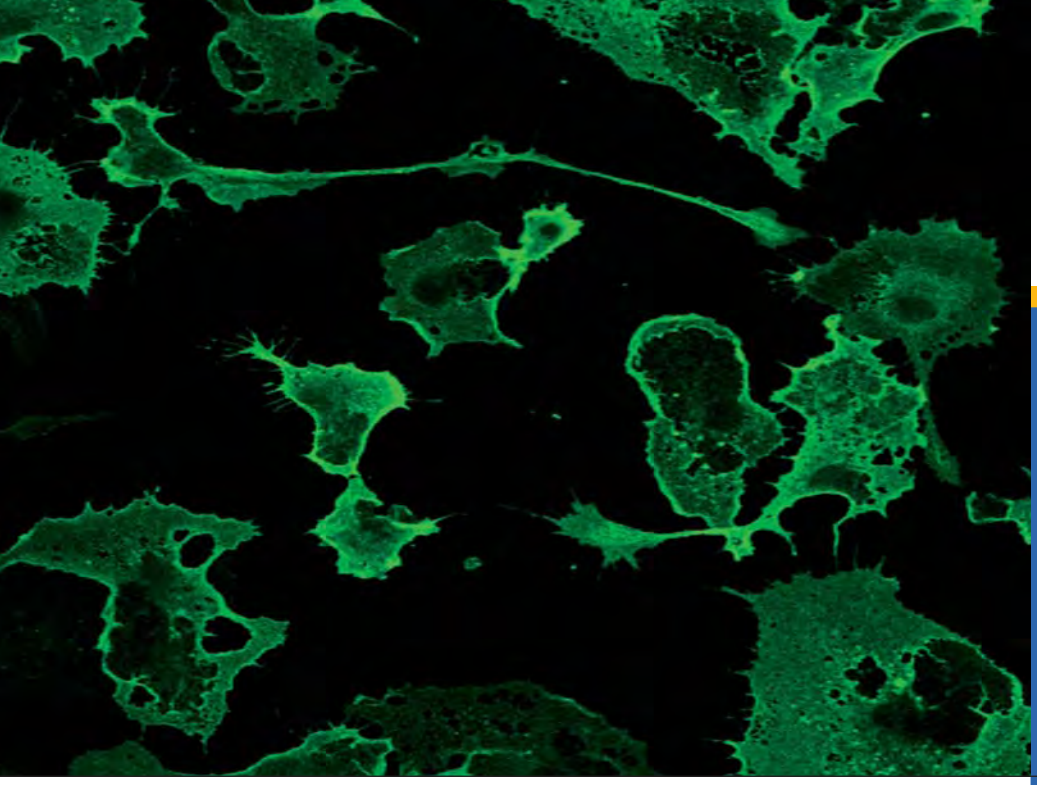

Anna Kiialainen

Pathogenic Mechanisms of Polycystic Lipomembranous Osteodysplasia with Sclerosing Leukoencephalopathy (PLOSL)

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Helsinki 2007

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PATHOGENIC MECHANISMS OF POLYCYSTIC LIPOMEMBRANOUS OSTEODYSPLASIA WITH SCLEROSING LEUKOENCEPHALOPATHY (PLOSL)

A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Faculty of Biosciences, University of Helsinki, for public examination in the Small Hall, University Main Building, Fabianinkatu 33 (4th floor), on October 12th, 2007, at 12 noon.

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ABSTRACT

Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), also known as Nasu-Hakola disease, is a recessively inherited disease of brain and bone. PLOSL manifests as early-onset progressive dementia and bone fractures. Mutations in the *TYROBP* (*DAP12*) and *TREM2* genes have been identified as the primary cause of PLOSL. *DAP12* and *TREM2* encode important signalling molecules in cells of the innate immune system. The mechanism by which loss-of-function of the DAP12/TREM2 signalling complex leads to PLOSL is currently unknown.

The aim of this thesis work was to gain insight into the pathogenic mechanisms behind PLOSL. To first identify the central nervous system (CNS) cell types that express both Dap12 and Trem2, the expression patterns of Dap12 and Trem2 in mouse CNS were analyzed. Dap12 and Trem2 expression was seen from embryonic stage to adulthood and microglial cells and oligodendrocytes were identified as the major Dap12/Trem2 producing cells of the CNS. To subsequently identify the pathways and biological processes associated with DAP12/TREM2 mediated signalling in human cells, genome wide transcript analysis of *in vitro* differentiated dendritic cells (DCs) of PLOSL patients representing functional knockouts of either DAP12 or TREM2 was performed. Both DAP12 and TREM2 deficient cells differentiated into DCs and responded to pathogenic stimuli. However, the DCs showed morphological differences compared to control cells due to defects in the

actin filaments. Transcript profiles of the patient DCs showed differential expression of genes involved in immune response and for genes earlier associated with other disorders of the CNS as well as genes involved in the remodeling of bone, linking the findings with the tissue phenotype of PLOSL patients. To analyze the effect of Dap12 deficiency in the CNS, genome wide expression analysis of Dap12 deficient mouse brain and Dap12 deficient microglia as well as functional analysis of Dap12 deficient microglia was performed. Regulation of several pathways involved in synaptic function and transcripts coding for the myelin components was seen in Dap12 knockout mice. Decreased migration, morphological changes and shortened lifespan of the Dap12 knockout microglia was further observed.

Taken together, this thesis work showed that both Dap12 and Trem2 are expressed by CNS microglia and that Dap12 deficiency results in functional defects of these cells. Lack of Dap12 in the CNS also leads to synaptic abnormalities even before pathological changes are seen in the tissue level.This work further showed that loss-of-function of DAP12 or TREM2 leads to changes in morphology and gene expression in human dendritic cells. These data underline the functional diversity of the molecules of the innate immune system and implies their significant contribution also in demyelinating CNS disorders, including those resulting in dementia.

Keywords: PLOSL, Nasu-Hakola disease, neurodegeneration, DAP12, TREM2, innate immunity, dendritic cells, microglia

Anna Kiialainen, PLOSL:n (polykystinen lipomembranoottinen osteodysplasia ja sklerosoiva luekoenkefalopatia) tautimekanismit
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TIIVISTELMÄ

Polykystinen lipomembranoottinen osteodysplasia ja sklerosoiva leukoenkefalopatia (PLOSL) on autosomissa peittyvästi periytyvä luuston ja aivojen sairaus. PLOSL:sta käytetään myös nimeä Nasu-Hakolan tauti. PLOSL aiheuttaa luuston heikkenemistä ja varhaisella iällä alkavan dementian. Mutaatiot *TYROBP* (*DAP12*) ja *TREM2* geeneissä johtavat PLOSL:n. DAP12 ja TREM2 proteiinit ovat tärkeitä immuunijärjestelmän solujen signaalinvälityksessä. Mekanismi, jolla DAP12/TREM2 välitteisten signaalien puute johtaa PLOSL:n, on kuitenkin selvittämättä.

Tämän väitöskirjatutkimuksen tavoitteena oli selvittää PLOSL:n tautimekanismeja. Aluksi tarkasteltiin Dap12 ja Trem2 lähetti RNA:n ja proteiinien ilmentymistä hiiren keskushermostossa. Molempia ilmennettiin samoilla aivoalueilla sikiökaudelta lähtien aina aikuisuuteen saakka. Mikrogliat ja oligodendrosyytit tunnistettiin Dap12 ja Trem2 ilmentäviksi keskushermoston solutyypeiksi. Seuraavaksi haluttiin selvittää mihin aineenvaihduntareitteihin ja solun toimintoihin DAP12 ja TREM2 puutos vaikuttaa ihmisen soluissa. Tätä tutkittiin genominlaajuisella ekspressioanalyysillä DAP12 ja TREM2 mutanteissa potilaiden dendriittisoluissa. Potilaiden dendriittisolut erilaistuivat ja reagoivat patogeenistimulaatioon lähes normaalisti. Solujen morfologiassa nähtiin kuitenkin muutoksia johtuen solujen tukirangan aktiinin epänormaalista järjestäytymisestä. Ekspressioanalyysissä nähtiin eroja sekä immuunipuolustukseen liittyvien geenien että keskushermostosairauksiin

ja luun muodostukseen liittyvien geenien ilmentymisessä potilaiden ja kontrollien välillä. Seuraavaksi tarkasteltiin Dap12 puutoksen seurauksia hiiren keskushermostossa genominlaajuisen ekspressioanalyysin avulla. Geenien ilmentymistä analysoitiin Dap12 poistogeenisten hiirten aivoissa ja mikroglia-soluissa. Aivoista eristetyillä mikroglia-soluilla tehtiin myös toiminnallisia kokeita. Synapsien toimintaan ja myeliinin muodostukseen liittyvien geenien ilmentymisessä nähtiin eroja Dap12 poistogeenisten ja kontrollihiirten aivoissa. Dap12 poistogeeniset mikroglia-solut liikkuivat huonommin ja kuolivat aikaisemmin kuin villityypin mikrogliat.

Tässä väitöskirjatyössä havaittiin että mikrogliat ilmentävät Dap12:ta ja Trem2:ta keskushermostossa ja että Dap12:n puute johtaa mikrogliojen vialliseen toimintaan. Dap12:n puute aivoissa johtaa myös muutoksiin synapsien toimintaan liittyvien geenien ilmentymisessä jo ennen kuin aivoissa nähdään muutoksia kudostasolla. DAP12:n ja TREM2:n puute johtaa lisäksi morfologisiin ja geenien ilmentymisen muutoksiin ihmisen dendriittisoluissa. Tutkimuksen tulokset korostavat synnynnäiseen immuniteettiin liittyvien molekyylien toiminnan monimuotoisuutta ja viittaavat siihen, että niillä olisi tärkeä rooli myös keskushermostoa rappeuttavissa sairauksissa.

