JE, ADLER WH, ANDRES R, 1986. Absolute peripheral blood lymphocyte count and subsequent mortality of elderly men. The Baltimore Longitudinal Study of Aging. J Am Geriatr Soc 34: 649 –54.

BERKENBOSCH, F., WOLVERS, D.A., AND DERIJK, R., 1991. Neuroendocrine and immunological mechanisms in stress-induced immunomodulation. J.Steroid Biochem.Mol.Biol. 40, 639-647.

BERLETT BS, STADTMAN ER, 1997. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 272, 20313-20316.

BERRIDGE MJ, 1997. Lymphocyte activation in health and disease. Crit Rev Immunol 17: 155–78.

BESSLER H, WEIZMAN R, GAVISH M, NOTTI I, DJALDETTI M, 1992. Immunomodulatory effect of peripheral benzodiazepine receptor ligands on human mononuclear cells. J Neuroimmunol 38: 19-26

BHASIN R, VAN NOSTRAND WE, SAITOH T, DONETS MA, BARNES EA, QUITSCHKE WW, GOLDGABER D, 1991. Expression of active secreted forms of human amyloid beta-protein precursor by recombinant baculovirus-infected insect cells. Proc Natl Acad Sci U S A 88: 10307-11.

BIANCA VD, DUSI S, BIANCHINI E, DAL PRA I, ROSSI F, 1999. beta-amyloid activates the O-2 forming NADPH oxidase in microglia, monocytes, and neutrophils. A possible inflammatory mechanism of neuronal damage in Alzheimer's disease. J Biol Chem 274: 15493-9.

BICKEL H, 1999. Alzheimer Demenz: Grundlagen, Klinik und Therapie. edited by Förstl, H., Bickel, H. and Kurz, A. Springer Verlag, Berlin.

BLACKER D, HAINES JL, RODES L, TERWEDOW H, GO RC, HARRELL LE, PERRY RT, BASSETT SS, CHASE G, MEYERS D, ALBERT, MS, TANZI R., 1997. ApoE-4 and age at onset of Alzheimer's disease: the NIMH genetics initiative. Neurology 48: 139-147.

BLAZER D, WILLIAMS CD, 1980. Epidemiology of dysphoria and depression in an elderly population. Am J Psychiatry 137: 439-44.

BONGIOANNI P, BOCCARDI B, BORGNA M, CASTAGNA M, MONDINO C, 1997a. T-cell interferon gamma binding in patients with dementia of the Alzheimer type. Arch Neurol 54: 457-62;

BONGIOANNI P, ROMANO MR, SPOSITO R, CASTAGNA M, BOCCARDI B, BORGNA M, 1997b. T-cell tumour necrosis factor-alpha receptor binding in demented patients. J Neurol 244:418-25

BONGIOANNI P, CASTAGNA M, MONDINO C, BOCCARDI B, BORGNA M, 1997c. Platelet and lymphocyte benzodiazepine binding in patients with Alzheimer's disease. Exp Neurol 146: 560-6.

BORTH W, 1992. Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. FASEB J 6: 3345-53.

BÖTTNER M, UNSICKER K, SUTER-CRAZZOLARA C, 1996. Expression of TGF-beta type II receptor mRNA in the CNS. Neuroreport 7: 2903-7.

BREITNER JC, WELSH KA, HELMS MJ, GASKELL PC, GAU BA, ROSES AD, PERICAK-VANCE MA, SAUNDERS AM, 1995. Delayed onset of Alzheimer's disease with nonsteroidal anti-inflammatory and histamine H2 blocking drugs. Neurobiol Aging 16: 523-30.

BREITNER JC, GAU BA, WELSH KA, PLASSMAN BL, MCDONALD WM, HELMS MJ, ANTHONY JC, 1994. Inverse association of anti-inflammatory treatments and Alzheimer's disease: initial results of a co-twin control study. Neurology 44: 227-32.

BROE GA, HENDERSON AS, CREASEY H, MCCUSKER E, KORTEN AE, J ORM AF, LONGLEY W, ANTHONY JC, 1990. A case-control study of Alzheimer's disease in Australia. Neurology 40: 1698-707.

BRUCE AJ, BOLING W, KINDY MS, PESCHON J, KRAEMER PJ, CARPENTER MK, HOLTSBERG FW, MATTSON MP, 1996. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. Nat Med 2: 788-94.

BRUUNSGAARD H, PEDERSEN AN, SCHROLL M, SKINHOJ P, PEDERSEN BK, 2000a. TNF-alpha, leptin, and lymphocyte function in human aging. Life Sci. 67, 2721-2731.

BRUUNSGAARD H, PEDERSEN AN, SCHROLL M, SKINHOJ P, PEDERSEN BK, 2000b. Proliferative responses of blood mononuclear cells (BMNC) in a cohort of elderly humans: role of lymphocyte phenotype and cytokine production. Clin.Exp.Immunol.119, 433-440.

BULLIDO MJ, MUNOZ-FERNANDEZ MA, RECUERO M, FRESNO M, VALDIVIESO F, 1996. Alzheimer's amyloid precursor protein is expressed on the surface of hematopoietic cells upon activation. Biochim Biophys Acta 1313: 54-62.

BURNS EA, LUM LG, L'HOMMEDIEU G, GOODWIN JS, 1993. Specific humoral immunity in the elderly: in vivo and in vitro response to vaccination. J.Gerontol. 48, B231-B236.

BUSCIGLIO J, LORENZO A, YEH J, YANKNER BA, 1995. beta-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. Neuron 14: 879-88.

BUTTKE TM, SANDSTROM PA, 1994. Oxidative stress as a mediator of apoptosis. Immunol Today 15: 7–10.

BUXBAUM JD, CHOI EK, LUO Y, LILLIEHOOK C, CROWLEY AC, MERRIAM DE, WASCO W, 1998b. Calsenilin: a calcium-binding protein that interacts with the presenilins and regulates the levels of a presenilin fragment. Nat Med 4:1177-81

BUXBAUM JD, LIU KN, LUO Y, SLACK JL, STOCKING KL, PESCHON JJ, JOHNSON RS, CASTNER BJ, CERRETTI DP, BLACK RA, 1998a. Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. J Biol Chem 273: 27765-7.

BUXBAUM JD, OISHI M, CHEN HI, PINKAS-KRAMARSKI R, JAFFE EA, GANDY SE, GREENGARD P, 1992. Cholinergic agonists and interleukin 1 regulate processing and secretion of the Alzheimer beta/A4 amyloid protein precursor. Proc Natl Acad Sci U S A 89: 10075-8.

CALLAHAN JE, KAPPLER JW, MARRACK P, 1993. Unexpected Expansions of CD8-Bearing Cells in Old Mice. J Immunol 151: 6657-69.

CARDINAUX JR, ALLAMAN I, MAGISTRETTI PJ, 2000. Pro-inflammatory cytokines induce the transcription factors C/EBPbeta and C/EBPdelta in astrocytes. Glia 29: 91-7.

CARSON MJ, REILLY CR, SUTCLIFFE JG, LO D, 1999. Disproportionate recruitment of CD8+ T cells into the central nervous system by professional antigen-presenting cells. Am J Pathol 154: 481-94.

CARSON, M.J. AND SUTCLIFFE, J.G., 1999. Balancing function vs. self defense: the CNS as an active regulator of immune responses. J.Neurosci Res. 55, 1-8.

CECCHI C, LATORRACA S, SORBI S, IANTOMASI T, FAVILLI F, VINCENZINI MT, LIGURI G, 1999. Gluthatione level is altered in lymphoblasts from patients with familial Alzheimer's disease. Neurosci Lett 275: 152-4.

CERUTI S, BARBIERI D, VERONESE E, CATTABENI F, COSSARIZZA A, GIAMMARIOLI AM, MALORNI W, FRANCESCHI C, ABBRACCHIO MP, 1997. Different pathways of apoptosis revealed by 2-chloro-adenosine and deoxy-D-ribose in mammalian astroglial cells. J Neurosci Res 47:372-383.

CHAO CC, ALA TA, HU S, CROSSLEY KB, SHERMAN RE, PETERSON PK, FREY WH 2ND, 1994a. Serum cytokine levels in patients with Alzheimer's disease. Clin Diagn Lab Immunol 1: 433-6.

CHAO CC, HU S, FREY WH 2ND, ALA TA, TOURTELLOTTE WW, PETERSON PK, 1994b. Transforming growth factor beta in Alzheimer's disease. Clin Diagn Lab Immunol 1: 109-10.

CHAO DT, KORSMEYER SJ, 1998. BCL-2 family: regulators of cell death. Annu Rev Immunol 16: 395-419.

CHEN C, WEI T, GAO Z, ZHAO B, HOU J, XU H, XIN W, PACKER L, 1999. Different effects of the constituents of EGb761 on apoptosis in rat cerebellar granule cells induced by hydroxyl radicals. Biochem Mol Biol Int 47: 397-405.

CHUI DH, TANAHASHI H, OZAWA K, IKEDA S, CHECLER F, UEDA O, SUZUKI H, ARAKI W, INOUE H, SHIROTANI K, TAKAHASHI K, GALLYAS F, TABIRA T, 1999. Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. Nat Med 5: 560-4.

CHUN JT, WANG L, PASINETTI GM, FINCH CE, ZLOKOVIC BV, 1999. Glycoprotein 330/megalin (LRP-2) has low prevalence as mRNA and protein in brain microvessels and choroid plexus. Exp Neurol 157: 194-201.

CITRON M, 2000. Secretases as targets for the treatment of Alzheimer's disease. Mol Med Today 6: 392-7.

CITRON M, ECKMAN CB, DIEHL TS, CORCORAN C, OSTASZEWSKI BL, XIA W, LEVESQUE G, ST GEORGE HYSLOP P, YOUNKIN SG, SELKOE DJ, 1998. Additive effects of PS1 and APP mutations on secretion of the 42-residue amyloid beta-protein. Neurobiol Dis 5: 107-16.

CITRON M, OLTERSDORF T, HAASS C, MCCONLOGUE L, HUNG AY, SEUBERT P, VIGO-PELFREY C, LIEBERBURG I, SELKOE DJ, 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360: 672-4.

COOK DG, SUNG JC, GOLDE TE, FELSENSTEIN KM, WOJCZYK BS, TANZI RE, TROJANOWSKI JQ, LEE VM, DOMS RW, 1996. Expression and analysis of presenilin 1 in a human neuronal system: localization in cell bodies and dendrites. Proc Natl Acad Sci U S A 93: 9223-8.

COTMAN CW, ANDERSON AJ, 2000. The brain's microenvironment, early functional loss, and the conversion to Alzheimer's disease. Ann N Y Acad Sci 924:112-6.

COTMAN CW, SU JH, 1996. Mechanisms of neuronal death in Alzheimer's disease. Brain Pathol 6: 493-506.

COTMAN CW, TENNER AJ, CUMMINGS BJ, 1996. beta-Amyloid converts an acute phase injury response to chronic injury responses. Neurobiol Aging 17: 723-31.

COTMAN CW, ANDERSON AJ, 1995. A potential role for apoptosis in neurode-generation and Alzheimer's disease. Mol Neurobiol 10: 19 –45.CRISI GM, TSIAGBE VK, RUSSO C, BASCH RS, THORBECKE GJ, 1996. Evaluation of presence and functional activity of potentially self-reactive T cells in aged mice. Int Immunol 8: 387-95.

CRUCIAN, B., DUNNE, P., FRIEDMAN, H., RAGSDALE, R., PROSS, S., AND WIDEN, R., 1995a. Alterations in peripheral blood mononuclear cell cytokine production in response to phytohemagglutinin in multiple sclerosis patients. Clin.Diagn.Lab.Immunol. 2, 766-769.

CUNNINGHAM DD, VAN NOSTRAND WE, FARROW JS, WAGNER SL, BHASIN R, GOLDGABER D, COTMAN CW, 1991. The predominant form of the amyloid beta-protein precursor in human brain is protease nexin 2. Proc Natl Acad Sci U S A 88: 10302-6.

CZECH C, LESORT M, TREMP G, TERRO F, BLANCHARD V, SCHOMBERT B, CARPENTIER N, DREISLER S, BONICI B, TAKASHIMA A, MOUSSAOUI S, HUGON J, PRADIER L, 1998. Characterization of human presenilin 1 transgenic rats: increased sensitivity to apoptosis in primary neuronal cultures. Neuroscience 87: 325-36.

CZECH C, DELAERE P, MACQ AF, REIBAUD M, DREISLER S, TOUCHET N, SCHOMBERT B, MAZADIER M, MERCKEN L, THEISEN M, PRADIER L, OCTAVE JN, BEYREUTHER K, TREMP G, 1997. Proteolytical processing of mutated human amyloid precursor protein in transgenic mice. Brain Res Mol Brain Res 47: 108-16.

DALTON TP, SHERTZER HG, PUGA A, 1999. Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol 39: 67–101.

DAMLE NK, MOHAGHEGHPOUR N, HANSEN JA, ENGLEMAN EG, 1983. Alloantigen-specific cytotoxic and suppressor T lymphocytes are derived from phenotypically distinct precursors. J Immunol 131: 2296-300.

DAS S, POTTER H, 1995. Expression of the Alzheimer amyloid-promoting factor antichymotrypsin is induced in human astrocytes by IL-1. Neuron 14: 447-56.

DAVOUST N, JONES J, STAHEL PF, AMES RS, BARNUM SR, 1999. Receptor for the C3a anaphylatoxin is expressed by neurons and glial cells. Glia 26: 201-11.

DE GREY AD, 1997. A proposed refinement of the mitochondrial free radical theory of aging. BioEssays 19:161–6.

DE LA MONTE SM, SOHN YK, WANDS JR, 1997. Correlates of p53- and Fas (CD95)-mediated apoptosis in Alzheimer's disease. J Neurol Sci 152: 73-83.

DE LOS ANGELES ROBINSON-AGRAMONTE M, DORTA-CONTRERAS A, SERRANO- SÁNCHEZ T, MCRAE A, 1998. T Activation Markers From Cerebrospinal Fluid of Alzheimer's Disease Patients. Alz Dis Rev 3: 113-6 (http://www.mc.uky.edu/adreview/jamaica97.htm).

DE VOS K, GOOSSENS V, BOONE E, VERCAMMEN D, VANCOMPERNOLLE K, VANDENABEELE P, HAEGEMAN G, FIERS W, GROOTEN J, 1998. The 55-kDa tumor necrosis factor receptor induces clustering of mitochondria through its membrane-proximal region. J Biol Chem 273: 9673-80.

DEL BO R, ANGERETTI N, LUCCA E, DE SIMONI MG, FORLONI G, 1995. Reciprocal control of inflammatory cytokines, IL-1 and IL-6, and beta-amyloid production in cultures. Neurosci Lett 188: 70-4.

DOBBS, R.J., CHARLETT, A., PURKISS, A.G., DOBBS, S.M., WELLER, C., AND PETERSON, D.W., 1999. Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism. Acta Neurol.Scand. 100, 34-41.

DRAGUNOW M, FAULL RL, LAWLOR P, BEILHARZ EJ, SINGLETON K, WALKER EB, MEE E, 1995. In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. Neuroreport 6: 1053-7.

DROGE W, SCHULZE–OSTHOFF K, MIHM S, ET AL, 1994. Functions of glutathione and glutathione disulfide in immunology and immunopathology. FASEB J. 8: 1131–8.

DU YAN S, ZHU H, FU J, YAN SF, ROHER A, TOURTELLOTTE WW, RAJAVASHISTH T, CHEN X, GODMAN GC, STERN D, SCHMIDT AM, 1997. Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. Proc Natl Acad Sci U S A 94: 5296-5301.

EBSTEIN RP, NEMANOV L, LUBARSKI G, DANO M, TREVIS T, KORCZYN AD, 1996. Changes in expression of lymphocyte amyloid precursor protein mRNA isoforms in normal aging and Alzheimer's disease. Brain Res Mol Brain Res 35: 260-8.

ECKERT, A., OSTER, M., ZERFASS, R., HENNERICI, M., AND MÜLLER, W.E., 2001a. Elevated levels of fragmented DNA nucleosomes in native and activated lymphocytes indicate an enhanced sensitivity to apoptosis in sporadic Alzheimer's disease. Dement Geriatr Cogn Disord.12, 98-105.

ECKERT A, SCHINDOWSKI K, LEUTNER S, LUCKHAUS C, TOUCHET N, CZECH C, MULLER WE, 2001b. Alzheimer's disease-like alterations in peripheral cells from presenilin-1 transgenic mice. Neurobiol Dis 8: 331-42.

ECKERT A, STEINER B, MARQUES C, LEUTZ S, ROMIG H, HAASS C, MÜLLER WE, 2001c. Elevated vulnerability to oxidative stress-induced cell death and activation of caspase-3 by the Swedish amyloid precursor protein mutation. J Neurosci Res 64: 183-92.

ECKERT A, COTMAN CW, ZERFASS R, HENNERICI M, MÜLLER WE 1998a. Enhanced vulnerability to apoptotic cell death in sporadic Alzheimer's disease. Neuroreport **9**, 2443-6,.

ECKERT, A., FORSTL, H., ZERFASS, R., OSTER, M., HENNERICI, M., AND MÜLLER, W.E., 1998b. Changes of intracellular calcium regulation in Alzheimer's disease and vascular dementia. J.Neural Transm.Suppl. 54, 201-10.

ECKERT A, FORSTL H, ZERFASS R, HENNERICI M, MÜLLER WE, 1997a. Free intracellular calcium in peripheral cells in Alzheimer's disease. Neurobiol Aging 18: 281-4.

ECKERT A, OSTER M, FORSTL H, HENNERICI M, MULLER WE, 1997b. Impaired calcium regulation in subcortical vascular encephalopathy. Stroke 28: 1351-6.

ECKERT A, FORSTL H, HARTMANN H, CZECH C, MONNING U, BEYREUTHER K, MÜLLER WE, 1995a. The amplifying effect of beta-amyloid on cellular calcium signalling is reduced in Alzheimer's disease. Neuroreport 6: 1199-202.

ECKERT A, FORSTL H, ZERFASS R, HARTMANN H, MULLER WE, 1995b. Lymphocytes and neutrophils as peripheral models to study the effect of beta-amyloid on cellular calcium signalling in Alzheimer's disease. LifeSci 96: 499-510.

ECKERT, A., HARTMANN, H., FORSTL, H., AND MÜLLER, W.E., 1994. Alterations of intracellular calcium regulation during aging and Alzheimer's disease in nonneuronal cells. Life Sci. 55, 2019-29.

ECKERT A, HARTMANN H, MULLER WE, 1993. beta-Amyloid protein enhances the mitogen-induced calcium response in circulating human lymphocytes. FEBS Lett 330: 49-52.

EL KHOURY J, HICKMAN SE, THOMAS CA, LOIKE JD, SILVERSTEIN SC, 1998. Microglia, scavenger receptors, and the pathogenesis of Alzheimer's disease. Neurobiol Aging 19(Suppl):S81-4

ELLIS HM, HORVITZ HR, 1986. Genetic control of programmed cell death in the nematode C. elegans. Cell 44: 817-29.

ERIKSSON S, JANCIAUSKIENE S, LANNFELT L, 1995. Alpha 1-antichymotrypsin regulates Alzheimer beta-amyloid peptide fibril formation. Proc Natl Acad Sci U S A 92: 2313-7.

ESPEY MG, CHERNYSHEV ON, REINHARD JF JR, NAMBOODIRI MA, COLTON CA, 1997. Activated human microglia produce the excitotoxin quinolinic acid. Neuroreport 8: 431-4.

ETCHEBERRIGARAY R, ITO E, KIM CS, ALKON DL, 1994. Soluble beta-amyloid induction of Alzheimer's phenotype for human fibroblast K+ channels. Science 264: 276-9.

FAHEY, J.L., SCHNELLE, J.F., BOSCARDIN, J., THOMAS, J.K., GORRE, M.E., AZIZ, N., SADEGHI, H., AND NISHANIAN, P., 2000. Distinct categories of immunologic changes in frail elderly. Mech.Ageing Dev. 115, 1-20.

FERGUSON FG, WIKBY A, MAXSON P, OLSSON J, JOHANSSON B, 1995. Immune parameters in a longitudinal study of a very old population of Swedish people: A comparison between survivors and nonsurvivors. J Gerontol Ser A-Biol Sci Med 50: B378-82.

FERRERO P, ROCCA P, EVA C, BENNA P, REBAUDENGO N, RAVIZZA L, GENAZZANI E, BERGAMASCO B, 1991. An analysis of lymphocyte 3H-N-methyl-scopolamine binding in neurological patients. Evidence of altered binding in Alzheimer's disease. Brain 114 (Pt 4):1759-70.

FIALA M, 2000. Mononuclear cell transmigration across the blood-brain barrier (BBB) induced by amyloid beta. (abstract 2.20). Neuroinflammation in Alzheimer's disease, Washington D.C. (USA) 7-8 July 2000.

FILLIT H, DING WH, BUEE L, KALMAN J, ALTSTIEL L, LAWLOR B, WOLF-KLEIN G, 1991. Elevated circulating tumor necrosis factor levels in Alzheimer's disease. Neurosci Lett 129: 318-20.

FRANCESCHI C, MONTI D, SANSONI P, COSSARIZZA A, 1995. The immunology of exceptional individuals: the lesson of centenarians. Immunol Today 16: 12-6.

FREEDMAN RS, TOMASOVIC B, TEMPLIN S, ATKINSON EN, KUDELKA A, EDWARDS CL, PLATSOUCAS CD, 1994. Large-scale expansion in interleukin-2 of tumor-infiltrating lymphocytes from patients with ovarian carcinoma for adoptive immunotherapy. J Immunol Methods 167: 145-60.

FRENCH LR, SCHUMAN LM, MORTIMER JA, HUTTON JT, BOATMAN RA, CHRISTIANS B, 1985. A case-control study of dementia of the Alzheimer type Am J Epidemiol 121: 414-21.

FUKUYAMA R, MURAKAWA Y, RAPOPORT SI, 1994. Induction of gene expression of amyloid precursor protein (APP) in activated human lymphoblastoid cells and lymphocytes. Mol Chem Neuropathol 23: 93-101.

FULOP TJ, LEBLANC, C., LACOMBE, G., AND DUPUIS, G., 1995. Cellular distribution of protein kinase C isozymes in CD3-mediated stimulation of human T lymphocytes with aging. FEBS Lett. 375: 69-74.

GAINES, H., ANDERSSON, L., AND BIBERFELD, G., 1996. A new method for measuring lymphoproliferation at the single-cell level in whole blood cultures by flow cytometry. J.Immunol.Methods 195, 63-72.

GARIBALDI, D.C. AND ZHANG, K., 1999. Molecular genetics of macular degeneration. Int.Ophthalmol.Clin. 39, 117-142.

GASPARINI L, RACCHI M, BINETTI G, TRABUCCHI M, SOLERTE SB, ALKON D, ETCHEBERRIGARAY R, GIBSON G, BLASS J, PAOLETTI R, GOVONI S, 1998. Peripheral markers in testing pathophysiological hypotheses and diagnosing Alzheimer's disease. FASEB J. 12, 17–34.

GASQUE P, SINGHRAO SK, NEAL JW, WANG P, SAYAH S, FONTAINE M, MORGAN BP, 1998. The receptor for complement anaphylatoxin C3a is expressed by myeloid cells and nonmyeloid cells in inflamed human central nervous system: analysis in multiple sclerosis and bacterial meningitis. J Immunol 160: 3543-54.

GERVAIS FG, XU D, ROBERTSON GS, VAILLANCOURT JP, ZHU Y, HUANG J, LEBLANC A, SMITH D, RIGBY M, SHEARMAN MS, CLARKE EE, ZHENG H, VAN DER PLOEG LH, RUFFOLO SC, THORNBERRY NA, XANTHOUDAKIS S, ZAMBONI RJ, ROY S, NICHOLSON DW, 1999. Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. Cell 97: 395-406.

GHISO J, TAGLIAVINI F, TIMMERS WF, FRANGIONE B, 1989 Alzheimer's disease amyloid precursor protein is present in senile plaques and cerebrospinal fluid: immunohistochemical and biochemical characterization. Biochem Biophys Res Commun 163: 430-7.

GIANNAKOPOULOS P, KOVARI E, SAVIOZ A, DE BILBAO F, DUBOIS-DAUPHIN M, HOF PR, BOURAS C, 1999. Differential distribution of presenilin-1, Bax, and Bcl-X(L) in Alzheimer's disease and frontotemporal dementia. Acta Neuropathol (Berl) 98: 141-9.

GIETZEN DW, FREGEAU D, GOODMAN T, WEILER PG, GRAF K, MAGLIOZZI J, 1989. Lymphocyte beta-adrenoceptor/effector complex in aging and dementia of the Alzheimer type. Alzheimer Dis Assoc Disord 3: 132-42.

GIULIAN D, HAVERKAMP LJ, YU JH, KARSHIN W, TOM D, LI J, KIRKPATRICK J, KUO LM, ROHER AE, 1996. Specific domains of beta-amyloid from Alzheimer plaque elicit neuron killing in human microglia. J Neurosci 16: 6021-37.

GOATE A, CHARTIER-HARLIN MC, MULLAN M, BROWN J, CROWFORD F, FIDANI L, GIUFFRA A, HAYNES A, IRVING N, JAMES L, MANT R, NEWTON P, ROOKE P, ROGES P, TALBOT C, PERICAK-VANCE M, ROSES A, WILLIAMSON R, ROSSER M, OWEN M, HARDY J, 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349, 704-6.

GOEDERT M, JAKES R, SPILLANTINI MG, HASEGAWA M, SMITH MJ, CROWTHER RA, 1996. Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. Nature 383: 550-3.

GOOD PF, WERNER P, HSU A, OLANOW CW, PERL DP, 1996. Evidence of neuronal oxidative damage in Alzheimer's disease. Am J Pathol 149: 21-8.

GOODMAN Y, MATTSON MP, 1994. Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury. Exp Neurol 128: 1-12.

GRAVES AB, WHITE E, KOEPSELL TD, REIFLER BV, VAN BELLE G, LARSON EB, RASKIND M, 1990. A case-control study of Alzheimer's disease. Ann Neurol 28: 766-74 .

GREENBERG CS, BIRCKBICHLER PJ, RICE RH, 1991. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J 5: 3071-7.

GRIFFIN WS, SHENG JG, ROYSTON MC, GENTLEMAN SM, MCKENZIE JE, GRAHAM DI, ROBERTS GW, MRAK RE, 1998. Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. Brain Pathol 8: 65-72.

GRIFFIN WS, SHENG JG, ROBERTS GW, MRAK RE, 1995. Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. J Neuropathol Exp Neurol 54: 276-81.

GRIFFITH, W.H., JASEK, M.C., BAIN, S.H., MURCHISON, D., 2000. Modification of ion channels and calcium homeostasis of basal forebrain neurons during aging. Behav. Brain Res. 115, 219-233.

GROSSMANN A, KUKULL WA, JINNEMAN JC, BIRD TD, VILLACRES EC, IARSON EB, RABINOVITCH PS, 1993. Intracellular calcium response is reduced in CD4+ lymphocytes in Alzheimer's disease and in older persons with Down's syndrome. Neurobiol Aging 14: 177-85.