Avansanat: PLOSL, Nasu-Hakolan tauti, keskushermoston rappeutuminen, DAP12, TREM2, synnynnäinen immuniteetti, dendriittisolu, mikroglia

CONTENTS

ABBREVIATIONS

AD	Alzheimer's disease
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	antigen presenting cell
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BMDM	bone marrow derived macrophages
BM-MC	bone marrow derived myeloid cells
bp	base pair
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CNS	central nervous system
DAP12	DNAX activation protein of 12 kDa
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ES cell	embryonic stem cell
FcR	Fc receptor
FTD	frontotemporal dementia
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GO	gene ontology
HE	hematoxylin-eosin

HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	intracellular tyrosine based activation motif
ITIM	intracellular tyrosine based inhibitory motif
KARAP	killer cell activating receptor-associated protein
kb	kilobase
kDa	kilo Dalton
KIR	killer cell inhibitory receptor
LPS	lipopolysaccharide
LTP	long-term potentiation
mAb	monoclonal antibody
MBP	myelin basic protein
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex
mIPSC	miniature inhibitory postsynaptic current
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
MS	multiple sclerosis
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
NKR	natural killer receptor
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NO	nitric oxide
PBMC	peripheral blood mononuclear cells

PCR	polymerase chain reaction
PLOSL	polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
PTK	protein tyrosine kinase
RANKL	receptor activator of NF-κB ligand
RT-PCR	reverse transcriptase-PCR
SAPK	stress-activated protein kinase
SNP	single nucleotide polymorphism
Syk	spleen tyrosine kinase
TLR	Toll like receptor
TNF	tumor necrosis factor
TREM	triggering receptor expressed on myeloid cells
TrkB	tyrosine kinase receptor B
TYROBP	TYRO protein tyrosine kinase binding protein
ZAP-70	zeta-associated protein of 70 kDa

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Anna Kiialainen, Karine Hovanes, Juha Paloneva, Outi Kopra, Leena Peltonen. Dap12 and Trem2, molecules involved in innate immunity and neurodegeneration, are co-expressed in the CNS. *Neurobiol Dis* 2005 Mar 18(2):314-22

II Anna Kiialainen, Ville Veckman, Juha Saharinen, Juha Paloneva, Massimiliano Gentile, Panu Hakola, Dimitri Hemelsoet, Basil Ridha, Outi Kopra, Ilkka Julkunen, Leena Peltonen. Transcript profiles of dendritic cells of PLOSL patients link demyelinating CNS disorders with abnormalities in pathways of actin bundling and immune response. *J Mol Med* 2007 Sep 85(9):971-83

III Anna Kiialainen, Henna Linturi, Juha Saharinen, Lewis L. Lanier, Outi Kopra, Leena Peltonen. Dap12 (Tyrobp)-deficient mice show defects in microglial cell function and abnormal synaptic properties. Submitted

1 INTRODUCTION

Diseases of the central nervous system (CNS), especially dementia, become more common in aging populations. They thus present increasing medical, social, and financial challenge to the society. Both, genetic and environmental factors, contribute to the complex process of neurodegeneration. Understanding the disease mechanisms is essential in developing treatments or helping to prevent these diseases. We have currently more means and experience in studying monogenic than complex diseases. In addition to being interesting at their own right, monogenic diseases can serve as models for understanding the pathogenic mechanisms of more common disorders.

Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) is a recessively inherited disease, which causes early onset progressive dementia and bone fractures. Molecular basis of PLOSL is known, since mutations in *DAP12* and *TREM2* have been identified as the primary cause of the disease (Paloneva et al. 2000; Paloneva et al. 2002). Together, DAP12 and TREM2 form a signalling receptor complex in cells of the myeloid lineage (Bouchon et al. 2001b; Daws et al. 2001). Microglia and osteoclasts are the myeloid cells of the brain and bone, respectively, suggesting their involvement in PLOSL pathogenesis. The mechanism by which loss of DAP12/TREM2 mediated signalling in myeloid cells leads to the dementia and neurodegeneration observed in PLOSL is currently not known.

Animal models are important tools for studying disease mechanisms, especially in the CNS where tissue samples from patients at early stages of the disease are not readily available. Cell models, especially patient cells, are also valuable for analyzing the cellular processes affected by disease causing mutations. Novel high-throughput methods, such as genome wide expression analysis, have made it possible to analyse these models more efficiently than before.

The aim of this thesis work was to gain insight into the pathogenic mechanisms behind PLOSL. To achieve this goal, expression of Dap12 and Trem2 in the developing mouse CNS as well as in primary cells derived from the CNS was first analyzed both in RNA and protein level. Next, the genome-wide gene expression patterns as well as functional responses of cells collected from PLOSL patients homozygous for loss-of-function mutations of DAP12 or TREM2 and those of Dap12 knockout mice were analyzed.

2 REVIEW OF THE LITERATURE

2.1 Dementia

Dementia is defined as "the development of multiple cognitive deficits that include memory impairment and at least one of the following cognitive disturbances: aphasia, apraxia, agnosia or a disturbance in executive functioning. The cognitive deficits must be sufficiently severe to cause impairment in the occupational or social functioning and must represent a decline from a previously higher level of functioning" (American Psychiatric Association 1994). Dementia is a complex clinical phenotype with multiple causes. Dementia can be caused by primary neurodegenerative disorders, such as Alzheimers's disease, frontotemporal dementias, dementia with Lewy bodies, Parkinson's disease, and Huntington's disease; vascular diseases, such as familial amyloid angiopathy, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL); immunological disorders, such as HIV-dementia and prion disorders; toxic and metabolic disorders, such as alcoholism and vitamin B_{12} deficiency; as well as brain tumours and traumatic brain injury (Mirra and Hyman 2002). Some 5 to 10% of multiple sclerosis (MS) patients also present with dementia (DeSousa et al. 2002; Benedict and Bobholz 2007).

2.2 Genetics of dementia

Alzheimer's disease (AD) is the most common cause of dementia in Europe and North America (Mirra and Hyman 2002). It has both sporadic and familial forms. Familial AD is caused by autosomal dominant mutations in *amyloid precursor protein (APP)*, *presenilin 1* (*PS1*), or *presenilin 2* (*PS2*). Mutations in each of these genes lead to increased amount of the amyloidogenic Aβ42 peptide. Although the familial cases of AD are rare, the genetic analysis of these cases has provided insight into the

pathogenic mechanisms of AD in general. According to the amyloid hypothesis, the Aβ peptide, which is produced by cleavage of the APP protein, aggregates in the brain to form amyloid plaques, which are considered as the triggering factor of AD development. PS1 and PS2 are involved in the cleavage of APP. A variant of *apolipoprotein E* (*apoE*) has been shown to be a risk factor for the sporadic form of AD. *ApoE* is inherited in three forms: ε2, ε3, and ε4. Individuals with one or two copies of the ε4 allele have increased risk of AD, whereas the ε2 allele is protective. In families with *APP* mutations, genetic variability in *apoE* modifies the age of onset of AD in such a way that the ε4 allele decreases the age of onset. *ApoE* encodes for an Aβ binding protein, linking the mechanisms of familial and sporadic forms. (Hardy and Gwinn-Hardy 1998; Mirra and Hyman 2002; Hardy 2006)

Inspite of these advances, the exact mechanism of AD remains unresolved. Genetic bases of many other dementing neurodegenerative disorders have also been defined. Some examples are presented in Table 1.

TABLE 1. Examples of inherited dementias and their causative genes. (Mirra and Hyman 2002; Baker et al. 2006)

Disease	Gene	Consequence
CADASIL	*NOTCH3*	unknown
Frontotemporal dementia (FTD)	*Progranulin* (*PGRN*)	unknown
FTD with parkinsonism linked to chromosome 17 (FTDP-17)	Microtubule-associated protein tau (*MAPT*)	Tau protein inclusions in neurons and/or glia
Huntington's disease	*Huntingtin* (expanded CAG repeat)	Nuclear inclusions of polyglutamine
Prion disorders	*PRNP*	Aggregation of misfolded Prion protein (PrP)

Although many genes in neurodegenerative diseases have been identified, there is still a long way to go to solve the exact pathogenic mechanisms of these disorders and develop therapies to treat or prevent them.

2.3 Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL)

Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL; MIM221770), also known as Nasu-Hakola disease, is a progressive early onset dementia with bone fractures. First patients were described in the 1960's in Finland and Japan (Terayama 1961; Järvi et al. 1964; Järvi et al. 1968; Hakola 1972; Nasu et al. 1973).

2.3.1 The clinical picture of PLOSL

Early development of the PLOSL patients is normal. Symptoms usually begin with pain and fractures in wrists and ankles after minor injuries at early adulthood. Neurological symptoms appear as behavioral and personality changes at the fourth decade of life. In time, these develop into fullblown frontal lobe syndrome (loss of judgement, euphoria, lack of social inhibition, disturbance of concentration, lack of insight, lack of libido, and motor persistence). Patients develop progressing memory disturbance, which onsets at around the same time as the personality changes, but is initially less severe. Upper motor neuron involvement and gait disturbances are also observed. Most patients have epileptic seizures. The disease culminates in profound dementia and a vegetative state and leads to death before age 50 (Hakola et al. 1970; Paloneva et al. 2001; Klunemann et al. 2005).