GRÜNBERG J, WALTER J, LOETSCHER H, DEUSCHLE U, JACOBSEN H, HAASS C, 1998. Alzheimer's disease associated presenilin-1 holoprotein and its 18-20 kDa C-terminal fragment are death substrates for proteases of the caspase family. Biochemistry 37: 2263-70.

GUIDI, L., TRICERRI, A., FRASCA, D., VANGELI, M., ERRANI, A.R., AND BARTOLONI, C., 1998. Psychoneuroimmunology and aging.. Gerontology 44, 247-26.

GUO Q, FU W, HOLTSBERG FW, STEINER SM, MATTSON MP, 1999a. Superoxide mediates the cell-death-enhancing action of presenilin-1 mutations. J Neurosci Res 56: 457-70.

GUO Q, FU W, SOPHER BL, MILLER MW, WARE CB, MARTIN GM, MATTSON MP, 1999b. Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. Nat Med 5: 101-6.

GUO Q, SOPHER BL, FURUKAWA K, PHAM DG, ROBINSON N, MARTIN GM, MATTSON M, 1997. Alzheimer's presenilin mutation sensitizes neural cells to apoptosis induced by trophic factor withdrawal and amyloid beta-peptide: involvement of calcium and oxyradicals. J Neurosci 17: 4212-22.

GUYTON, K.Z., GOROSPE, M., WANG, X., MOCK, Y.D., KOKKONEN, G.C., LIU, Y., ROTH, G.S., AND HOLBROOK, N.J., 1998. Age-related changes in activation of mitogen-activated protein kinase cascades by oxidative stress. J.Investig.Dermatol.Symp.Proc. 3, 23-27.

HAASS, C. 1999. Molekulare Mechanismen der Alzheimer Erkrankung. In: Alzheimer Demenz: Grundlagen, Klinik und Therapie. edited by Förstl, H., Bickel, H. and Kurz, A. Springer Verlag, Berlin.

HAASS C, 1996. Presenile because of presenilin: the presenilin genes and early onset Alzheimer's disease. Curr Opin Neurol 9: 254-9.

HAASS C, HUNG AY, SELKOE DJ, TEPLOW DB, 1994. Mutations associated with a locus for familial Alzheimer's disease result in alternative processing of amyloid beta-protein precursor. J Biol Chem 269: 17741-8.

HAMANN, J., FIEBIG, H., AND STRAUSS, M., 1998. Expression cloning of the early activation antigen CD69, a type II integral membrane protein with a C-type lectin domain. J.Immunol. 150, 4920-4927, 1993.Eckert, A., Cotman, C.W., Zerfass, R., Hennerici, M., and Muller, W.E., Enhanced vulnerability to apoptotic cell death in sporadic Alzheimer's disease. Neuroreport 9, 2443-2446.

HAMPEL H, SUNDERLAND T, KOTTER HU, SCHNEIDER C, TEIPEL SJ, PADBERG F, DUKOFF R, LEVY J, MOLLER HJ, 1998. Decreased soluble interleukin-6 receptor in cerebrospinal fluid of patients with Alzheimer's disease. Brain Res 780: 356-9.

HAN, D., HOSOKAWA, T., AOIKE, A., AND KAWAI, K., 1995. Age-related enhancement of tumor necrosis factor (TNF) production in mice. Mech.Ageing Dev. 84, 39-54.

HANISCH, U.K., NEUHAUS, J., QUIRION, R., AND KETTENMANN, H., 1996. Neurotoxicity induced by interleukin-2: involvement of infiltrating immune cells. Synapse 24, 104-114.

HARTMANN H, ECKERT A, VELBINGER K, REWSIN M, MULLER WE, 1996. Down-regulation of free intracellular calcium in dissociated brain cells of aged mice and rats. Life Sci 59: 435–49.

HARTMANN H, ECKERT A, MULLER WE, 1994a. Apolipoprotein E and cholesterol affect neuronal calcium signalling: the possible relationship to beta-amyloid neurotoxicity. Biochem Biophys Res Commun 200: 1185-92

HARTMANN H, ECKERT A, FORSTL H, MULLER WE, 1994b. Similar age-related changes of free intracellular calcium in lymphocytes and central neurons: effects of Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci 243: 218-23.

HARTMANN H, ECKERT A, MULLER WE, 1994c. Disturbances of the neuronal calcium homeostasis in the aging nervous system. Life Sci 55: 2011-8.

HENDRICKS, L.C. AND HEIDRICK, M.L., 1998. Susceptibility to lipid peroxidation and accumulation of fluorescent products with age is greater in T-cells than B-cells. Free Radic.Biol.Med. 5, 145-154.

HERNDON FJ, HSU HC, MOUNTZ JD, 1997. Increased apoptosis of CD45RO(-) T cells with aging. Mech Ageing Dev 94: 123-34.

HEYMAN A, WILKINSON WE, STAFFORD JA, HELMS MJ, SIGMON AH, WEINBERG T, 1984. Alzheimer's disease: a study of epidemiological aspects. Ann Neurol 15: 335-41.

HICKEY WF, KIMURA H, 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science 239: 290-2

HIJMANS W, RADL J, BOTTAZZO GF, DONIACH D, 1984. Autoantibodies in highly aged humans. Mech.Ageing Dev. 26, 83-89.

HIRANO T, NAKAJIMA K, HIBI M, 1997. Signaling mechanisms through gp130: a model of the cytokine system. Cytokine Growth Factor Rev 8 :241-52.

HO L, OSAKA H, AISEN PS, PASINETTI GM, 1999. Induction of cyclooxygenase (COX)-2 but not COX-1 gene expression in apoptotic cell death. J Neuroimmunol 89: 142-9.

HOCKENBERY DM, OLTVAI ZN, YIN XM, MILLIMAN CL, KORSMEYER SJ, 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75: 241-51.

HORIUCHI S, WILMOTH JR, 1997. Age patterns of the life table aging rate for major causes of death in Japan, 1951-1990. J Gerontol Ser A-Biol Sci Med 52, B67-77.

HOSHINO T, YAMADA A, HONDA J, IMAI Y, NAKAO M, INOUE M, SAGAWA K, YOKOYAMA MM, OIZUMI K, ITOH K, 1993. Tissue-Specific Distribution and Age-Dependent Increase of Human CD11b+T-Cells. J Immunol 151: 2237-46.

HSU H, SHU HB, PAN MG, GOEDDEL DV, 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84: 299-308.

HU GR, WALLS RS, CREASEY H, MCCUSKER E, BROE GA, 1995. Peripheral blood lymphocyte subset distribution and function in patients with Alzheimer's disease and other dementias. Aust N Z J Med 25: 212-7.

HUBERT, P., GRENOT, P., AUTRAN, B., AND DEBRE, P., 1997. Analysis by flow cytometry of tyrosine-phosphorylated proteins in activated T-cell subsets on whole blood samples. Cytometry 29, 83-91.

HUPPERT FA, SOLOMOU W, O'CONNOR S, MORGAN K, SUSSAMS P, BRAYNE C, 1998. Aging and lymphocyte subpopulations: Whole-blood analysis of immune markers in a large population sample of healthy elderly individuals. Exp Gerontol 33: 593-600.

HYMAN BT, WEST HL, REBECK GW, LAI F, MANN DM, 1995. Neuropathological changes in Down's syndrome hippocampal formation. Effect of age and apolipoprotein E genotype. Arch Neurol 52: 373-8.

IBARRETA D, PARRILLA R, AYUSO MS, 1997. Altered Ca2+ homeostasis in lymphoblasts from patients with late-onset Alzheimer disease. Alzheimer Dis Assoc Disord 11: 220-7.

IKEDA T, YAMAMOTO K, TAKAHASHI K, YAMADA M., 1991a. Immune system-associated antigens on the surface of peripheral blood lymphocytes in patients with Alzheimer's disease. Acta Psychiatr Scand 83: 444-8

IKEDA T, YAMAMOTO K, TAKAHASHI K, KANEYUKI H, YAMADA M, 1991b. Interleukin-2 receptor in peripheral blood lymphocytes of Alzheimer's disease patients. Acta Psychiatr Scand 84: 262-5.

IN 'T VELD BA, LAUNER LJ, HOES AW, OTT A, HOFMAN A, BRETELER MM, STRICKER BH, 1998. NSAIDs and incident Alzheimer's disease. The Rotterdam Study. Neurobiol Aging 19: 607-11.

IRWIN, M., DANIELS, M., BLOOM, E.T., SMITH, T.L., AND WEINER, H., 1987. Life events, depressive symptoms, and immune function. Am.J.Psychiatry 144, 437-441.

ITAGAKI S, MCGEER PL, AKIYAMA H, 1988. Presence of T-cytotoxic suppressor and leucocyte common antigen positive cells in Alzheimer's disease brain tissue. Neurosci Lett 91: 259-64

JANEWAY CA AND TRAVERS P, 1997. Immunologie. 2nd edition, Spektrum Akademischer Verlag GmbH, Heidelberg.

JENKINSON ML, BLISS MR, BRAIN AT, SCOTT DL, 1989. Rheumatoid arthritis and senile dementia of the Alzheimer's type. Br J Rheumatol 28: 86-8.

JIANG H, BURDICK D, GLABE CG, COTMAN CW, TENNER AJ, 1994. beta-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. J Immunol 152: 5050-9.

JIMENEZ-JIMENEZ FJ, MOLINA JA, GOMEZ P, VARGAS C, DE BUSTOS F, BENITO-LEON J, TALLON-BARRANCO A, ORTI-PAREJA M, GASALLA T, ARENAS J, 1998. Neurotransmitter amino acids in cerebrospinal fluid of patients with Alzheimer's disease. J Neural Transm 105: 269-77.

JOHNSON GVW , HARTIGAN JA, 1998. Tau Protein in Normal and Alzheimer's Disease Brain: An Update. Alzheimer's Disease Review 3, 125-141. (http://www.mc.uky.edu/adreview/Vol3/Johnson/Johnson-ab.htm).

JUNG SS, GAUTHIER S, CASHMAN NR, 1999. Beta-amyloid precursor protein is detectable on monocytes and is increased in Alzheimer's disease. Neurobiol Aging 20: 249-57.

KALTSCHMIDT B, UHEREK M, VOLK B, BAEUERLE PA, KALTSCHMIDT C, 1997. Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease. Proc Natl Acad Sci U S A 94: 2642-7.

KAMBOH MI, SANGHERA DK, FERRELL RE, DEKOSKY ST, 1995. APOE*4-associated Alzheimer's disease risk is modified by alpha 1-antichymotrypsin polymorphism. Nat Genet 10: 486-8.

KANG J, IEMAIRE HG, UNTERBECK A, SALBAUM JM, MASTERS CL, GRZESCHNIK KH, MULTHAUP G, BAYREUTHER K, MÜLLER-HILL B, 1987. The precursor of alzheimer's disease amyloid A4 protein resembles a cell surface receptor. Nature 325: 733-6.

KANOWSKI S, HERRMANN WM, STEPHAN K, WIERICH W, HORR R, 1996. Proof of efficacy of the ginkgo biloba special extract EGb 761 in outpatients suffering from mild to moderate primary degenerative dementia of the Alzheimer type or multi-infarct dementia. Pharmacopsychiatry. 29:47-56.

KERR JF, WYLLIE AH, CURRIE AR, 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-57.

KIM JW, CHANG TS, LEE JE, HUH SH, YEON SW, YANG WS, JOE CO, MOOK-JUNG I, TANZI RE, KIM TW, CHOI EJ, 2001. Negative regulation of the SAPK/JNK signaling pathway by presenilin 1. J Cell Biol 153: 457-63.

KING CM, GILLESPIE ES, MCKENNA PG, BARNETT YA, 1994. AN INVESTIGATION OF MUTATION AS A FUNCTION OF AGE IN HUMANS. MUTATION RESEARCH 316: 79-90.

KITAMURA Y, SHIMOHAMA S, KAMOSHIMA W, ET AL, 1998. Alteration of proteins regulating apoptosis, Bcl-2, Bcl-x, Bax, Bak, Bad, ICH-1 and CPP32, in Alzheimer's disease. Brain Res 780: 260 –9.

KLETSAS D, BARBIERI D, STATHAKOS D, ET AL, 1998. The highly reducing sugar 2-deoxy-D-ribose induces apoptosis in human fibroblasts by reduced glutathione depletion and cytoskeletal disruption. Biochem Biophys Res Commun 243: 416 –25.

KORNHUBER, J., HUNDACKER, I., MALER, M., OTTO, M., WILTFANG, J. 1999. Neurotransmitterveränderungen bei der Alzheimer Demenz. In: Alzheimer Demenz: Grundlagen, Klinik und Therapie. edited by Förstl, H., Bickel, H. and Kurz, A. Springer Verlag, Berlin.

KOSE K, DOGAN P, ASCIOGLU M, ASCIOGLU O, 1997. In vitro antioxidant effect of Ginkgo biloba extract (EGb 761) on lipoperoxidation induced by hydrogen peroxide in erythrocytes of Behcet's patients. Jpn J Pharmacol 75: 253-8.

KOVACS DM, FAUSETT HJ, PAGE KJ, KIM TW, MOIR RD, MERRIAM DE, HOLLISTER RD, HALLMARK OG, MANCINI R, FELSENSTEIN KM, HYMAN BT, TANZI RE, WASCO W, 1996. Alzheimer-associated presenilins

1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. Nat Med 2: 224-9.

KRIEGLSTEIN K, RUFER M, SUTER-CRAZZOLARA C, UNSICKER K, 1995. Neural functions of the transforming growth factors beta. Int J Dev Neurosci 13: 301-15.