2.3.2 Histopathology of PLOSL

PLOSL patients develop osteoporosis (loss of bone material). In radiography, cystic lesions in the bones of the extremities are observed (Paloneva et al. 2001). The cystic lesions of the bones are filled with convoluted lipid membranes, amorphous lipid material, and fat cells (Nasu et al. 1973). In neuroimaging, the patients show cerebral atrophy, calcification of the basal ganglia, and diffuse white matter changes (Paloneva et al. 2001; Klunemann et al. 2005). Neuropathological analysis demonstrates reduced brain weight, and frontally accentuated loss of the white matter. Histology shows an advanced loss of axons and myelin, activation of microglia and astrocytes, and vascular changes in PLOSL. The vascular changes consist of thickening of the vascular wall with narrowing of the lumen. No intraneuronal or glial pathologic inclusions have been observed in PLOSL, when stained for phosphorylated tau, α-synuclein, or ubiquitin. No Lewy bodies, plaques, congophilic angiopathy, phosphorylated neurofilament protein, or α-B-crystallin has been observed either (Paloneva et al. 2001).

Two hypotheses on the pathogenic mechanism behind PLOSL have been proposed. The first hypothesis suggested that an error in systemic lipid metabolism would lead to breakdown of the myelin sheats and be responsible for PLOSL (Nasu et al. 1973; Wood 1978). The second hypothesis considered vascular damage as the primary defect that would lead to breakdown of the blood-brain barrier and chronic brain edema (Kalimo et al. 1994). Vascular alterations in the brain of PLOSL patients are observed, but it is not known whether they are primary or secondary (Paloneva et al. 2001). Neither of these hypotheses has been proven right, so we turn to genetics to look for an answer.

2.3.3 Genetics of PLOSL

It was already noted on the first reports of the Finnish patients that PLOSL is an inherited disease (Hakola et al. 1970; Hakola 1972). PLOSL has an autosomal recessive pattern of inheritance. Although PLOSL is globally distributed, it is enriched in the Finnish population with an estimated population prevalence of 1-2 x 10^{-6} (Hakola 1990). PLOSL is one of the diseases of the Finnish disease heritage.

2.3.3.1 Finnish Disease Heritage

The concept of Finnish disease heritage was established in 1973 (Norio et al. 1973). Rare inherited disorders that are overrepresented in Finland make up this entity. The group of disorders has grown from the twenty originally described to nerly fourty described to date. Most of the diseases have an autosomal recessive mode of inheritance. Two of the diseases are X-chromosomal and two autosomal dominant. The origin of enrichment of some rare diseases is in the population history of Finland. It has shaped the Finnish gene pool and resulted in the enrichment of some diseases in the Finnish population. At the same time, alleles for other diseases have

disappeared. The homogeneity of the Finnish gene pool has proven as a valuable tool in finding disease genes (Peltonen et al. 1999; Norio 2003).

2.3.3.2 Identification of *TYROBP* as the first PLOSL gene

PLOSL locus was assigned to chromosome 19q13.1 in Finnish patients by genome-wide linkage, linkage disequlibrium, and haplotype analyses (Pekkarinen et al. 1998a; Pekkarinen et al. 1998b). The PLOSL gene in this region was later identified as *TYROBP* (also known as DAP12 or KARAP) by sequencing the coding regions of the candidate genes in the region. All of the Finnish patients were shown to have a homozygous 5265 bp genetic deletion encompassing the exons 1-4 of the 5 exons of the *TYROBP* gene. No TYROBP mRNA or protein expression was detected in patient lymphoid cells by Northern and Western blots. A homozygous single base deletion in exon 3 of a Japanese patient was also identified (Paloneva et al. 2000). Additional mutations in *TYROBP* have been described since (TABLE 1, Fig. 1). Some non-Finnish PLOSL families were not linked to chromosome 19q13 indicating genetic heterogeneity in PLOSL (Pekkarinen et al. 1998a).

2.3.3.3 Identification of *TREM2* as the second PLOSL gene

Candidate gene approach was used to look for a second PLOSL gene in patients without mutations in *TYROBP*. The genes, the products of which were known to interact with the TYROBP protein, were considered as candidate genes. Analysis of segregation of the marker haplotypes flanking the candidate genes revealed co-segregation of the 6p21.2 region with PLOSL. This region still contained three of the candidate genes: *TREM1*, *TREM2*, and *NKP44*. *TREM2* was identified as the second PLOSL gene by sequence analysis of the genomic DNA in the non-Finnish PLOSL families (Paloneva et al. 2002). Several mutations in *TREM2* have been described to date (TABLE 1, Fig. 1). Most of the mutations in *TREM2* are point

mutations. Some of the mutations introduce premature stop codons while others change amino acids.

With respect to neurologic and skeletal problems, mutations in *TYROBP* and *TREM2* cause a similar clinical phenotype. However, the age of onset of bone pain, fractures, and dementia varies, even between patients with identical mutations (Klunemann et al. 2005).

2.3.3.4 *TYROBP* and *TREM2* in other neurodegenerative disorders

Early onset dementia and involvement of frontal regions are also observed in neurodegenerative disorders other than PLOSL. Thus the question of *TYROBP* and *TREM2* polymorphisms affecting other neurodegenerative disorders has been raised. *TREM2* polymorphisms were analyzed in a study of Italian patients with Alzheimer's disease and Frontotemporal Lobar Degeneration. None of the three single nucleotide polymorphisms (SNPs) analyzed were polymorphic and no new polymorphisms were found by sequencing the exons of *TREM2* (Fenoglio et al. 2007). Possible role of *TYROBP* and *TREM2* polymorphisms in diseases other than PLOSL thus remains to be determined.

TABLE 1. PLOSL mutations.

Mutation	Country	Reference
TYROBP		
Del 5.3 kb ($PLOSL_{Fin}$)	Finlad, Sweden, Norway	(Paloneva et al. 2000; Tranebjaerg et al. 2000)
Del 8 kb	Brazil	(Paloneva et al. 2002)
2T>C / Met1Thr	Japan	(Kondo et al. 2002)
141delG / FS and termination at aa 52	Japan	(Paloneva et al. 2000; Kondo et al. 2002)
145G>C / Gly49Arg	Portugal	(Baeta et al. 2002)
154-155ins42nt	United Kingdom (Scotland), Germany	(Klunemann et al. 2005) and not reported
262G>T / Glu87Stp	Japan	(Kuroda et al. 2007)
TREM2		
40G>T / Glu14Stp	Germany	(Paloneva et al. 2003)
97C>T / Gln33Stp	Belgium, Italy	(Soragna et al. 2003; Klunemann et al. 2005)
132G>A / Trp44Stp	Bolivia	(Paloneva et al. 2002)
233G>A / Trp78Stp	Sweden	(Paloneva et al. 2002)
267delG / FS	France (Turkey)	(Klunemann et al. 2005)
313delG / FS	Germany	(Klunemann et al. 2005)
377T>G / Val126Gly	Canada, UK (Sri Lanka)	(Klunemann et al. 2005)
401A>G / Asp134Gly	USA (Slovakia)	(Paloneva et al. 2002)
482+2T>C / SP	Italy	(Paloneva et al. 2002)
558G>T / Lys186Asn	Norway	(Paloneva et al. 2002)

TP, truncated protein; FS, frameshift; SP, splicing mutation.

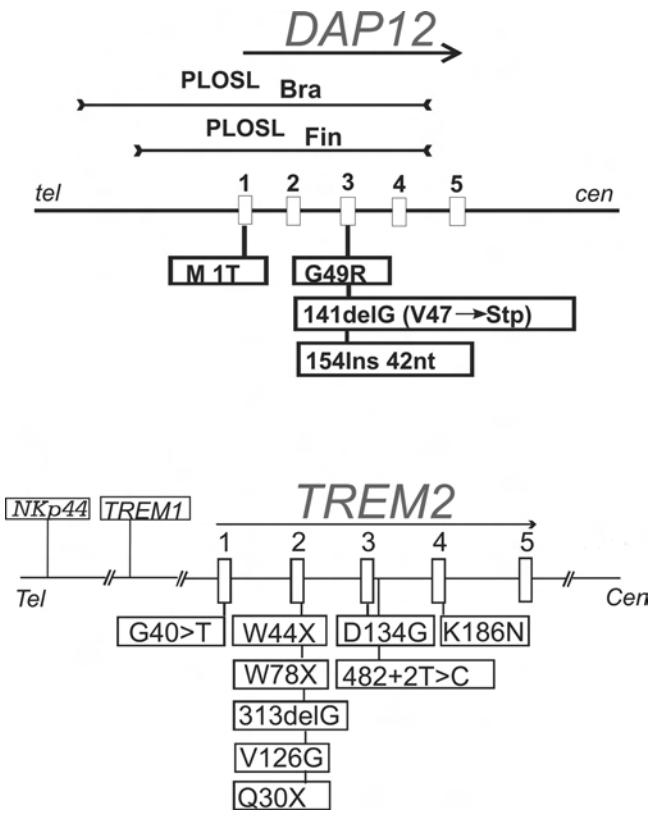

Figure 1. ***DAP12 and TREM2 genes and PLOSL mutations.***

2.4 DAP12 protein

The first PLOSL gene, *TYROBP*, encodes a 12 kDa DAP12 protein in humans. DAP12 is a type I transmembrane protein of 113 amino acids (Lanier et al. 1998). It contains a 27 amino acid leader segment, a 14 amino acid extracellular domain, a 24 amino acid transmembrane domain, and a 48 amino acid intracellular domain. DAP12 is a signalling adapter protein. In its intracellular domain, DAP12 contains an intracellular tyrosine based activation motif (ITAM, D/ExxYxxL/I-x_{6-8}-YxxL/I (Reth 1989)) with the sequence **E**SP**Y**QE**L**QGQRSDV**Y**SD**L** and potential

phosphorylation sites for protein kinase C and casein kinase II (Fig. 2). In its transmembrane region, DAP12 contains a conserved aspartic acid (D) residue, important for its association with receptor molecules (Lanier et al. 1998).