KRISHNARAJ, R. AND BLANDFORD, G., 1987. Age-associated alterations in human natural killer cells. 1. Increased activity as per conventional and kinetic analysis. Clin.Immunol.Immunopathol. 45, 268-285.

KROHN AJ, PREIS E, PREHN JH, 1998. Staurosporine-induced apoptosis of cultured rat hippocampal neurons involves caspase-1-like proteases as upstream initiators and increased production of superoxide as a main downstream effector. J Neurosci 18: 8186-97.

KUO CT, LEIDEN JM, 1999. Transcriptional regulation of T lymphocyte development and function. Annu.Rev.Immunol. 17: 149-187.

LADU MJ, GILLIGAN SM, IUKENS JR, CABANA VG, REARDON CA, VAN ELDIK LJ, HOLTZMAN DM, 1998. Nascent astrocyte particles differ from lipoproteins in CSF. J Neurochem 70: 2070-81.

LASKOWITZ DT, MATTHEW WD, BENNETT ER, SCHMECHEL D, HERBSTREITH MH, GOEL S, MCMILLIAN MK, 1998. Endogenous apolipoprotein E suppresses LPS-stimulated microglial nitric oxide production. Neuroreport 9: 615-8.

LASKOWITZ DT, GOEL S, BENNETT ER, MATTHEW WD, 1997. Apolipoprotein E suppresses glial cell secretion of TNF alpha. J Neuroimmunol 76: 70-4.

LE BARS PL, KATZ MM, BERMAN N, ITIL TM, FREEDMAN AM, SCHATZBERG AF, 1997. A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo biloba for dementia. North American EGb Study Group. JAMA 278:1327-1332.

LEBLHUBER F, WALLI J, TILZ GP, WACHTER H, FUCHS D, 1998. Systemic changes of the immune system in patients with Alzheimer's dementia. Dtsch Med Wochenschr 123: 787-91.

LECHNER H, AMORT M, STEGER MM, MACZEK C, GRUBECK–LOEBENSTEIN B, 1996. Regulation of CD95 (APO-1) expression and the induction of apoptosis in human T cells: changes in old age. Int Arch Allergy Immunol 110: 238 –43.

LEDOUX S, BERGERON C, NALBANTOGLU J, GAUTHIER S, CASHMAN NR , 1995. Polymerase chain reaction quantification of lymphoid amyloid precursor protein mRNAs in Alzheimer's disease and Down's syndrome. Neurosci Lett 193: 137-9.

LEDOUX S, REBAI N, DAGENAIS A, SHAW IT, NALBANTOGLU J, SEKALY RP, CASHMAN NR, 1993. Amyloid precursor protein in peripheral mononuclear cells is up-regulated with cell activation. J Immunol 150: 5566-75.

LEE RK, WURTMAN RJ, 2000. Regulation of APP synthesis and secretion by neuroimmunophilin ligands and cyclooxygenase inhibitors. Ann N Y Acad Sci 920: 261-8.

LEE SC, DICKSON DW, LIU W, BROSNAN CF, 1993. Induction of nitric oxide synthase activity in human astrocytes by interleukin-1 beta and interferon-gamma. J Neuroimmunol 46: 19-24.

LEFFELL MS, IUMSDEN L, STEIGER WA, 1985. An analysis of T lymphocyte subpopulations in patients with Alzheimer's disease. J Am Geriatr Soc 33: 4-8.

LEMKE MR, GLATZEL M, HENNEBERG AE, 1999. Antimicroglia antibodies in sera of Alzheimer's disease patients. Biol Psychiatry 45: 508-11

LENARDO MJ, 1991. IL-2 programs mouse alpha beta T lymphocytes for apoptosis. Nature 353: 858 -61.

LENKEI, R., BRATT, G., HOLMBERG, V., MUIRHEAD, K., AND SANDSTROM, E., 1998. Indicators of T-cell activation: correlation between quantitative CD38 expression and soluble CD8 levels in asymptomatic HIV+ individuals and healthy controls. Cytometry 33, 115-122.

LEUTNER S, 2001. thesis: Metabolismus reaktiver Sauerstoffspezies im Alter und bei der Alzheimer Demenz. Johann Wolfgang-Goethe Universität, Frankfurt am Main

LEUTNER S, CZECH C, SCHINDOWSKI K, TOUCHET N, ECKERT A, MÜLLER WE, 2000. Reduced antioxidant enzyme activity in brains of mice transgenic for human presenilin-1 with single or multiple mutations. Neurosci Lett 292: 87-90.

LEVITAN D, GREENWALD I, 1995. Reduced antioxidant enzyme activity in brains of mice transgenic for human presenilin-1 with single or multiple mutations. Neurosci Lett 292: 87-90.

LEVY-LEHAD ET AL., 1995. The structure of the presenilin 1 (S182) gene and identification of six novel mutations in early onset AD families. Alzheimer's Disease Collaborative Group. Nat Genet 11: 219-22.

L'HERNAULT SW, ARDUENGO PM, 1992. Mutation of a putative sperm membrane protein in Caenorhabditis elegans prevents sperm differentiation but not its associated meiotic divisions. J Cell Biol 119: 55-68.

LI G, SHEN YC, LI YT, CHEN CH, ZHAU YW, SILVERMAN JM, 1992. A case-control study of Alzheimer's disease in China. Neurology 42: 1481-8.

LI J, MA J, POTTER H, 1995. Identification and expression analysis of a potential familial Alzheimer disease gene on chromosome 1 related to AD3. Proc Natl Acad Sci U S A 92: 12180-4.

LIGTHART GJ, CORBERAND JX, FOURNIER C, GALANAUD P, HIJMANS W, KENNES B, MULLER-HERMELINK HK, AND STEINMANN GG, 1984. Admission criteria for immunogerontological studies in man: the SENIEUR protocol. Mech.Ageing Dev. 28, 47-55.

LINDER E, LEHTO VP, STENMAN S, 1979. Activation of complement by cytoskeletal intermediate filaments. Nature 278: 176-8.

LING EA, WONG WC, 1993. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. Glia 7: 9-18.

LIO D, BALISTRERI CR, CANDORE G, D'ANNA C, DI LORENZO G, GERVASI F, LISTI F, SCOLA L, AND CARUSO C, 2000. In vitro treatment with interleukin-2 normalizes type-1 cytokine production by lymphocytes from elderly. Immunopharmacol Immunotoxicol 22,195-203.

LOMBARDI VR, GARCIA M, REY L, CACABELOS R, 1999. Characterization of cytokine production, screening of lymphocyte subset patterns and in vitro apoptosis in healthy and Alzheimer's Disease (AD) individuals. J Neuroimmunol 97: 163-71.

LOMBARDI VR, GARCIA M, CACABELOS R, 1998. Microglial activation induced by factor(s) contained in sera from Alzheimer-related ApoE genotypes. J Neurosci Res 54: 539-53.

LU DC, RABIZADEH S, CHANDRA S, SHAYYA RF, ELLERBY LM, YE X, SALVESEN GS, KOO EH, BREDESEN DE, 2000. A second cytotoxic proteolytic peptide derived from amyloid beta-protein precursor. Nat Med 6: 397-404.

LUCCA U, TETTAMANTI M, FORLONI G, SPAGNOLI A, 1994. Nonsteroidal antiinflammatory drug use in Alzheimer's disease. Biol Psychiatry 36: 854-6.

LUE LF, KUO YM, ROHER AE, BRACHOVA L, SHEN Y, SUE L, BEACH T, KURTH JH, RYDEL RE, ROGERS J, 1999. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol 155: 853-62.

LUO J, LANG JA, MILLER MW, 1998. Transforming growth factor beta1 regulates the expression of cyclooxygenase in cultured cortical astrocytes and neurons. J Neurochem 71: 526-34.

MACHO A, HIRSCH T, MARZO I, MARCHETTI P, DALLAPORTA B, SUSIN SA, ZAMZAMI N, KROEMER G, 1997. Glutathione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. J Immunol 158: 4612-9.

MACKENZIE IR, 2000. Anti-inflammatory drugs and Alzheimer-type pathology in aging. Neurology 54: 732-4.

MACKENZIE IR, MUNOZ DG, 1998. Nonsteroidal anti-inflammatory drug use and Alzheimer-type pathology in aging. Neurology 50: 986-90.

MACKENZIE IR, HAO C, MUNOZ DG, 1995. Role of microglia in senile plaque formation. Neurobiol Aging 16: 797-804.

MAESAKA JK, PALAIA T, CHOWDHURY SA, SHIMAMURA T, FISHBANE S, REICHMAN W, COYNE A, O'REAR JJ, EL-SABBAN ME, 1999. Partial characterization of apoptotic factor in Alzheimer plasma. Am J Physiol 276(4 Pt 2): F521-7.

MARKESBERY WR, CARNEY JM, 1999. Oxidative alterations in Alzheimer's disease. Brain Pathol 9: 133-46.

MATIBA B, MARIANI SM, KRAMMER PH, 1997. The CD95 system and the death of a lymphocyte. Semin Immunol 9: 59 –68.

MATTSON MP, CHAN SL, CAMANDOLA S, 2001. Presenilin mutations and calcium signaling defects in the nervous and immune systems. BioEssays 23:733-44.

MATTSON MP, 2000. Emerging neuroprotective strategies for Alzheimer's disease: dietary restriction, telomerase activation, and stem cell therapy. Exp Gerontol 35: 489-502.

MATTSON MP, PEDERSEN WA, 1998. Effects of amyloid precursor protein derivatives and oxidative stress on basal forebrain cholinergic systems in Alzheimer's disease. Int J Dev Neurosci 16: 737-53.

MATTSON MP, RYDEL RE, LIEBERBURG I, SMITH-SWINTOSKY VL, 1993a. Altered calcium signaling and neuronal injury: stroke and Alzheimer's disease as examples. Ann N Y Acad Sci 679: 1-21.

MATTSON MP, BARGER SW, 1993b. Roles for calcium signaling in structural plasticity and pathology in the hippocampal system.. Hippocampus 3: 73-87

MATTSON MP, CHENG B, DAVIS D, BRYANT K, LIEBERBURG I, RYDEL RE, 1992. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci 12: 376-89.

MAZARI L, LESOURD BM, 1998. Nutritional influences on immune response in healthy aged persons. Mech Ageing Dev 104: 25-40.

MCGEER PL, SCHULZER M, MCGEER EG, 1996. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. Neurology 47: 425-32.

MCGEER PL, MCGEER EG, 1995. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases.Brain Res Rev 21: 195-218.

MCGEER PL, MCGEER E, ROGERS J, SIBLEY J, 1990. Anti-inflammatory drugs and Alzheimer disease. Lancet 335: 1037.

MCGEER PL, AKIYAMA H, ITAGAKI S, MCGEER EG, 1989. Immune system response in Alzheimer's disease. Can J Neurol Sci 16 Suppl: 516-27

MCKEE AC, KOWALL NW, SCHUMACHER JS, BEAL MF, 1998. The neurotoxicity of amyloid beta protein in aged primates. Amyloid 5: 1-9.

MECOCCI P, CHERUBINI A, SENIN U, 1997. Increased oxidative damage in lymphocytes of Alzheimer's disease patients. J Am Geriatr Soc; 45:1536 –7.

MECOCCI P, MACGARVEY U, BEAL MF, 1994. Oxidative damage to mitochon-drial DNA is increased in Alzheimer's disease. Ann Neurol 36: 747–51.

MEHLER MF, GOKHAN S, 2001. Developmental mechanisms in the pathogenesis of neurodegenerative diseases. Prog Neurobiol 63: 337-63

MERRILL JE, MURPHY SP, 1997. Inflammatory events at the blood brain barrier: regulation of adhesion molecules, cytokines, and chemokines by reactive nitrogen and oxygen species. Brain Behav Immunity 11:245-63

MESULAM MM, 2000, A plasticity-based theory of the pathogenesis of Alzheimer's disease. Ann NY Acad Sci Ann N Y Acad Sci 924: 42-52

MEVORACH D, 1999, The immune response to apoptotic cells.. Ann NY Acad Sci, 887: 191-8

MILLER AE, 1980. Selective decline in cellular immune response to varicella-zoster in the elderly. Neurology 30, 582-587.

MILLER RA, 1997. Age-related changes in T cell surface markers: A longitudinal analysis in genetically heterogeneous mice. Mech Ageing Dev 96: 181-96.

MIQUEL J, ECONOMOS AC, FLEMING J, JOHNSON JE JR, 1980. Mitochondrial role in cell aging. Exp Gerontol, 15:575-91.

MONNING U, SANDBRINK R, BANATI RB, MASTERS CL, BEYREUTHER K, 1994. Transforming growth factor beta mediates increase of mature transmembrane amyloid precursor protein in microglial cells. FEBS Lett 342: 267-72.

MONNING U, KONIG G, BANATI RB, MECHLER H, CZECH C, GEHRMANN J, SCHREITER-GASSER U, MASTERS CL, BEYREUTHER K, 1992. Alzheimer beta A4-amyloid protein precursor in immunocompetent cells. J Biol Chem 267: 23950-6

MONNING U, KONIG G, PRIOR R, MECHLER H, SCHREITER-GASSER U, MASTERS CL, BEYREUTHER K, 1990. Synthesis and secretion of Alzheimer amyloid beta A4 precursor protein by stimulated human peripheral blood leucocytes. FEBS Lett 277: 261-6.

MOUSSAOUI S, BLANCHARD V, BONICI B, CZECH C, TREMP GL, TOUCHET N, PRADIER L, IMPERATO A, 1999. Progression of neuropathology in several transgenic mice expressing a single gene or two linked to familial Alzheimer's disease: APP, PS-1 or both proteins. Soc Neurosci 25: 1046 (abtract 426.7).

MRAK RE, SHENG JG, GRIFFIN WS, 1996. Correlation of astrocytic S100 beta expression with dystrophic neurites in amyloid plaques of Alzheimer's disease. J Neuropathol Exp Neurol 55: 273-9.

MULLA M, CRAWFORD F, AXELMAN K, HOULDEN H, LILIUS L, WINBLAD B, LANNFELT, L, 1992.. A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1, 345-7.

MULLAN M, CRAWFORD F, AXELMAN K, HOULDEN H, LILIUS L, WINBLAD B, LANNFELT L, 1992. A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1: 345-7.

MULLBERG J, DURIE FH, OTTEN-EVANS C, ALDERSON MR, ROSE-JOHN S, COSMAN D, BLACK RA, MOHLER KM, 1995. A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. J Immunol 155: 5198-205.

MÜLLER WE, ECKERT A, HARTMANN H, VELBINGER K, FORSTL H, 1996a. The calcium hypothesis of brain aging. Nervenarzt 67: 15-24.

MÜLLER WE, HARTMANN H, ECKERT A, VELBINGER K, FORSTL H, 1996b. Free intracellular calcium in aging and Alzheimer's disease. Ann.N.Y.Acad.Sci. 786, 305-320.

MURASKO, D.M., WEINER, P., AND KAYE, D., 1987. Decline in mitogen induced proliferation of lymphocytes with increasing age. Clin.Exp.Immunol. 70, 440-448.

MURASKO, D.M., NELSON, B.J., SILVER, R., MATOUR, D., AND KAYE, 1986. D., Immunologic response in an elderly population with a mean age of 85. Am.J.Med. 81, 612-618.

MYLLYKANGAS-LUOSUJARVI R, ISOMAKI H, 1994. Alzheimer's disease and rheumatoid arthritis. Br J Rheumatol 33: 501-2.

NAGEL, J.E., CHOPRA, R.K., CHREST, F.J., MCCOY, M.T., SCHNEIDER, E.L., HOLBROOK, N.J., AND ADLER, W.H., 1988. Decreased proliferation, interleukin 2 synthesis, and interleukin 2 receptor expression are accompanied by decreased mRNA expression in phytohemagglutinin-stimulated cells from elderly donors. J.Clin.Invest. 81, 1096-1102.

NARITA M, HOLTZMAN DM, SCHWARTZ AL, BU G, 1997. Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide via cell surface low-density lipoprotein receptor-related protein. J Neurochem 69: 1904-11.

NEUROINFLAMMATION WORKING GROUP: AKIYAMA H, BARGER S, BARNUM S, BRADT B, BAUER J, COLE GM, COOPER NR, EIKELENBOOM P, EMMERLING M, FIEBICH BL, FINCH CE, FRAUTSCHY S, GRIFFIN WS, HAMPEL H, HULL M, LANDRETH G, LUE L, MRAK R, MACKENZIE IR, MCGEER PL, O'BANION MK, PACHTER J, PASINETTI G, PLATA-SALAMAN C, ROGERS J, RYDEL R, SHEN Y, STREIT W, STROHMEYER R, TOOYOMA I, VAN MUISWINKEL FL, VEERHUIS R, WALKER D, WEBSTER S, WEGRZYNIAK B, WENK G, WYSS-CORAY T, 2000. Inflammation and Alzheimer's disease. Neurobiol Aging 21: 383-421.

NI Y, ZHAO B, HOU J, XIN W, 1996. Preventive effect of Ginkgo biloba extract on apoptosis in rat cerebellar neuronal cells induced by hydroxyl radicals. Neurosci Lett 214:115-118.

NICANOR X, AUSTRIACO NR JR, GUARENTE LP, 1997. Changes of telomere length cause reciprocal changes in the lifespan of mother cells in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 94: 9768-72.

NICOLETTI I, MIGLIORATI G, PAGLIACCI MC, GRIGNANI F, RICCARDI C, 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139: 271-9.

NIIJKAMP FP AND PARNHAM MJ, 1999. Principles of immunopharmacology. Birkhäuser Verlag, Basel.

NISHIMURA T, AKIYAMA H, YONEHARA S, KONDO H, IKEDA K, KATO M, ISEKI E, KOSAKA K., 1995. Fas antigen expression in brains of patients with Alzheimer-type dementia. Brain Res 695: 137-45

OGATA K, YOKOSE N, TAMURA H, AN E, NAKAMURA K, DAN K, NOMURA T, 1997. Natural killer cells in the late decades of human life. Clin Immunol Immunopathol 84: 269-75.

OJAIMI J, MASTERS CL, OPESKIN K, MCKELVIE P, BYRNE E, 1999. Mitochondrial respiratory chain activity in the human brain as a function of age. Mech Ageing Dev 111: 39-47.

OKA A, TAKASHIMA S, 1997. Induction of cyclo-oxygenase 2 in brains of patients with Down's syndrome and dementia of Alzheimer type: specific localization in affected neurones and axons. Neuroreport 8: 1161-4.

O'LEARY, J.J., FOX, R., BERGH, N., RODYSILL, K.J., AND HALLGREN, H.M., 1988. Expression of the human T cell antigen receptor complex in advanced age. Mech.Ageing Dev. 45, 239-252.

PADBERG F, FENEBERG W, SCHMIDT S, SCHWARZ MJ, KORSCHENHAUSEN D, GREENBERG BD, NOLDE T, MULLER N, TRAPMANN H, KONIG N, MOLLER HJ, HAMPEL H, 1999. CSF and serum levels of soluble interleukin-6 receptors (sIL-6R and sgp130), but not of interleukin-6 are altered in multiple sclerosis. J Neuroimmunol 99: 218-23.

PAGANELLI, R., QUINTI, I., FAGIOLO, U., COSSARIZZA, A., ORTOLANI, C., GUERRA, E., SANSONI, P., PUCILLO, L.P., SCALA, E., AND COZZI, E., 1992. Changes in circulating B cells and immunoglobulin classes and subclasses in a healthy aged population. Clin.Exp.Immunol. 90, 351-354.

PALLISTER C, JUNG SS, SHAW I, NALBANTOGLU J, GAUTHIER S, CASHMAN NR. 1997. Lymphocyte content of amyloid precursor protein is increased in Down's syndrome and aging. Neurobiol Aging 18: 97-103.

PARK E, ALBERTI J, MEHTA P, DALTON A, SERSEN E, SCHULLER-LEVIS G, 2000. Partial impairment of immune functions in peripheral blood leukocytes from aged men with Down's syndrome. Clin Immunol 95: 62-9.

PARSHAD RP, SANFORD KK, PRICE FM, MELNICK LK, NEE LE, SCHAPIRO MB, TARONE RE, ROBBINS JH, 1996. Fluorescent light-induced chromatid breaks distinguish Alzheimer disease cells from normal cells in tissue culture. Proc Natl Acad Sci USA 93, 5146–5150.

PASCALE A, ETCHEBERRIGARAY R, 1999. Calcium alterations in Alzheimer's disease: pathophysiology, models and therapeutic opportunities. Pharmacol Res 39: 81-8.

PASSER BJ, PELLEGRINI L, VITO P, GANJEI JK, D'ADAMIO L, 1999. Interaction of Alzheimer's presenilin-1 and presenilin-2 with Bcl-X(L). A potential role in modulating the threshold of cell death. J Biol Chem 274: 24007-13.

PAWELEC G, EFFROS RB, CARUSO C, REMARQUE E, BARNETT Y, SOLANA R, 1999. T cells and aging (update february 1999). Front Biosci 4: D216-69.

PAWELEC G, SANSOM D, REHBEIN A, ADIBZADEH M, BECKMAN I, 1996. Decreased proliferative capacity and increased susceptibility to activation-induced cell death in late-passage human CD4(+) TCR2(+) cultured T cell clones. Exp Gerontol 31: 655-68.

PELLEGRINI L, PASSER BJ, TABATON M, GANJEI JK, D'ADAMIO L , 1999. Alternative, non-secretase processing of Alzheimer's beta-amyloid precursor protein during apoptosis by caspase-6 and -8. J Biol Chem 274: 21011-6.

PENNINGER JM, KROEMER G, 1998. Molecular and cellular mechanisms of T lymphocyte apoptosis.. Adv Immunol 68: 51-144.

PERFETTO SP, HICKEY TE, BLAIR PJ, MAINO VC, WAGNER KF, ZHOU S, MAYERS DL, ST.LOUIS D, JUNE CH, SIEGEL JN, 1997. Measurement of CD69 induction in the assessment of immune function in asymptomatic HIV-infected individuals. Cytometry 30: 1-9.

PERICAK-VANCE MA, BASS MP, YAMAOKA LH, GASKELL PC, SCOTT WK, TERWEDOW HA, MENOLD MM, CONNEALLY PM, SMALL GW, VANCE JM, SAUNDERS AM, ROSES AD, HAINES JL, 1997. Complete genomic screen in late-onset familial Alzheimer disease. Evidence for a new locus on chromosome 12. JAMA 278: 1237-41.

PHARMINGEN, 1999, Pharmingen Catalog, Appendix: Technical Protocols,

PHELOUZAT MA, LAFORGE T, ARBOGAST A, QUADRI RA, BOUTET S, PROUST JJ, 1997. Susceptibility to apoptosis of T lymphocytes from elderly humans is associated with increased in vivo expression of functional Fas receptors. Mech Ageing Dev 96: 35–46.

PHELOUZAT MA, ARBOGAST A, LAFORGE TS, QUADRI RA, PROUST JJ, 1996. Excessive apoptosis of mature T lymphocytes is a characteristic feature of human immune senescence. Mech Ageing Dev 88: 25-38.

PIANI D, SPRANGER M, FREI K, SCHAFFNER A, FONTANA A, 1992. Macrophage-induced cytotoxicity of N-methyl-D-aspartate receptor positive neurons involves excitatory amino acids rather than reactive oxygen intermediates and cytokines.Eur J Immunol 22: 2429-36

PIETRI S, SEGUIN JR, D'ARBIGNY P, DRIEU K, CULCASI M , 1997. Ginkgo biloba extract (EGb 761) pretreatment limits free radical-induced oxidative stress in patients undergoing coronary bypass surgery. Cardiovasc Drugs Ther 11: 121-31.

PIKE CJ, BURDICK D, WALENCEWICZ AJ, GLABE CG, COTMAN CW, 1993. Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. J Neurosci 13: 1676-87.

PIRTTILA T, MATTINEN S, FREY H, 1992. The decrease of CD8-positive lymphocytes in Alzheimer's disease. J Neurol Sci 107: 160-5.

PODLISNY MB, MAMMEN AL, SCHLOSSMACHER MG, PALMERT MR, YOUNKIN SG, SELKOE DJ, 1990. Detection of soluble forms of the beta-amyloid precursor protein in human plasma. Biochem Biophys Res Commun 167: 1094-101.

POIRIER J, 2000. Apolipoprotein E and Alzheimer's disease. A role in amyloid catabolism. Ann N Y Acad Sci 924: 81-90.

POSNETT DN, SINHA R, KABAK S, RUSSO C, 1994. Clonal Populations of T Cells in Normal Elderly Humans -The T Cell Equivalent to Benign Monoclonal Gammapathy. J Exp Med 179: 609-18.

POTESTIO M, CARUSO C, GERVASI F, ET AL, 1998. Apoptosis and ageing. Mech Ageing Dev 102: 221–37.

PREHN JH, BINDOKAS VP, JORDAN J, GALINDO MF, GHADGE GD, ROOS RP, BOISE LH, THOMPSON CB, KRAJEWSKI S, REED JC, MILLER RJ, 1996. Protective effect of transforming growth factor-beta 1 on beta-amyloid neurotoxicity in rat hippocampal neurons. Mol Pharmacol 49: 319-28.

PRINCE HE, LAPE–NIXON M, 1995. Influence of specimen age and anticoag-ulant on flow cytometric evaluation of granulocyte oxidative burst generation. J Immunol Methods 188: 129 –38.

QUADRI RA, PLASTRE O, PHELOUZAT MA, ARBOGAST A, PROUST JJ, 1996. Age-related tyrosine-specific protein phosphorylation defect in human T lymphocytes activated through CD3, CD4, CD8 or the IL-2 receptor. Mech Aging Dev 88: 125–38.

RABINOWE SL, RUBIN IL, GEORGE KL, ADRI MN, EISENBARTH GS, 1989. Trisomy 21 (Down's syndrome): autoimmunity, aging and monoclonal antibody-defined T-cell abnormalities. J Autoimmun 2: 25-30

RABINOWICH, H., GOSES, Y., RESHEF, T., AND KLAJMAN, A., 1985. Interleukin-2 production and activity in aged humans. Mech.Ageing Dev. 32, 213-226.

RAPPUOLI R, 2000. Laudatory speech to mark the award of the Paul Ehrlich- und Ludwig Darmstaedter-Prize 2000 to HR Horvitz (Cambridge/USA) und JFR Kerr (Hamilton/Australien), 14.03.2000, Paulskirche, Frankfurt am Main, Germany.

REBECK GW, HARR SD, STRICKLAND DK, HYMAN BT, 1995. Multiple, diverse senile plaque-associated proteins are ligands of an apolipoprotein E receptor, the alpha 2-macroglobulin receptor/low-density-lipoprotein receptor-related protein. Ann Neurol 37: 211-7.

REED JC, MIYASHITA T, CUDDY M, CHO D, 1992. Regulation of p26-bcl-2 protein levels in human peripheral blood lymphocytes. Lab Invest 67: 443–9.

REMARQUE E, PAWELEC G, 1998. T cell immunosenescence and its clinical relevance in man. Reviews in Clinical Gerontology 8: 5-25.