Killer cell activating receptor-associated protein (KARAP) was identified as a disulfide-linked tyrosine-phosphorylated dimer that selectively associates with the activating natural killer receptors (NKRs) (Tomasello et al. 1998). Karap gene is localized on mouse chromosome 7, spans 3.56 kb, contains five exons, and produces an open reading frame of 342 bp. The predicted protein contains a 27 amino acid leader peptide, a 16 amino acid extracellular domain, a 24 amino acid transmembrane domain, and a 47 amino acid intracellular domain with an ITAM (Y^{65}QELQGQRPEVY^{76}SDLN). Karap also contains cysteines in the extracellular region and a charged amino acid (D25) in its transmembrane domain. Karap is a 9.6 kDa type I transmembrane protein. The Karap polypeptide was found to have 73% amino acid identity with and to be orthologous to the human DAP12 (Tomasello et al. 1998).

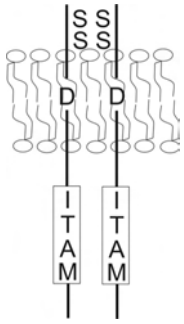

Figure 2. ***Schematic representation of the DAP12 protein dimer. The negatively charged aspartic acid (D) residue is needed for the association of Dap12 with receptor molecules. Intracellular tyrosine based activation motif (ITAM) is needed for signal transduction.***

2.4.1 DAP12 mediated signal transduction

Natural killer (NK) cells are lymphocytes of the innate immune system. They are involved in the early defense against foreign cells as well as cells undergoing stress such as viral and bacterial infection, parasites, or malignant transformation. NK cells exert direct cytotoxicity and produce cytokines and chemokines. The activation status of NK cells is controlled by a dynamic equilibrium between excitatory and inhibitory signals (Vivier et al. 2004). DAP12 is expressed as a disulphide-bonded homodimer in NK cells (Lanier et al. 1998). It associates non-covalently with killer-cell inhibitory receptor (KIR) family members that do not contain intracellular tyrosine based inhibitory motifs (ITIMs) in their cytoplasmic domains. Crosslinking of KIR-DAP12 complexes leads to phosphorylation of the DAP12 ITAM and cellular activation, as demonstrated by tyrosine phosphorylation of cellular proteins and upregulation of early-activation antigens (Fig. 3). The mechanism of activation might be explained by the binding of ZAP-70 and Syk protein tyrosine kinases to phosphorylated DAP12 intracellular peptides (Lanier et al. 1998).

In murine NK cells, Dap12 associates with activating Ly49D and H isoforms (McVicar et al. 1998). Ly49 receptors are a family of mouse NK cell receptors with both inhibitory and activating members. Ligation of the Ly49D-Dap12 complex results in tyrosine phosphorylation of phosphoplipase Cγ1, Cbl, and p44/p42 mitogen-activated protein kinase, as well as calcium mobilization. It also leads to activation of Syk but not Zap-70 (McVicar et al. 1998). Syk plays a critical role in the NK cell lytic pathway whereas Zap-70 is dispensable (Brumbaugh et al. 1997). Inspite of the involvement of DAP12 in NK cell activation, DAP12 deficient human NK cells are able to kill K562 erythroleukaemia cells as efficiently as control NK cells (Paloneva et al. 2000).

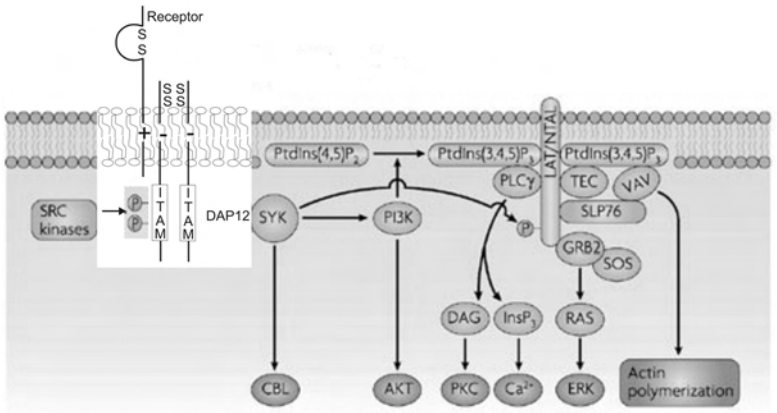

Figure 3. ***Dap12 mediated signal transduction. Modified from (Turnbull and Colonna 2007)***

In addition to NK cells, DAP12 is expressed in multiple other hematopoietic cell types. In neutrophils and macrophages, DAP12 has been shown to be involved in integrin signalling (Mocsai et al. 2006). Integrin signalling leads to phosphorylation of the tyrosine residues of the DAP12 ITAM by Src kinases and activation of Syk. DAP12 does not associate directly with the integrins, but the association is most likely mediated by one or some of the DAP12 associated receptors (Mocsai et al. 2006). To date, DAP12 has been shown to associate with several receptors in human and mouse (TABLE 2.).

TABLE 2. Dap12 associated receptors. Modified from (Takaki et al. 2006).

Common name	Expression	Structure
Human and mouse		
CD94/NKG2C	NK, T-cell subset	C-type lectin, heterodimer
PILRβ	NK, granylocyte, macrophage, DC	Ig-domain (1)
SIRPβ1	Granulocyte, monocyte, macrophage, DC	Ig-domain (3)
MDL-1	Monocyte, macrophage	C-type lectin, homodimer
TREM-1	Monocyte, macrophage, granulocyte, neutrophil	Ig-domain (1)
TREM-2	Macrophage, DC, osteoclast, microglia	Ig-domain (1)
Human		
NKp44	Activated NK, IPC subset, rare γδ-T-cell	Ig-domain (1)
KIR3DS1	NK, T-cell subset	Ig-domain (3)
KIR2DS1, 2DS2, 2DS4	NK, T-cell subset	Ig-domain (2)
IREM-2/CLM2	Monocyte, DC precursor	Ig-domain (1)
Mouse		
Ly49D, H, L, M, P, R, U, W	NK	C-type lectin, homodimer
NKG2D-S	NK, T-cell subset	C-type lectin, homodimer
Siglec-H	IFN-producing cells (IPC)	Ig-domain (2)
MAIR-II	Mast cell, granulocyte, macrophage, DC	Ig-domain (1)
CD200R3	Mast cell, basophil	Ig-domain (2)
CD200R4	NK, monocyte, macrophage	Ig-domain (2)
TREM-3	Macrophage	Ig-domain (1)

Number of Ig-domains of the receptor is in brackets.

2.5 TREM2 protein

Triggering receptor expressed on myeloid cells-2 (TREM2), encoded by the second PLOSL gene, is a transmembrane receptor of the Ig-superfamily. The TREM family of cell surface receptors participate in a variety of cellular functions in myeloid cells. TREM family members associate with DAP12 for signal transduction. TREM1, the member of the TREM family that was identified first, activates neutrophils and monocytes (Bouchon et al. 2000; Bouchon et al. 2001a). TREM2 was identified in human *in vitro* induced dendritic cells (Bouchon et al. 2000). It is a ~40 kDa glycoprotein. Its size is reduced to 26 kDa after N-deglycosylation. TREM2 has a single variable (V)-type extracellular domain, a charged lysine residue (K) in its transmembrane domain, and a short cytoplasmic tail with no known signalling motifs (Fig. 4). The charged lysine in the transmembrane domain of TREM2 is needed for its association with DAP12 (Bouchon et al. 2000; Bouchon et al. 2001b).

Mouse Trem2 was identified in macrophages as a transmembrane receptor with a single Ig (V) domain, a positively charged lysine in its transmembrane domain, and a short cytoplasmic tail (Daws et al. 2001). Mouse Trem2 associates with Dap12 and its crosslinking leads to nitric oxide (NO) release by macrophages. The mouse *Trem2* gene is located on mouse chromosome 17.

A splice variant of Trem2, which lacks the transmembrane domain and probably encodes a soluble form of the protein, was described in mouse microglia (Schmid et al. 2002). This svTrem2 differs from Trem2 by having a 55 bp insertion between exons 3 and 4. Sequence analysis indicates that splicing of exon 3 to an alternative splice site located 55 nucleotides upstream of exon 4 forms this variant. The insertion causes a frameshift generating a putative svTrem2 protein that lacks the transmembrane domain and is 22 amino acids longer than Trem2 (Schmid et al. 2002). In our RT-PCR experiments we have observed a similar transcript in the C57BL/6 mouse CNS (A. Kiialainen, unpublished observation). No further reports on the properties or function of the svTrem2 have been published.

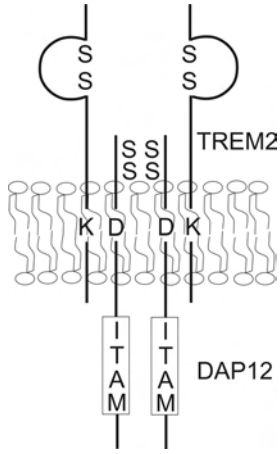

Figure 4. ***The DAP12/TREM2 receptor complex.***

2.6 Expression and function of the DAP12/TREM2 complex

DAP12 was originally cloned from a dendritic cell (DC) library (Lanier et al. 1998). Expression of DAP12 transcripts was detected in human peripheral blood leukocytes, spleen, and NK cell lines. DAP12 expression was also detected in cDNA libraries from peripheral blood mononuclear cells, DCs, peripheral blood monocytes and NK cells (Lanier et al. 1998). Karap expression was detected in NK cells, T cells, B cells, mast cells, endothelial and epithelial cells as well as neural cell lines (Tomasello et al. 1998). TREM2 was also first identified in DCs (Bouchon et al. 2000). Whereas DAP12 is expressed on several hematopoietic cell types, TREM2 expression seems to be restricted to myeloid cells. When *DAP12* was identified as the first PLOSL gene, it was already suggested that myeloid origin of microglia in the brain and osteoclasts in the bone could explain the tissue specificity of the PLOSL phenotype (Paloneva et al. 2000).

2.6.1 Myeloid cells

Myeloid cells are derived from hematopoietic precursors of the bone marrow (Fig. 5). Hematopoietic stem cells first differentiate into myeloid progenitors, which then give rice to monocytes, osteoclast precursors, and microglia. Monocytes further differentiate into macrophages and myeloid dendritic cells. Pre-osteoclasts fuse into mature osteoclasts (Colonna 2003).

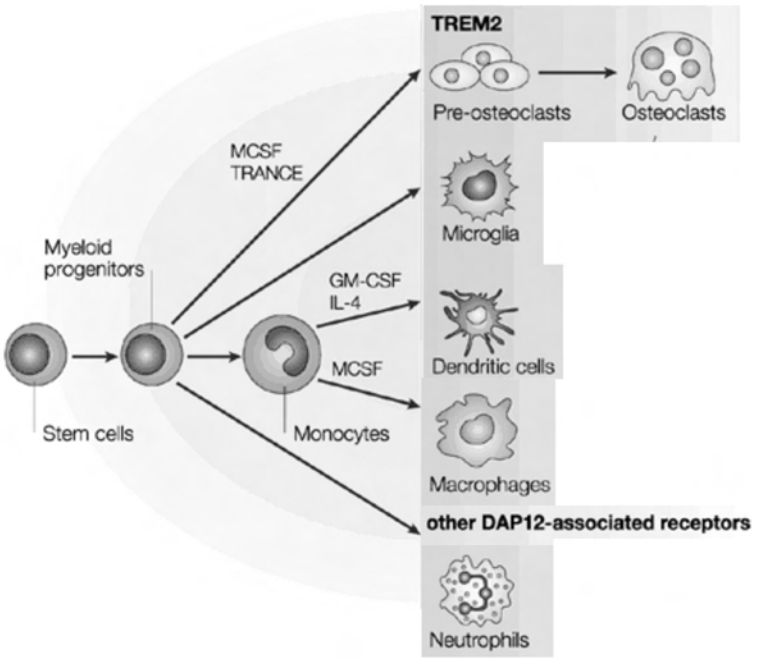

Figure 5. ***Myeloid cells expressing DAP12 and TREM2. Modified from (Colonna 2003)***

2.6.2 Dap12 and Trem2 in dendritic cells and macrophages

TREM2 associates with DAP12 in human monocyte-derived DCs (Bouchon et al. 2001b). TREM2 is not expressed on monocytes and is completely downregulated on DCs when DC maturation is induced by lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, CD40L-expressing cells, interleukin (IL)-1β, CpG oligonucleotodes, or aggregated immunoglobulin (Ig) G. Ligation of TREM2 with a monoclonal antibody (mAb) resulted in rapid rise in intracellular calcium levels of DCs indicating activation. Crosslinking of TREM2 also led to tyrosine phosphorylation of extracellular signal-regulated kinase (ERK1/2) and prolonged survival of DCs. This effect was blocked by ERK inhibitor, indicating that TREM2 induces survival of DCs through activation of the ERK pathway. In DCs, the DAP12/TREM2 complex promoted upregulation of CC chemokine receptor 7. Ligation of TREM2 also induced increased expression of MHC class II, CD40, and CD86 (B7.2), which are all involved in T cell stimulation. TREM2 induced activation was dependent on protein tyrosine kinase (PTK), partially dependent on ERK and independent of nuclear factor κ-B (NF-κB) and stress-activated protein kinase (p38/SAPK). Thus TREM2 seems to be an activating receptor on DCs and was suggested to be important in normal homeostasis of DCs (Bouchon et al. 2001b).

When mouse Trem2 was described, its expression was detected in macrophage cell lines (Daws et al. 2001). Dap12 and Trem2 protein expression has been detected in mouse bone marrow derived macrophages (Hamerman et al. 2006). *In vivo*, Trem2 was induced on tissue resident macrophages in the presence of type-II inflammation and on macrophages newly differentiated from monocytes leaving the circulation (Turnbull et al. 2006). It has been recently proposed that the Dap12/Trem2 complex has an inhibitory rather than activating role in macrophages (Hamerman et al. 2006; Turnbull et al. 2006). It was first noted that Dap12 deficient macrophages produce more inflammatory cytokines in response to Toll-like receptor (TLR) stimuli than wild type macrophages. It was thus suggested that Dap12 mediated signals

negatively regulate TLR signalling (Hamerman et al. 2005). Similar to Dap12 knockout macrophages, Trem2 knockdown in mouse macrophages increased TLR induced TNF production. A chimeric protein composed of the extracellular domain of Trem2 and the intracellular domain of Dap12 inhibited TLR and Fc receptor (FcR) induced TNF production in Dap12 deficient macrophages and rescued it to the wild type level. It was thus concluded that Trem2 is the Dap12 associated receptor involved in inhibitory signalling (Hamerman et al. 2006). Using Trem2 knockout macrophages it was also found that Trem2 inhibits cytokine production in response to TLR ligands (Turnbull et al. 2006). These studies show that the Dap12/Trem2 complex inhibits macrophage activation (Fig. 6).

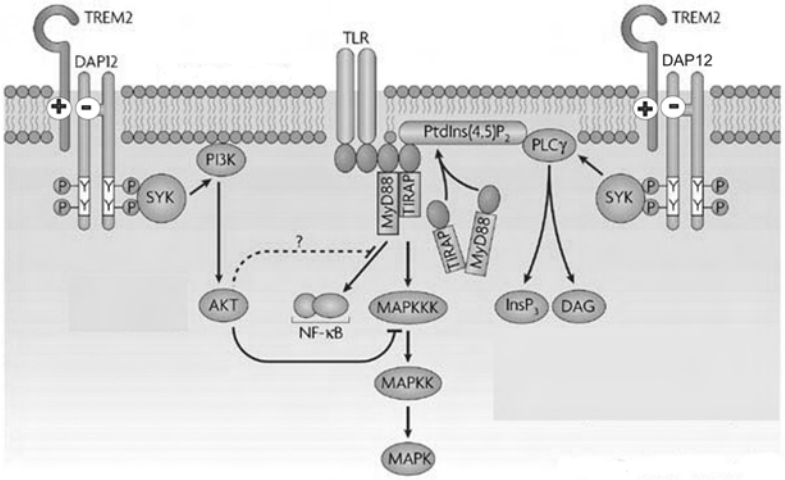

Figure 6. ***Inhibitory signalling through the DAP12/TREM2 complex. Modified from (Turnbull and Colonna 2007)***

2.6.3 Function of the DAP12/TREM2 complex in osteoclasts

Osteoclasts are multinucleated giant cells involved in bone resorption and homeostasis (Teitelbaum 2000; Väänänen et al. 2000). Studies by others and us have shown that DAP12 and TREM2 deficient peripheral blood mononuclear cells (PBMC) isolated from PLOSL patients fail to differentiate into multinucleated osteoclats *in vitro* (Cella et al. 2003; Paloneva et al. 2003). DAP12 and TREM2 deficient osteoclast-like cells show reduced bone resorption activity and impaired actin reorganization. Dap12 deficient mouse cells are also incapable of forming multinucleated osteoclasts *in vitro*. Osteoclast formation is restored when the cells are retrovirally reconstituted with Dap12 (Humphrey et al. 2004). Dap12 or Trem2 stimulation on RAW264.7 cells (a tumor cell line capable of forming osteoclasts) leads to increased formation of osteoclast-like multinucleated cells (Humphrey et al. 2004). Trem2 regulates multinucleation as well as resorption and migration of mature osteoclasts *in vitro* (Humphrey et al. 2006). Thus, the Dap12/Trem2 complex plays an important role in osteoclast differentiation. This is interesting considering the bone phenotype in PLOSL, but the mechanism of development of the bone abnormalities still requires further study.