RICH JB, RASMUSSON DX, FOLSTEIN MF, CARSON KA, KAWAS C, BRANDT J, 1995. Nonsteroidal anti-inflammatory drugs in Alzheimer's disease. Neurology 45: 51-5.

ROBAKIS NK, ANDERSON JP, REFOLO LM, WALLACE W, 1990. Expression of the Alzheimer amyloid precursor in brain tissue and effects of NGF and EGF on its metabolism.. Clin Neuropharmacol Suppl 1: S15-23.

ROBINSON AGRAMONTE MD M, DORTA-CONTRERAS AJ, LORIGADOS PEDRE L, 2001. Immune events in central nervous system of early and late onset Alzheimer's disease patients. Rev Neurol 32: 901-904.

ROGAEV EI, SHERRINGTON R, ROGAEVA EA, LEVESQUE G, IKEDA M, LIANG Y, CHI H, LIN C, HOLMAN K, TSUDA T, ET AL, 1995. Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 376: 775-8.

ROGERS J, LUBER-NAROD J, STYREN SD, CIVIN WH, 1998a. Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. Neurobiol Aging 9: 339-49.

ROGERS J, LUE LF, YANG LB ET AL., 1998b. Complement activation by neurofibrillary tangels in Alzheimer's disease. Soc Neuroscience 24: 1268 (Abstract)

ROGERS J, MUFSON EJ, 1990. Demonstrating immune-related antigens in Alzheimer's disease brain tissue. Neurobiol Aging 11: 477-9.

ROSTAING, L., TKACZUK, J., DURAND, M., PERES, C., DURAND, D., DE PREVAL, C., OHAYON, E., AND ABBAL, M., 1999. Kinetics of intracytoplasmic Th1 and Th2 cytokine production assessed by flow cytometry following in vitro activation of peripheral blood mononuclear cells. Cytometry 35, 318-328.

ROTHE G, VALET G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 29,79-dichlorofluores-cin. J Leukocyte Biol 1990;47:440 –8.

RUFF MR, PERT CB, WEBER RJ, WAHL LM, WAHL SM, PAUL SM, 1985. Benzodiazepine receptor-mediated chemotaxis of human monocytes. Science 229: 1281-3

RUTELLA S, RUMI C, LUCIA MB, BARBERI T, PUGGIONI PL, LAI M, ROMANO A, CAUDA R, IEONE G, 1999. Induction of CD69 antigen on normal CD4+ and CD8+ lymphocyte subsets and its relationship with the phenotype of responding T-cells. Cytometry 38: 95-101.

SADEGHI, H.M., SCHNELLE, J.F., THOMA, J.K., NISHANIAN, P., AND FAHEY, J.L., 1999. Phenotypic and functional characteristics of circulating monocytes of elderly persons. Exp.Gerontol. 34, 959-970.

SAKATA-KANEKO, S., WAKATSUKI, Y., MATSUNAGA, Y., USUI, T., AND KITA, T., 2000. Altered Th1/Th2 commitment in human CD4+T cells with ageing. Clin.Exp.Immunol. 120, 267-273.

SANSONI, P., FAGNONI, F., VESCOVINI, R., MAZZOLA, M., BRIANTI, V., BOLOGNA, G., NIGRO, E., LAVAGETTO, G., COSSARIZZA, A., MONTI, D., FRANCESCHI, C., AND PASSERI, M., 1997. T lymphocyte proliferative capability to defined stimuli and costimulatory CD28 pathway is not impaired in healthy centenarians. Mech.Ageing Dev. 96, 127-136.

SANSONI, P., BRIANTI, V., FAGNONI, F., SNELLI, G., MARCATO, A., PASSERI, G., MONTI, D., COSSARIZZA, A., AND FRANCESCHI, C., 1992. NK cell activity and T-lymphocyte proliferation in healthy centenarians. Ann.N.Y.Acad.Sci. 663, 505-507.

SATOU T, CUMMINGS BJ, COTMAN CW, 1995. Immunoreactivity for Bcl-2 protein within neurons in the Alzheimer's disease brain increases with disease severity. Brain Res 697: 35-43

SAVILL J, FADOK V, HENSON P, HASLETT C, 1993. Phagocyte recognition of cells undergoing apoptosis. Immunol Today, 14: 131-6.

SAWA A. 1999. Neuronal cell death in Down's syndrome. J Neural Transm Suppl 57: 87-97.

SCHEFFLER B, HORN M, BLUMCKE I, LAYWELL ED, COOMES D, KUKEKOV VG, STEINDLER DA, 1999. Marrow-mindedness: a perspective on neuropoiesis. Trends Neurosci 22: 348-57

SCHENK D, BARBOUR R, DUNN W, GORDON G, GRAJEDA H, GUIDO T, HU K, HUANG J, JOHNSON-WOOD K, KHAN K, KHOLODENKO D, LEE M, LIAO Z, LIEBERBURG I, MOTTER R, MUTTER L, SORIANO F, SHOPP G, VASQUEZ N, VANDEVERT C, WALKER S, WOGULIS M, YEDNOCK T, GAMES D, SEUBERT P, 1999. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature, 400:173-7

SCHEUNER D, ECKMAN C, JENSEN M, SONG X, CITRON M, SUZUKI N, BIRD TD, HARDY J, HUTTON M, KUKULL W, LARSON E, LEVY-LAHAD E, VIITANEN M, PESKIND E, POORKAJ P, SCHELLENBERG G, TANZI R, WASCO W, LANNFELT L, SELKOE D, YOUNKIN S, 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med 2: 864-70.

SCHINDOWSKI K, LEUTNER S, MÜLLER WE, ECKERT A, 2000. Age-related changes of apoptotic cell death in human lymphocytes. Neurobiol Aging 21: 661-70.

SCHINDOWSKI K., LEUTNER S, ECKERT A, MÜLLER WE; 1999. Prevention of apoptotic cell death in human lymphocytes by antioxidants. Naunyn-Schmiedebergs' Archieves for Pharmacology, 359 (Suppl.3): 44.

SCHLUNCK T, SCHRAUT W, RIETHMULLER G, ZIEGLER-HEITBROCK HW, 1990. Inverse relationship of CA2+ mobilization and cell proliferation in CD8+ memory and virgin T cells. Eur J Immunol 20:1957-63

SCHMID I, UITTENBOGAART CH, GIORGI JV, 1994a. Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. Cytometry 15: 12-20.

SCHMID I, UITTENBOGAART CH, KELD B, GIORGI JV, 1994b. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. J Immunol Methods 170:145–57.

SCHMITT TL, STEGER MM, PAVELKA M, GRUBECK-LOEBENSTEIN B, 1997. Interactions of the Alzheimer beta amyloid fragment (25-35) with peripheral blood dendritic cells. Mech Ageing Dev 94: 223-32.

SCHUBERT D, LACORBIERE M, SAITOH T, COLE G, 1989. Characterization of an amyloid beta precursor protein that binds heparin and contains tyrosine sulfate. Proc Natl Acad Sci U S A 86: 2066-9.

SCHUBERT W, PRIOR R, WEIDEMANN A, DIRCKSEN H, MULTHAUP G, MASTERS CL, BEYREUTHER K, 1991. Localization of Alzheimer beta A4 amyloid precursor protein at central and peripheral synaptic sites. Brain Res 563: 184-94.

SEDGWICK JD, MOSSNER R, SCHWENDER S, TER MEULEN V, 1991. Major histocompatibility complex-expressing nonhematopoietic astroglial cells prime only CD8+ T lymphocytes: astroglial cells as perpetuators but not initiators of CD4+ T cell responses in the central nervous system. J Exp Med 173: 1235-46.

SEIDL R, BIDMON B, BAJO M, YOO PC, CAIRNS N, LACASSE EC, LUBEC G., 2001. Evidence for apoptosis in the fetal down syndrome brain. J Child Neurol 16: 438-42

SEISHIMA M, TAKEMURA M, SAITO K, ET AL. Highly sensitive ELISA for soluble Fas in serum: increased soluble Fas in the elderly. Clin Chem 1996;42:1911–4.

SELKOE DJ, 1994. Normal and abnormal biology of the beta-amyloid precursor protein. Annu Rev Neurosci 17: 489-517.

SELKOE DJ, HAASS C, LEMERE CA, CAPELL A, CITRON M, SEUBERT P, SCHENK D, LANNFELT L, 1995. The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. Nat Med 1: 1291-6.

SERRA JA, FERNANDEZ–GUTIERREZ B, HERNANDEZ–GARCIA C, ET AL. Early T-cell activation in elderly humans. Age Ageing 1996;25:470 –8.

SHALIT F, SREDNI B, BRODIE C, KOTT E, HUBERMAN M. T lymphocyte subpopulations and activation markers correlate with severity of Alz-heimer's disease. Clin Immunol Immunopathol 1995;75:246 –50.

SHEN Y, LI R, MCGEER EG, MCGEER PL, 1997. Neuronal expression of mRNAs for complement proteins of the classical pathway in Alzheimer brain. Brain Res 769: 391-5.

SHENG JG, ITO K, SKINNER RD, MRAK RE, ROVNAGHI CR, VAN ELDIK LJ, GRIFFIN WS, 1996. In vivo and in vitro evidence supporting a role for the inflammatory cytokine interleukin-1 as a driving force in Alzheimer pathogenesis. Neurobiol Aging 17: 761-6.

SHERRINGTON R, ROGAEV EI, LIANG Y, ROGAEVA EA, LEVESQUE G, IKEDA M, CHI H, LIN C, LI G, HOLMAN K, ET AL, 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375: 754-60.

SIMONIAN NA, COYLE JT. Oxidative stress in neurodegenerative dis-eases. Annu Rev Pharmacol Toxicol 1996;36:83–106.

SINGH VK, 1994. Studies of neuroimmune markers in Alzheimer's disease. Mol.Neurobiol. 9, 73-81.

SINGH VK, 1990. Neuroimmune axis as a basis of therapy in Alzheimer's disease. Prog Drug Res 34: 383-93.

SLUNT HH, DOAN A, THINAKARAN G, BORCHELT DR, RATOVITSKY T, PODLISNY M, SELKOE DJ, SEEGER M, GANDY SE, PRICE DL, SISODIA SS, 1995. Protein topology of presenilin 1. Neuron 17: 1023-30.

SNOW AD, MAR H, NOCHLIN D, KIMATA K, KATO M, SUZUKI S, HASSELL J, WIGHT TN, 1988. The presence of heparan sulfate proteoglycans in the neuritic plaques and congophilic angiopathy in Alzheimer's disease. Am J Pathol 133: 456-63.

SNYDER EY, PARK KI, FLAX JD, LIU S, ROSARIO CM, YANDAVA BD, AURORA S, 1997. Potential of neural "stem-like" cells for gene therapy and repair of the degenerating central nervous system. Proc Natl Acad Sci U S A 1997 94: 663-8

SOLANA, R. AND MARIANI, E., 2000. NK and NK/T cells in human senescence. Vaccine 18, 1613-1620.

SOLERTE SB, CRAVELLO L, FERRARI E, FIORAVANTI M, 2000. Overproduction of IFN-gamma and TNF-alpha from natural killer (NK) cells is associated with abnormal NK reactivity and cognitive derangement in Alzheimer's disease. Ann N Y Acad Sci 917 :331-40.

SOLERTE SB, FIORAVANTI M, PASCALE A, FERRARI E, GOVONI S, BATTAINI F, 1998. Increased natural killer cell cytotoxicity in Alzheimer's disease may involve protein kinase C dysregulation. Neurobiol Aging 19: 191-9.

SOLERTE SB, FIORAVANTI M, SEVERGNINI S, LOCATELLI M, RENZULLO M, PEZZA N, CERUTTI N, FERRARI E, 1996. Enhanced cytotoxic response of natural killer cells to interleukin-2 in Alzheimer's disease. Dementia 7: 343-8.

SONG L, STEPHENS JM, KITTUR S, ET AL. Expression of c-fos, c-jun and jun B in peripheral blood lymphocytes from young and elderly adults. Mech Ageing Dev 1992;65:149 –56.

SPARKS DL, 1997. Coronary artery disease, hypertension, ApoE, and cholesterol: a link to Alzheimer's disease? Ann NY Acad Sci 826: 128-46.

STADTMAN, E.R., 1992. Protein oxidation and aging. Science 257, 1220-1224.

STEINMAN HM. The Bcl-2 oncoprotein functions as a pro-oxidant. J Biol Chem 1995;270:3487–90.

STEWART WF, KAWAS C, CORRADA M, METTER EJ, 1997. Risk of Alzheimer's disease and duration of NSAID use. Neurology 48: 626-32.

STRAUSS S, BAUER J, GANTER U, JONAS U, BERGER M, VOLK B, 1992. Detection of interleukin-6 and alpha 2-macroglobulin immunoreactivity in cortex and hippocampus of Alzheimer's disease patients. Lab Invest66: 223-30.

STREIT WJ, KINCAID-COLTON CA, 1995, The brain's immune system. Sci Am 273: 54-5, 58-61.

STREIT WJ, WALTER SA, PENNELL NA, 1999. Reactive microgliosis. Prog Neurobiol 57: 563-81.

STRITTMATTER WJ, WEISGRABER KH, HUANG DY, DONG LM, SALVESEN GS, PERICAK-VANCE M, SCHMECHEL D, SAUNDERS AM, GOLDGABER D, ROSES AD, 1993. Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. Proc Natl Acad Sci U S A 90: 8098-102.

STULNIG T, MACZEK C, BOCK G, MAJDIC O, WICK G. Reference intervals for human peripheral blood lymphocyte subpopulations from 'healthy' young and aged subjects. Int Arch Allergy Immunol 1995; 108:205–10.

STURCHLER-PIERRAT C, STAUFENBIEL M, 2000. Pathogenic mechanisms of Alzheimer's disease analyzed in the APP23 transgenic mouse model. Ann NY Acad Sci, 920: 134-9.