2.6.4 Dap12 and Trem2 expression in microglia

Microglia are the resident immune cells of the CNS (Aloisi 2001). Microglia were shown to express Trem2 transcripts (Schmid et al. 2002). Others and we have detected Dap12 and Trem2 protein expression in microglia (Kaifu et al. 2003; Roumier et al. 2004; Takahashi et al. 2005)(I). Role of the DAP12/TREM2 complex in microglia and its consequences to PLOSL pathogenesis will be further considered in the Discussion.

2.7 Trem2 ligand

Since it is clear that the Dap12/Trem2 signalling is disturbed in PLOSL, it would be of interest to know what triggers this signalling pathway. The ligand for Trem2 is currently unknown. It has been suggested that Trem2 can bind both self- and pathogen-expressed ligands. It was shown that a Trem2-IgG_1Fc fusion protein binds specifically to gram-negative and gram-positive bacteria and yeast. Fluorescently labelled *Escherichia coli* and *Staphylococcus aureus* bound to Trem2 transfected cells. Bacterial products such as LPS and peptidoglycan inhibited fusion protein binding. The fusion protein also bound to a number of astrocytoma cell lines. It was suggested that the ligand recognition is partly based on charge (Daws et al. 2003). It was recently suggested that macrophages express Trem2 ligand, since they bind a Trem2-Fc fusion protein, and that Trem2 binding to its ligand in the same cell would lead to internalization of the complex (Hamerman et al. 2006). It was also proposed, but not shown, that the same would apply to microglia.

2.8 Mouse as a model in biomedical research

In order to study disease mechanisms, animal models are needed in addition to cells and *in vitro* experiments to analyze what happens in whole organisms. Although a lot has been learned by studying lower organisms such as bacteria, yeast, worms, and flies, mammalian models are also needed to understand disease processes in human. Mouse is widely used as a mammalian model animal, because of its small size, short lifespan, and short generation time. Most of the human genes have a mouse homolog, which can be manipulated to produce a mouse model of a human disease. Genetic manipulation of mouse is made possible by developments in different transgenic technologies for targeted gene manipulation and the availability of embryonic stem (ES) cell lines (Glaser et al. 2005). Availability of inbred mouse lines, phenotypic databases, and the mouse genome sequence are important tools for current biomedical research (Waterston et al. 2002; Guenet 2005).

2.9 DAP12 deficient mice

Three different Dap12 deficient mice have been described to date (Bakker et al. 2000; Tomasello et al. 2000; Kaifu et al. 2003). Immunological as well as central nervous system and bone phenotypes have been described in the mice.

2.9.1 Immunological phenotypes in Dap12 deficient mice

Bakker et al. generated Dap12 knockout mice using the cre-lox strategy to delete exons 3 and 4 of the *Tyrobp* gene on mouse chromosome 7 (Fig. 7A) (Bakker et al. 2000). 129/SV ES cells were introduced into the C57BL background. The mice had normal viability, weight, fertility, growth, and gross anatomy. Since Dap12 is implicated in NK and myeloid cell function, the immune cells of the mice were analyzed in detail. The mice had normally developed hematological compartment, except for increased number of major histocompatibility (MHC) class II positive DCs in the dermis compared to heterozygous controls. The activating Ly49D NK cell receptors that associate with Dap12 were inactive in these mice, but the NK cells of the mice were still able to lyse tumor cell lines. Thus Dap12 was found non-essential for cytotoxicity against tumor cells in mice. Dap12 knockout peritoneal macrophages as well as dendritic cells isolated from spleen or derived from bone marrow were identical to those derived from heterozygous controls. (Bakker et al. 2000)

Interestingly, Dap12 knockout mice were found to be resistant to experimental autoimmune encephalomyelitis (EAE) induced with myelin oligodendrocyte glycoprotein (MOG) peptide. EAE resembles some of the characteristics of the human disease multiple sclerosis (MS) and is studied as a mouse model of autoimmune demyelination. Because the $CD4^+$ T cells of the Dap12 knockout mice did not produce interferon-γ (IFNγ) when restimulated with the MOG peptide, it was suggested that the resistance to EAE was due to inadequate T cell priming in Dap12 knockout animals. (Bakker et al. 2000)

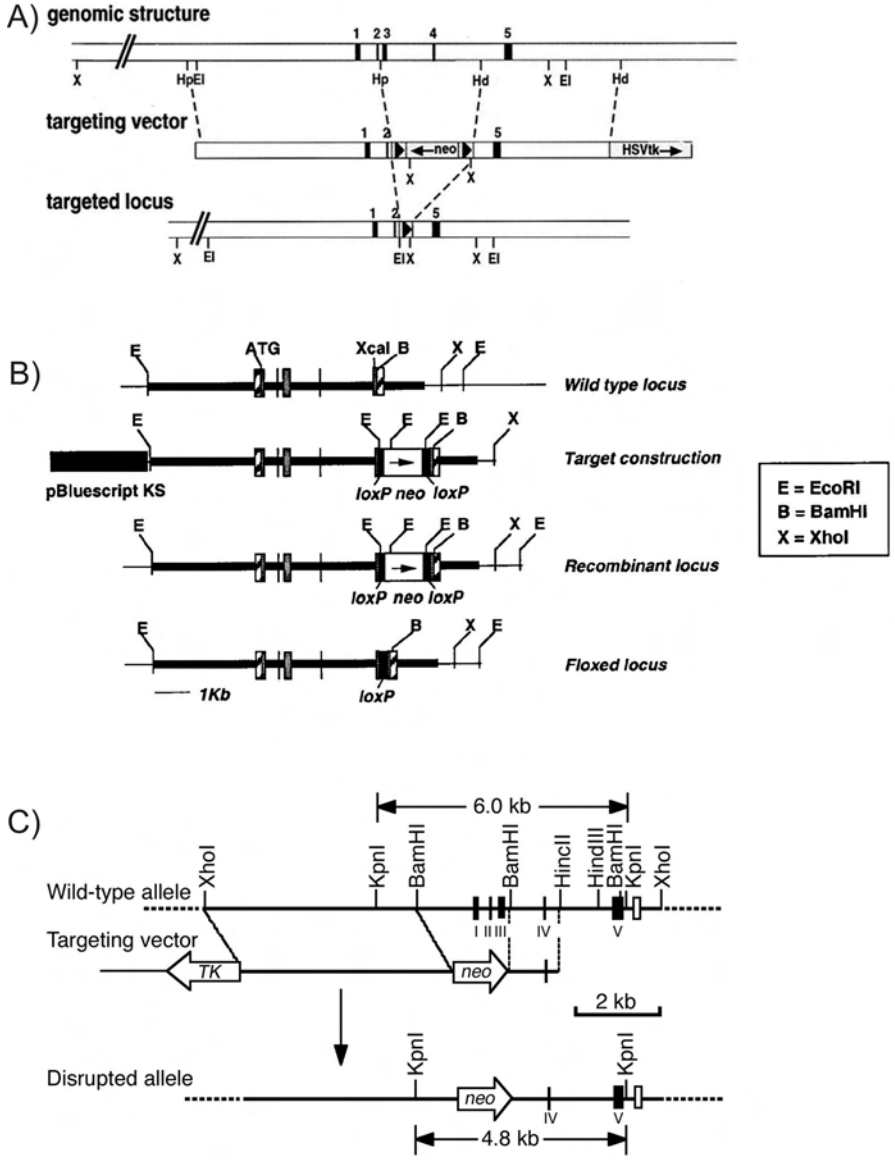

Figure 7. ***Constructs used to generate Dap12 deficient mice by A) Bakker et al. (2000), B) Tomasello et al. (2000), and C) Kaifu et al. (2003)***

Tomasello et al. generated Dap12 knockin mice with a non-functional ITAM (KΔY75/KΔY75, Fig. 7B) (Tomasello et al. 2000). The KΔY75/KΔY75 mice lack the Y75 residue of the ITAM and the following C-terminal amino acids, which are replaced by other amino acids that do not correspond to any known protein. The mutation was introduced into the exon 5 of the *Tyrobp* gene using the cre-lox strategy. 129 Ola ES cells were introduced into Balb/c background, which was then crossed with C57BL/6. The KΔY75/KΔY75 mice developed normally and were fertile. The knockin mice showed similar numbers of lymphoid and myeloid cell subsets as heterozygous and wild type controls. NK cells of these mice expressed the activating Ly49D and Ly49H receptors on the cell surface, but the receptors were non-functional. The natural cytotoxicity of NK cells from KΔY75/KΔY75 mice towards macrophage cell lines, but no other targets, was impaired. (Tomasello et al. 2000)

The distribution of DCs in KΔY75/KΔY75 mice was also studied. An accumulation of myeloid DCs in mucosal tissues was observed, but no detectable changes in phenotype or distribution of DCs in secondary lymphoid organs was seen. DCs were also cultured from bone marrow progenitors with granulocyte monocyte colony stimulating factor (GM-CSF). The *in vitro* derived DCs exhibited normal maturation process and LPS response as well as allostimulatory property for naïve $CD4^+$ T cells. The migratory capacity of skin DCs of the Dap12 knockin mice was comparable to normal, but hapten induced contact sensitivity was impaired indicating a defect in priming of hapten-specific $CD8^+$ T cells, which are responsible for the contact sensitivity, by skin DCs. (Tomasello et al. 2000)

These studies showed that Dap12 is essential for normal DC function in mice and especially for the ability of DCs to prime T cells.