SU JH, DENG G, COTMAN CW, 1997. Bax protein expression is increased in Alzheimer's brain: correlations with DNA damage, Bcl-2 expression, and brain pathology. J Neuropathol Exp Neurol 56: 86-93

SU JH, SATOU T, ANDERSON AJ, COTMAN CW, 1996. Up-regulation of Bcl-2 is associated with neuronal DNA damage in Alzheimer's disease. Neuroreport 7: 437-40.

SUH YH, CHONG YH, KIM SH, CHOI W, MIN K, JEONG SJ, FRASER SP, DJAMGOZ MB, 1996. Molecular physiology, biochemistry, and pharmacology of Alzheimer's amyloid precursor protein (APP). Ann N Y Acad Sci 786: 169-83.

SURH CD, SPRENT J, 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature 372: 100-103.

TAMATANI M, CHE YH, MATSUZAKI H, OGAWA S, OKADO H, MIYAKE S, MIZUNO T, TOHYAMA M, 1999. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. J Biol Chem 274: 8531-8.

TANCHOT C, LEMONNIER FA, PERARNAU B, FREITAS AA, ROCHA B, 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. Science 276: 2057-62.

TANII H, ANKARCRONA M, FLOOD F, NILSBERTH C, MEHTA ND, PEREZ-TUR J, WINBLAD B, BENEDIKZ E, COWBURN RF, 2000. Alzheimer's disease presenilin-1 exon 9 deletion and L250S mutations sensitize SH-SY5Y neuroblastoma cells to hyperosmotic stress-induced apoptosis. Neuroscience 95: 593-601.

TANZI RE,. KOVACS DM, KIM TW,.MOIR RD, GUENETTE SY, WASCO W, 1996, The Presenilin Genes and Their Role in Early-Onset Familial Alzheimer's Disease . Alzheimer's Disease Review 1, 91-98. (http://www.mc.uky.edu/adreview/Tanzi/Tanzi.htm).

TANZI RE, BUSH AI, WASCO W, 1994. Genetic studies of Alzheimer's disease: lessons learned and future imperatives Neurobiol Aging Suppl 2: S145-8.

TANZI RE, VAULA G, ROMANO DM, MORTILLA M, HUANG TL, TUPLER RG, WASCO W, HYMAN BT, HAINES JL, JENKINS BJ, ET AL, 1992. Assessment of amyloid beta-protein precursor gene mutations in a large set of familial and sporadic Alzheimer disease cases. Am J Hum Genet 51: 273-82.

TARKOWSKI E, BLENNOW K, WALLIN A, TARKOWSKI A, 1999. Intracerebral production of tumor necrosis factor-alpha, a local neuroprotective agent, in Alzheimer disease and vascular dementia. J Clin Immunol 19: 223-30.

THE CANADIAN STUDY OF HEALTH AND AGING: risk factors for Alzheimer's disease in Canada, 1994. Neurology 44: 2073-80.

THINAKARAN G, BORCHELT DR, LEE MK, SLUNT HH, SPITZER L, KIM G, RATOVITSKY T, DAVENPORT F, NORDSTEDT C, SEEGER M, HARDY J, LEVEY AI, GANDY SE, JENKINS NA, COPELAND NG, PRICE DL, SISODIA SS, 1996. Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. Neuron 17: 181-90.

THOMAN ML, WEIGLE WO, 1989. The cellular and subcellular bases of immunosenescence. Adv Immunol 46: 221-62.

TIELEN FJ, VAN VLIET AC, DE GEUS B, NAGELKERKEN L, ROZING J, 1993. Age-related changes in CD4+ T-cell subsets associated with prolonged skin graft survival in aging rats. Transplant Proc 25, 2872-4

TINKLE, C.W., LIPSCHITZ, D., AND PONNAPPAN, U., 1998. Decreased association of p56lck with CD4 may account for lowered tyrosine kinase activity in mitogen-activated human T lymphocytes during aging. Cell Immunol. 186, 154-160.

TORTOSA A, LOPEZ E, FERRER I, 1998. Bcl-2 and Bax protein expression in Alzheimer's disease. Acta Neuropathol (Berl) 95: 407-12.

TREBILCOCK, G.U. AND PONNAPPAN, U., 1996. Induction and regulation of NFkappaB during aging: role of protein kinases. Clin.Immunol.Immunopathol. 79, 87-91.

TRIEB K, RANSMAYR G, SGONC R, LASSMANN H, GRUBECK-LOEBENSTEIN B, 1996. APP peptides stimulate lymphocyte proliferation in normals, but not in patients with Alzheimer's disease. Neurobiol Aging 17: 541-7.

TRITSCHLER HJ, PACKER L, MEDORI R, 1994. Oxidative stress and mitochon-drial dysfunction in neurodegeneration. Biochem Mol Biol Int 34:169 –81.

UNSICKER K, FLANDERS KC, CISSEL DS, LAFYATIS R, SPORN MB, 1991. Transforming growth factor beta isoforms in the adult rat central and peripheral nervous system. Neuroscience 44: 613-25.

VALLIERES L, RIVEST S, 1997. Regulation of the genes encoding interleukin-6, its receptor, and gp130 in the rat brain in response to the immune activator lipopolysaccharide and the proinflammatory cytokine interleukin-1beta. J Neurochem 69: 1668-83.

VAN ELDIK LJ, GRIFFIN WS, 1994. S100 beta expression in Alzheimer's disease: relation to neuropathology in brain regions. Biochim Biophys Acta 1223: 398-403.

VAN LIESHOUT EM, PETERS WH, 1998. Age and gender dependent levels of glutathione and glutathione S-transferases in human lymphocytes. Carcinogenesis 19: 1873–5.

VAN NOSTRAND WE, WAGNER SL, FARROW JS, CUNNINGHAM DD, 1990. Immunopurification and protease inhibitory properties of protease nexin-2/amyloid beta-protein precursor. J Biol Chem 265: 9591-4.

VAN NOSTRAND WE, WAGNER SL, SUZUKI M, CHOI BH, FARROW JS, GEDDES JW, COTMAN CW, CUNNINGHAM DD, 1989. Protease nexin-II, a potent antichymotrypsin, shows identity to amyloid beta-protein precursor. Nature 341: 546-9.

VAN WAGONER NJ, OH JW, REPOVIC P, BENVENISTE EN., 1999. Interleukin-6 (IL-6) production by astrocytes: autocrine regulation by IL-6 and the soluble IL-6 receptor. J Neurosci 19: 5236-44.

VAZIRI H, SCHACHTER F, UCHIDA I, WEI L, ZHU X, EFFROS R, COHEN D, HARLEY CB, 1993. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet 52:661-7

VELAZQUEZ P, CRIBBS DH, POULOS TL, TENNER AJ, 1997. Aspartate residue 7 in amyloid beta-protein is critical for classical complement pathway activation: implications for Alzheimer's disease pathogenesis. Nat Med 3: 77-9

VEZINA J, TSCHOPP C, ANDERSEN E, MULLER K, 1999. Overexpression of a C-terminal fragment of presenilin 1 delays anti-Fas induced apoptosis in Jurkat cells. Neurosci Lett 263: 65-8.

VITETTA, E.S., BERTON, M.T., BURGER, C., KEPRON, M., LEE, W.T., AND YIN, X.M., 1991. Memory B and T cells. Annu.Rev.Immunol. 9, 193-217.

VITO P, WOLOZIN B, GANJEI JK, IWASAKI K, LACANA E, D'ADAMIO L, 1996. Requirement of the familial Alzheimer's disease gene PS2 for apoptosis. Opposing effect of ALG-3. J Biol Chem 271: 31025-8.

WALLACE DC, 1992, Mitochondrial genetics: a paradigm for aging and degenerative diseases? Science 256: 628-32.

WARNER HR, 1999. Apoptosis: a two-edged sword in aging. Ann N Y Acad Sci 887: 1-11.

WARNER HR, 1997. Aging and regulation of apoptosis. Curr Top Cell Regul 35: 107-21

WASCO W, PETTINGELL WP, JONDRO PD, SCHMIDT SD, GURUBHAGAVATULA S, RODES L, DIBLASI T, ROMANO DM, GUENETTE SY, KOVACS DM, ET AL., 1995. Familial Alzheimer's chromosome 14 mutations. Nat Med 1: 848.

WEBB DJ, ATKINS TL, CROOKSTON KP, BURMESTER JK, QIAN SW, GONIAS SL, 1994. Transforming growth factor beta isoform 2-specific high affinity binding to native alpha 2-macroglobulin. Chimeras identify a sequence that determines affinity for native but not activated alpha 2-macroglobulin. J Biol Chem 269: 30402-6.

WEBSTER S, BRADT B, ROGERS J, COOPER N, 1997. Aggregation state-dependent activation of the classical complement pathway by the amyloid beta peptide. J Neurochem 69: 388-98.

WEBSTER SD, TENNER AJ, POULOS TL, CRIBBS DH, 1999. The mouse C1q A-chain sequence alters beta-amyloid-induced complement activation. Neurobiol Aging 20: 297-304.

WEI, Y.H., LU, C.Y., LEE, H.C., PANG, C.Y., AND MA, Y.S., 1998. Oxidative damage and mutation to mitochondrial DNA and age-dependent decline of mitochondrial respiratory function. Ann.N.Y.Acad.Sci. 854, 155-170.

WEIDEMANN A, PALIGA K, D RRWANG U, REINHARD FB, SCHUCKERT O, EVIN G, MASTERS CL, 1999. Proteolytic processing of the Alzheimer's disease amyloid precursor protein within its cytoplasmic domain by caspase-like proteases. J Biol Chem 274: 5823-9.

WEIR DM, 1999 in: Niijkamp FP and Parnham MJ, 1999. Principles of immunopharmacology. Birkhäuser Verlag, Basel.

WEISS A, LITTMAN DR, 1994. Signal transduction by lymphocyte antigen receptors. Cell 76: 263-274.

WEKERLE H, SUN D, OROPEZA-WEKERLE RL, MEYERMANN R, 1986. Immune reactivity in the nervous system: modulation of T-lymphocyte activation by glial cells. J Exp Biol 132: 43-57.

WEVERLING-RIJNSBURGER AWE, BLAUW GJ, LAGAAY AM, KNOOK DL, MEINDERS AE, WESTENDORP RGJ, 1997. Total cholesterol and risk of mortality in the oldest old. Lancet 350: 1119-23.

WHISLER RL, BEIQING L, CHEN M. Age-related decreases in IL-2 production by human T cells are associated with impaired activation of nuclear transcriptional factors AP-1 and NF-AT. Cell Immunol 1996;169:185–95.

WODA, B.A., YGUERABIDE, J., AND FELDMAN, J.D., 1979. Mobility and density of AgB, "Ia", and Fc receptors on the surface of lymphocytes from young and old rats. J.Immunol. 123, 2161-2167.

WYSS-CORAY T, MASLIAH E, MALLORY M, MCCONLOGUE L, JOHNSON-WOOD K, LIN C, MUCKE L, 1997. Amyloidogenic role of cytokine TGF-beta1 in transgenic mice and in Alzheimer's disease. Nature 389: 603-6.

XU S, GASKIN F, 1997. Increased incidence of anti-beta-amyloid autoantibodies secreted by Epstein-Barr virus transformed B cell lines from patients with Alzheimer's disease. Mech Aging Dev 94: 213-22.

YAMAMOTO K, MIYOSHI T, YAE T, KAWASHIMA K, ARAKI H, HANADA K, OTERO DA, ROCH JM, SAITOH T, 1994. The survival of rat cerebral cortical neurons in the presence of trophic APP peptides. J Neurobiol 25: 585-94

YAMATSUJI T, MATSUI T, OKAMOTO T, ET AL., 1996. G protein-mediated neu-ronal DNA fragmentation induced by familial Alzheimer's disease-associated mutants of APP. Science 272: 1349 –52.

YAN R, BIENKOWSKI MJ, SHUCK ME, MIAO H, TORY MC, PAULEY AM, BRASHIER JR, STRATMAN NC, MATHEWS WR, BUHL AE, CARTER DB, TOMASSELLI AG, PARODI LA, HEINRIKSON RL, GURNEY ME, 1999. Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature 402: 533-7

YAN SD, CHEN X, FU J, CHEN M, ZHU H, ROHER A, SLATTERY T, ZHAO L, NAGASHIMA M, MORSER J, MIGHELI A, NAWROTH P, STERN D, SCHMIDT AM, 1996. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature 382: 685-91

YAN SD, YAN SF, CHEN X, FU J, CHEN M, KUPPUSAMY P, SMITH MA, PERRY G, GODMAN GC, NAWROTH P, ET AL, 1995. Non-enzymatically glycated tau in Alzheimer's disease induces neuronal oxidant stress resulting in cytokine gene expression and release of amyloid beta-peptide. Nature Med 1: 693-9.

YANG J, LIU X, BHALLA K, KIM CN, IBRADO AM, CAI J, PENG TI, JONES DP, WANG X, 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129-32.

YANKNER BA, DAWES LR, FISHER S, VILLA-KOMAROFF L, OSTER-GRANITE ML, NEVE RL, 1989. Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. Science 245: 417-20.

YEN, C.J., LIN, S.L., HUANG, K.T., AND LIN, R.H., 2000. Age-associated changes in interferon-gamma and interleukin-4 secretion by purified human CD4+ and CD8+ T cells. J.Biomed.Sci. 7, 317-321.

YOSHIKAWA TT, 1997. Perspective: Aging and infectious diseases: Past, present, and future. J Infect Dis 176: 1053-7.

ZAHARIA, M.D., RAVINDRAN, A.V., GRIFFITHS, J., MERALI, Z., AND ANISMAN, H., 1993. Lymphocyte proliferation among major depressive and dysthymic patients with typical or atypical features. J.Affect.Disord. 58, 1-10.

ZAMZAMI N, MARCHETTI P, CASTEDO M, DECAUDIN D, MACHO A, HIRSCH T, SUSIN SA, PETIT PX, MIGNOTTE B, KROEMER G, 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 182: 367-77.

ZAVALA X, ZUBENKO GS, WUSYLKO M, COHEN BM, BOLLER F, TEPLY I, 1987. Family study of platelet membrane fluidity in Alzheimer's disease. Science 238: 539-42

ZHANG C, LAMBERT MP, BUNCH C, BARBER K, WADE WS, KRAFFT GA, KLEIN WL, 1994. Focal adhesion kinase expressed by nerve cell lines shows increased tyrosine phosphorylation in response to Alzheimer's A beta peptide. J Biol Chem 269: 25247-50.

ZHENG L, FISHER G, MILLER RE, PESCHON J, LYNCH DH, LENARDO MJ, 1995. Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377: 348-51.

ZHOU T, EDWARDS CK, MOUNTZ JD, 1995. Prevention of age-related T cell apoptosis defect in CD2-fas-transgenic mice. J Exp Med 182: 129-37.

ZLOKOVIC BV, MARTEL CL, MATSUBARA E, MCCOMB JG, ZHENG G, MCCLUSKEY RT, FRANGIONE B, GHISO J, 1996. Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers. Proc Natl Acad Sci U S A 93: 4229-34.

Bibliography

Abstracts

Schindowski, K., Leutner, S., Eckert, A., Müller, W.E.; Age-related changes in apoptic cell death and oxidative stress of human peripheral cells.
First European Graduate Student Meeting, Frankfurt, Germany, 1998
Pharmazie, 53 (3), p,32, 1998.

Schindowski, K., Leutner, S., Eckert, A., Müller, W.E.; Enhanced apoptotic cell death in aging human peripheral lymphocytes.
German Society for Biological Psychiatry, Jena, Germany, 1998
Eur. Arch. Psychiatry Clin. Neurosci., 248 (Suppl.2), p.19, 1998

Schindowski, K., Leutner, S., Walter, J., Eckert, A., Müller, W.E.; Age-related alterations of apoptosis in human peripheral blood cells.
28th Annual Meeting Soc. Neuroscience, Los Angeles, USA, 1998
Soc. Neurosci., Abstracts 24, 585, 1998.

Schindowski, K., Leutner, S., Walter, J., Eckert, A, Müller, W.E.; Prevention of apoptotic cell death in human lymphocytes by antioxidants.
40. Frühjahrstagung der DGPht, Mainz, Germany, 1998
Naunyn-Schmiedebergs' Archieves for Pharmacology, 359 (Suppl.3), 44, 1999

Schindowski, K., Leutner, S., Eckert, A, Müller, W.E.; Prevention of apoptotic cell death in human lymphocytes by antioxidants.
German Graduate Student Meeting, Freiburg, Germany, 1999
Arch. Pharm. Pharm. Med. Chem., 332, (Suppl.1), p.64, 1999

Schindowski, K., Leutner, S., Eckert, A., Müller, W.E.; Ginkgo biloba extract 761 suppreses T-cell activation.
Jahrestagung der Deutschen Pharmazeutischen Gesellschaft e.V., Frankfurt, Germany, 1999
Arch. Pharm. Pharm. Med. Chem., 333, (Suppl.2), p.40, 1999

Schindowski, K., Leutner, S., Gorriz, C., Fröhlich, L., Maurer, K., Eckert, A., Müller, W.E.; Altered apoptosis in Alzheimer's diseases is not directly linked to changes in lymphocyte's subsets.
29th Annual Meeting Soc. Neuroscience, Miami, USA, 1999
Soc. Neurosci., Abstracts 25, 850.2, p.2124, 1999

Schindowski, K., Fröhlich, L., Maurer, K., Gorriz, C., Schramm, U., Weinandi, T., Peters, J., Czech, C., Touchet, N., Eckert, A, Müller, W.E.; Altered immunological features in lymphocytes from Alzheimer's diseases patients.
Neuroinflammation in Alzheimer's disease, Washington D.C., USA, 2000.
Abstract Ref. A066

Schindowski, K., Marques, C., Leutner, S., Eckert. A., Müller, W.E.; Anti-apoptotic action of C-terminal fragment of Alzheimer's disease Presenilin 1.
30th Annual Meeting Soc. Neuroscience, New Orleans, USA, 2000
Soc. Neurosci., Abstracts 26, 474.6, p.1281, 2000

Schindowski, K., Leutner, S., Frölich L., Maurer K., Gorriz C., Peters, J., Schramm, U., Weinandi, T., Eckert, A., Müller, WE.; Increased apoptosis and altered immunological features in lymphocytes from Alzheimer's disease patients: Evidence for enhanced generation of memory T cells in AD.
31st Annual Meeting Soc. Neuroscience, San Diego, USA, 2001
Soc. Neurosci., Abstract-CD ROM 27, 92.3, 2001

original papers

Eckert, A., Leutner, S., Schindowski, K., Müller, W.E.; Free radicals in aging and in Alzheimer's disease. In: The phytopharmaceuticals of the next century. Lopez, X., Gomez, E., Brunner, M (eds.), Mexico, IMSS, 1999.

Eckert, A., Eckert, G.P., Kastl. A., Leutner, S., Leutz, S., Schindowski, K., Steiner, B., Sych, M., Müller, W.E.; Vom unaufhaltsamen Niedergang der Hirnzellen: Wie die Alzheimer Demenz entsteht. *Forschung Frankfurt* 4: 60-66, 1999.

Leutner, S., Czech, C., Schindowski, K., Touchet, N., Eckert, A. and Müller, W.E.; Reduced antioxidant enzyme activity in brains of mice transgenic for human presenilin-1 with single or multiple mutations. *Neurosci. Lett.*, 292: 87-90, 2000.

Schindowski, K., Leutner, S., Müller, W.E. and Eckert, A.; Age-related changes of Apoptotic Cell Death in Human Lymphocytes. *Neurobiol. Aging*: 21, 661-670, 2000.

Eckert, A., Schindowski, K., Leutner, S., Luckhaus, C., Touchet, N., Czech, C., Müller, W.E.; Alzheimer's disease-like alterations in peripheral cells from presenilin-1 transgenic mice. *Neurobiol Dis* 8, 331-342, 2001.

Schindowski, K., Leutner, S., Kressmann, S., Eckert, A., Müller, W.E.; Age-related increase of oxidative stress-induced apoptosis in mice. Prevention by Ginkgo biloba extract (EGb761). *J Neural Trans* 108, 969-978, 2001.

Schindowski K, Fröhlich L, Maurer K, Müller WE, Eckert A.; Age-related impairment of human T lymphocytes' activation: Specific differences between $CD4^+$ and $CD8^+$ subsets. *Mech Aging Dev* 123, 375-390, 2002.

Schindowski K, Leutner S, Gorriz C, Peters J, Schramm U, Weinandi T, Kratsch T, Fröhlich L, Maurer K, Touchet N, Czech C, Eckert A, Müller WE.;. Incraesed apoptotic cell death in different lymphocyte's subsets in sporadic Alzheimer's disaese and APP/PS1 transgenic animals. *submitted.*

Schindowski K, Marques C, Touchet N, Czech C, Eckert A, Müller WE.;.Antiapoptotic action of C-terminal fragment of AD-related Presenilin-1 in PS1-transgenic mice. *in prep*

Schindowski K, Leutner S, Gorriz C, Peters J, Schramm U, Weinandi T, Kratsch T, Fröhlich L, Maurer K, Eckert A, Müller WE.;. Increased apoptosis and altered immunological action in T lymphocytes from sporadic Alzheimer's disease patients: Evidence for enhanced generation of memory T cells in AD? *in prep*

Curriculum Vitae

Katharina H. Schindowski

30.10.1972	geboren in Frankfurt am Main
1979 – 1983	Erich-Kästner-Schule, Frankfurt am Main
1983 – 1992	Anna-Schmidt-Gynasium, Frankfurt am Main
Juni 1992	Abitur, Note 1,9
1992 – 1996	Studium der Chemie an der Johann-Wolfgang-Goethe Universität, Frankfurt
Okt 1994	Quereinstieg in das 5.Semester des Studiengangs Biochemie an der Johann Wolfgang Goethe-Universität in Frankfurt parallel zum Chemiestudium.
Nov 1994	Vordiplom im Studiengang Chemie, Note 1
Feb 1995	Zwischenprüfung in Biochemie, Note 1.
Nov 1996	Diplomhauptprüfungen im Studiengang Biochemie
Dez96- Jun97	Diplomarbeit am Deutschen Krebsforschungszentrum (DKFZ) in Heidelberg in der Abteilung von Frau Prof. Dr. M. Zöller mit dem Thema: „Tetracyclin-regulierbare Expression von humanen Interleukin-2 in humanen Tumorzellen“. Abschluss: Diplom-Biochemikerin, Note 1.
seit Sept. 97	Promotionsarbeit an der Johann-Wolfgang-Goethe Universität am Pharmakologischen Institut von Prof. Dr. W.E. Müller mit dem Thema: „Mechanismen des apoptotischen Zelltodes an Lymphozyten im Alterungsprozess und in der Alzheimer Demenz“. Mitglied des Graduiertenkollegs „Arzneimittel: Entwicklung und Analytik”
seit Juni 2001	wissenschaftliche Mitarbeit am Institut für Anatomie und Zellbiologie III der Universität Heidelberg von Prof. Dr. K. Unsicker mit dem Thema: „Molekulare Schlaganfallforschung”. Mitglied des Interdisziplinären Zentrum für Neurowissenschaften (IZN) der Universität Heidelberg.

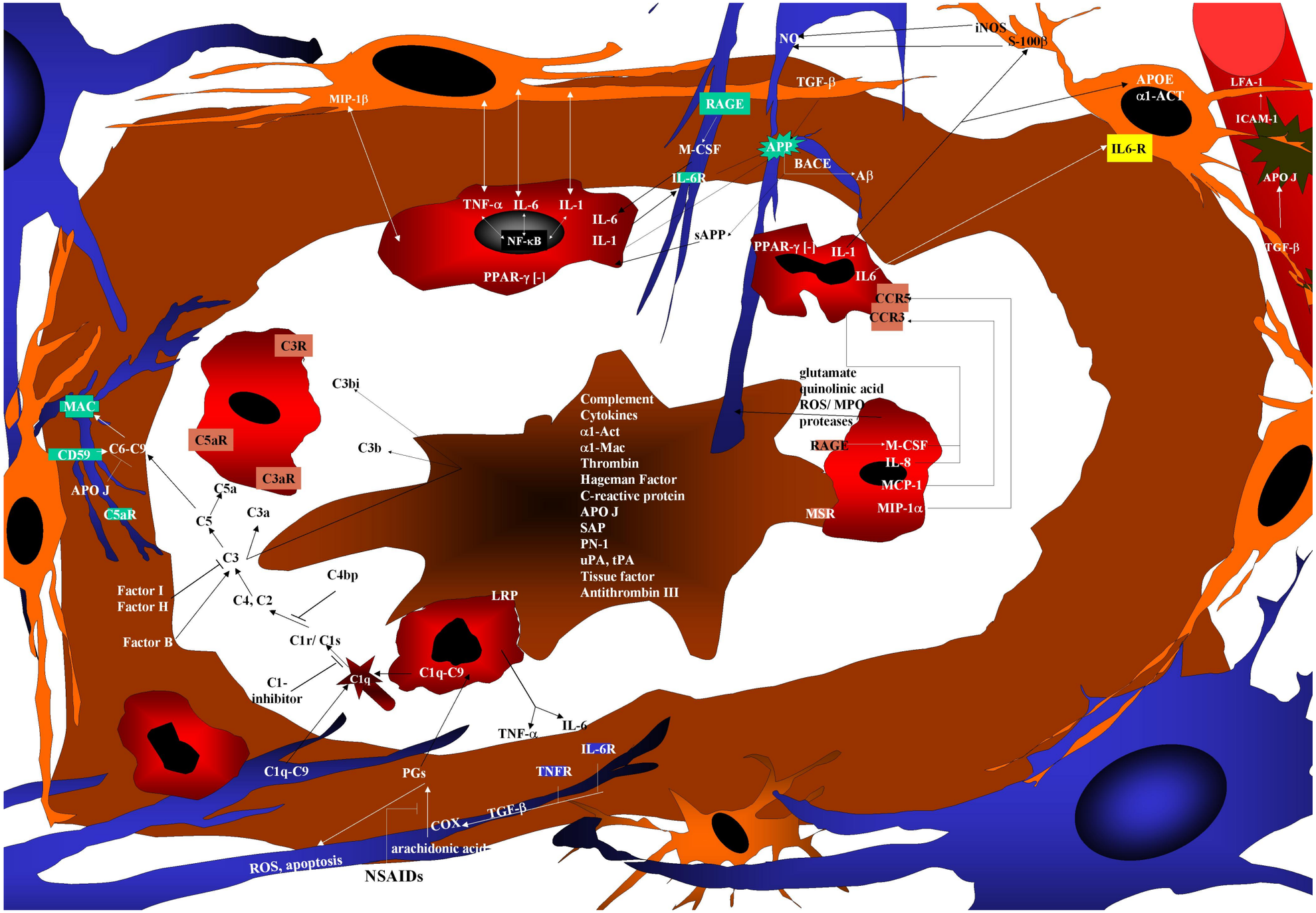


Figure 82. Comprehensive summary of inflammatory mechanisms in Alzheimer's disease (adapted from Neuroinflammation Working Group, 2000)