2.9.2 Neurological defects in Dap12 deficient mice

Kaifu et al. generated Dap12 knockout mice by replacing the putative promoter and exons one to three of the *Tyrobp* gene with the neo cassette (Fig. 7C) (Kaifu et al.

2003). Mice were generated into the 129/SVJ and C57BL/6 hybrid background. Knockout cells did not express any Dap12 protein. Mice grew normally, were fertile and did not show any gross behavioural abnormalities up to 24 months. Since Dap12 mutations had been shown to cause PLOSL in humans at this point, the effect of the knockout on the CNS of the mice was analyzed in detail. No differences were observed in microglial (F4/80), neuronal (Nissl), neurofilaments (NF), or astroglial (GFAP) immunohistochemical stainings between Dap12 knockout and wild type control mice. Further, no difference in the number of apoptotic neurons (Nissl+TUNEL staining) and no evidence for cerebral inflammation (HE staining) was observed. A reduced myelin basic protein (MBP) staining was observed in the thalamus of Dap12 knockout mice. Electron microscopy of the thalami showed reduction in the number of myelinated axons, intact endothelial cells and basement membranes, degenerated synapses and accumulated synaptic vesicles. No differences were seen in analysis of motor function, nociceptive responses, and learning of the Dap12 knockout mice. A reduced startle reflex to acoustic stimuli as well as significantly reduced prepulse inhibition were observed in the Dap12 knockout mice suggesting impairment of sensorimotor gating. Both processes involve thalamus and γ-aminobutyric acid (GABA) mediated inhibition. Also, impairment in developmental changes of the decay time constant in GABAergic miniature inhibitory postsynaptic currents (mIPSCs) of Dap12 knockout mice was observed. (Kaifu et al. 2003)

Roumier et al. (Roumier et al. 2004) analyzed synaptic function in the Dap12 deficient KΔY75 mice (Tomasello et al. 2000). Long-term potentiation (LTP) in these mice was enhanced and partly independent of the NMDA receptor (NMDAR). LTP induction requires postsynaptic Ca^{2+} influx, usually through NMDARs (Malenka and Nicoll 1993). It was concluded that by affecting LTP Dap12 deficiency impacts on hippocampal synaptic plasticity. The mice also showed changes in synaptic glutamate receptor content by electrophysiology and

biochemical analysis. The AMPA receptor GluR2 subunit expression was decreased in the postsynaptic densities, but not in the whole membrane fraction, demonstrating specific impairment of receptor accumulation into synapses. A dramatic decrease in the brain derived neurotrophic factor (BDNF) tyrosine kinase receptor B (TrkB) expression was also observed in the Dap12 mutant synapses. BDNF signalling affects NMDAR function and thus also LTP. Dap12 expression was detected only in microglia, but not in astrocytes or oligodendrocytes both in primary mixed glial cultures and *in vivo*. It was thus suggested that microglial Dap12 affects synaptic function and plasticity through a novel microglia-neuron interaction. It is also noted, but not shown, that these mice show thalamic hypomyelinosis in old animals similar to that described in Dap12 knockout mice by Kaifu et al (Kaifu et al. 2003). (Roumier et al. 2004)

Nataf et al. (Nataf et al. 2005) analyzed the brain pathology in the KΔ75 Dap12 deficient mice (Tomasello et al. 2000). Histological analysis of CNS of adult Dap12 deficient mice showed diffuse hypomyelination predominating in anterior brain regions not accompanied with oligodendrocyte degeneration or microglial activation. It was thus suggested that the hypomyelination was due to developmental defect of myelin formation. A dramatic reduction in microglial cell number in postnatal mutant mice as well as impairment of microglial cell differentiation *in vitro* was also observed. Microglia were differentiated from bone marrow precursors amplified with FLT3-ligand with glial cell conditioned medium (described in (Servet-Delprat et al. 2002)). (Nataf et al. 2005)

In light of these studies it seems that Dap12 deficiency affects myelin stability and synaptic function in the CNS, which are both interesting in light of the CNS symptoms in PLOSL (see Discussion).

2.9.3 Bone abnormalities in Dap12 deficient mice

In addition to CNS changes, Dap12 deficient mice show bone abnormalities. They develop increased bone mass (osteopetrosis) (Kaifu et al. 2003). Micro-CT 3D imaging showed an increase in trabecular bone mass of the tibia of 40-week-old mice and hematoxylin and eosin staining showed higher amounts of bone trabecula in femurs of six weeks old Dap12 deficient mice than littermates. Number and morphology of osteoclasts in tissue sections of femurs of six weeks old mice was similar in Dap12 deficient mice and control littermates. The *in vitro* differentiation of Dap12 knockout bone marrow cells into multinucleated osteoclasts in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) or TNF-α was severely impaired. The osteoclasts formed by the Dap12 knockout cells did not have an actin ring characteristic of mature and functional osteoclasts and their ability to form resorptive pits on dentin slices was significantly reduced (Kaifu et al. 2003). A block of *in vitro* osteoclast differentiation and altered bone remodelling were also observed in the KΔY75 mice (Roumier et al. 2004; Nataf et al. 2005). Increased bone mass was also reported in the Dap12 knockout mice described by Bakker et al. Cells from these mice were incapable of forming multinucleated osteoclasts *in vitro*. Osteoclast formation was restored when the cells were retrovirally reconstituted with Dap12 (Humphrey et al. 2004).

In both, human and mice, functional Dap12 is required for the formation of multinucleated and functional osteoclasts *in vitro* (Cella et al. 2003; Kaifu et al. 2003; Paloneva et al. 2003; Humphrey et al. 2004). Opposing *in vivo* phenotypes are observed in mouse and human, since Dap12 deficient mice develop osteopetrosis (increase in bone mass) and PLOSL patients suffer from osteoporosis (bone loss). Reasons for this remain to be solved.

2.10 TREM2 knockout mice

Trem2 knockout mice have been recently described (Turnbull et al. 2006). The mice were generated into mixed 129 and C57BL/6 background. Exons 3 and 4 of the *Trem2* gene were deleted using the cre-lox strategy (Fig. 8). This resulted in deletion of a part of the transmembrane and cytoplasmic domains of the protein. Mice were backcrossed to the C57BL/6 background. The mice did not show any gross abnormalities. Wild type mouse bone marrow derived macrophages (BMDM) expressed Trem2. BMDM of the knockout mice did not express any Trem2 protein. Trem2 knockout BMDM produced increased amounts of TNF-α and IL-6 in response to the Toll-like receptor (TLR) agonists LPS, zymosan, and CpG. Peritoneal macrophages isolated from Trem2 knockout mice also produced increased amounts of TNF-α and IL-6 in response to LPS but not zymosan. The increase in cytokine production was smaller in the peritoneal macrophages than in the *in vitro* derived BMDM. It was concluded that Trem2 functions to attenuate macrophage response to microbial products. (Turnbull et al. 2006)

Preliminary data from the Trem2 knockout mice suggests that they have accelerated osteoclastogenesis. More rapid fusion of the Trem2 knockout cells into osteoclasts capable of bone resorption was noted *in vitro*. The Trem2 knockout mice did not show osteopetrosis similar to that observed in Dap12 deficient mice (Klesney-Tait et al. 2006). It should be noted that, these data are presented as unpublished observations in a review and the original data has not been published. No reports on the CNS phenotype of the Trem2 knockout mice have been published to date.

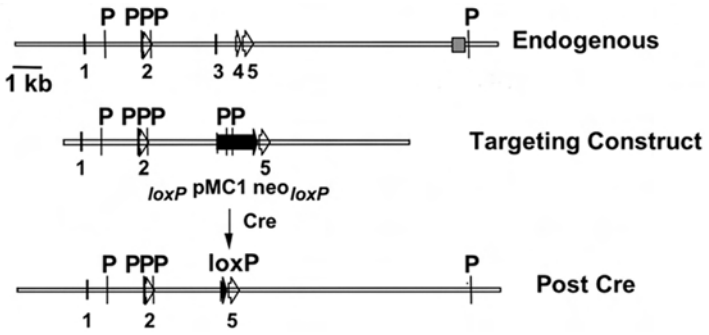

Figure 8. ***Construct used to generate Trem2 knockout mice. Modified from (Turnbull et al. 2006)***

2.11 Genome wide expression analysis

Completion of the human and mouse genome projects (Lander et al. 2001; Waterston et al. 2002), and development of the microarray hybridization technologies (Schena et al. 1995; Lockhart et al. 1996) has made it possible to analyze the expression of all of the genes of these organisms in one experiment. Instead of introducing bias by choosing candidate genes to study their expression between different conditions, we can now study the expression of all of the genes in the genome at once. This approach allows us to identify changes of expression in complete metabolic pathways in addition to individual genes, which makes it a valuable tool for studying disease mechanisms. The genome wide expression analysis has proven successful especially in cancer studies, where cancer subtypes with different drug response properties have been identified (Cheok and Evans 2006) and gene expression profiles have helped to predict disease outcome (Pomeroy et al. 2002).

3 AIMS OF THE STUDY

The general aim of this study was to gain insight into the pathogenic mechanisms of PLOSL. The more specific aims were:

First: to identify the spatial and temporal expression patterns of DAP12 and TREM2 in the CNS and to identify the CNS cell types, which express both of these gene products.

Second: to determine which pathways and cellular processes are affected by the lack of DAP12 and TREM2 in human cells that normally express these gene products.

Third: to determine which pathways and cellular processes are affected by the lack of DAP12 in the CNS and microglia of knockout mice.

4 MATERIALS AND METHODS

4.1 Methods

Methods used in this thesis are described in the original publications included.

TABLE 3. Methods.

Method	Original publication
Antibody production	I
Confocal and light microscopy	I, II, III
Dendritic cell culture	II
Enzyme-linked immunosorbent assay (ELISA)	II
Flow cytometry	II
Glial and neuronal primary cell culture	I, III
Immunofluorescence	I, II, III
Immunohistochemistry	III
In situ hybridization	I
Macrophage culture	II
Microarray analysis	II, III
Migration assay	III
Northern blot	I, II
PCR	II
Proliferation assay	III
Quantitative real time PCR	I, II
Reverse transcriptase-PCR (RT-PCR)	I
Sequencing	II
Western blot	III

4.2 Ethical aspects

Studies on PLOSL patient cells were approved by the Ethical Committee of the Hospital District of Helsinki and Uusimaa, Helsinki, Finland (II). Informed consent was obtained from all subjects. Animal studies were approved by the Chancellor's Animal Research Committee at the University of California Los Angeles (I), and the Ethical Committee for the use of Laboratory Animals at the National Public Health Institute, Helsinki, Finland (I, III).

4.3 Software and databases

Commercially and freely available software as well as software developed in our laboratory was used in this thesis. Expression data was submitted to the Gene expression omnibus (GEO) database and made freely available at publication (II).

TABLE 4. Software and databases.

Software/Database	Reference	Original publication
Cytoscape	www.cytoscape.org	III
Gene Expression Omnibus (GEO)	www.ncbi.nlm.nih.gov/geo	II
Genespring	Commercial (Agilent)	II, III
Gene Ontology Tree Machine (GOTM)	http://genereg.ornl.gov/gotm	II
GO2Cytoscape	Developed by Juha Saharinen at NPHI	III
Iterative Pathway Analysis	Developed by J.S. at NPHI	II, III
R	www.r-project.org	III
Statistical Package for Social Sciences (SPSS)	Commercial	II

5 RESULTS

5.1 Dap12 and Trem2 expression in the mouse central nervous system (CNS)

In the first part of the study we identified the cell types important for the CNS pathogenesis of PLOSL (I).

5.1.1 Dap12 and Trem2 are expressed in mouse brain from embryonic stage to adulthood

We first studied the expression of Dap12 and Trem2 in the developing mouse CNS by Northern blot and quantitative real-time PCR. Northern blot analysis of mouse brain at embryonic day 18, postnatal day one, day three, one week, two weeks, three weeks, one month, two months, three months, six months, and twelve months showed expression of both Dap12 and Trem2 mRNAs (Fig.1 in I). In order to quantitate the expression of Dap12 and Trem2 during mouse brain development, total RNA from the brain of C57/BL6 mice at embryonic day 17, one week, two weeks, one month, six months, and twelve months was analyzed with real-time quantitative PCR. Both Dap12 and Trem2 mRNAs were detected at all time points tested. Dap12 and Trem2 expression levels showed slight variation during development, but the changes in the steady state level were not significant at any time point up to twelve months. Thus, neither Dap12 nor Trem2 expression seem to be developmentally regulated, at least at the transcript level. Dap12 expression was higher than Trem2 at all developmental stages.

5.1.2 Dap12 and Trem2 transcripts show a glial expression pattern

We characterized the spatial expression pattern of Dap12 and Trem2 by *in situ* hybridization. Brain sections of neonatal (P1), two week, three month, and six month old mice were hybridized with Dap12 and Trem2 specific radioactively labeled probes. In the P1 brain sections, Dap12 and Trem2 transcripts showed a spatial co-localization in several brain regions, including the cerebral cortex (Ctx), the hippocampus (Hc) and the thalamus (Th) (Fig. 9). Dap12 and Trem2 expression patterns were very similar throughout the development. At P1, both Dap12 and Trem2 were mostly expressed in sub-cortical regions, especially in the thalamus. In the cortex, the expression was observed close to the white matter. The expression patterns imply glial expression of Dap12 and Trem2. In older mice expression of both transcripts was scattered throughout the CNS.

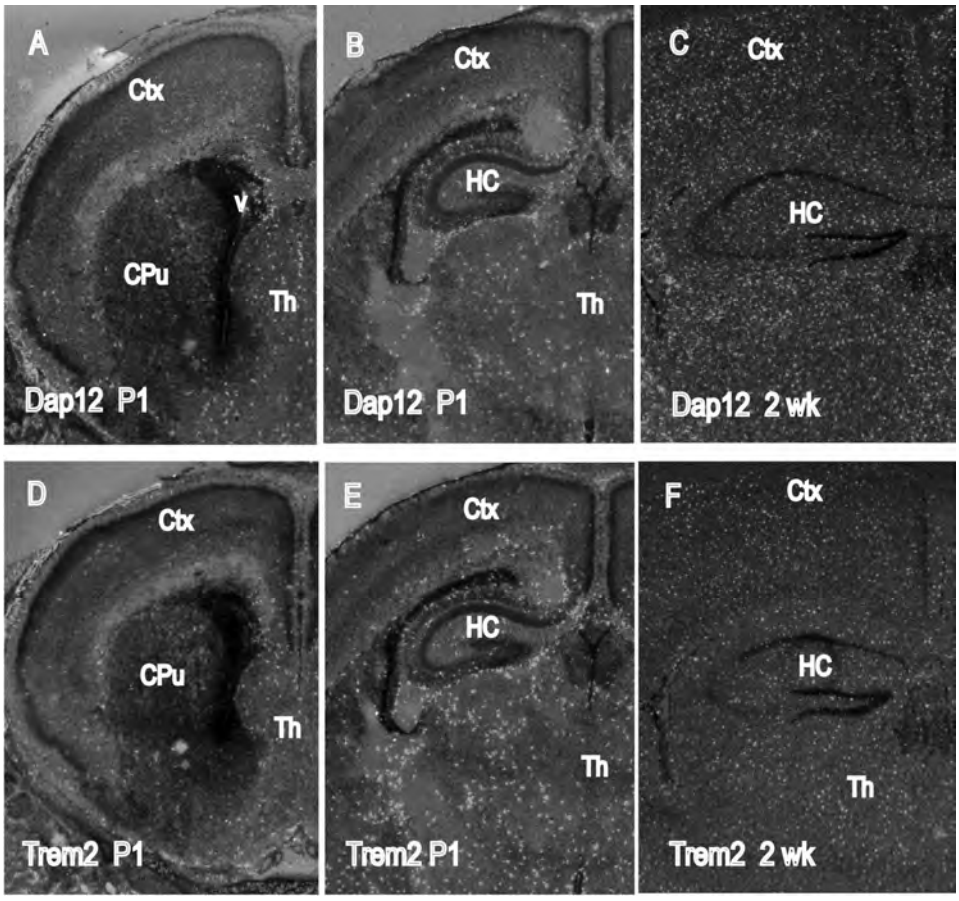

Figure 9. ***Dap12 and Trem2 expression in the developing mouse CNS. Dap12 and Trem2 are mostly expressed in the sub-cortical regions at postnatal day 1 (P1). At two weeks, the expression of both transcripts is scattered throughout the CNS. Ctx=cortex, CPu=caudate putamen, Hc=hippocampus, Th=thalamus, and V=ventricle.***

5.1.3 Microglial cells and oligodendrocytes express Dap12 and Trem2

In addition to neurons, CNS contains three types of glial cells: astrocytes, oligodendrocytes, and microglia. To characterize the specific cell type(s) expressing Dap12 and Trem2, we performed reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR analyses. Total RNA from the cerebral cortex, and CNS-derived primary neuronal and glial cell cultures of C57BL/6 mice were analysed by RT-PCR. Dap12 and Trem2 transcripts were detected in the cortex and in the mixed glial cell cultures (Fig. 10B). No Dap12 expression and little Trem2 expression were detected in neuronal cultures. RNA from rat primary cultures of astrocytes, oligodendrocytes and microglia were further analysed by real time quantitative PCR. Dap12 and Trem2 transcripts were detected in microglial cells and oligodendrocytes, but not in astrocytes (Fig. 10C). The relative expression levels of both Dap12 and Trem2 were considerably higher in microglial cells than in oligodendrocytes.

To monitor Dap12 and Trem2 expression at the protein level, primary glial cell cultures from C57BL6 mice were prepared and immunostained for Dap12 and Trem2. The cells were first characterized by immunostaining with cell type specific marker-antibodies: glial fibrillary acidic protein (GFAP) for astrocytes, O4, galactocerebroside (GalC), and myelin basic protein (MBP) for oligodendrocytes and F4/80-antigen for microglial cells. All three glial cell types were detected in the cultures. Subset of the primary glial cells showed immunopositivity for Dap12 and Trem2. Double staining showed co-localization of Dap12 and Trem2 in the same cells with F4/80-antigen. However, not all cells positive for F4/80-antigen expressed Dap12 or Trem2, and Dap12 and Trem2 staining was also observed in cells negative for F4/80-antigen.

We also prepared separate glial cell cultures. Microglial cells, oligodendrocytes and astrocytes can be separated by their different adhesion properties (McCarthy and de Vellis 1980; Cole R 1990). Mixed glial cell cultures were grown for two weeks and

microglial cells and oligodendrocyte progenitors then separated by shaking. Oligodendrocyte progenitors were proliferated and differentiated into mature oligodendrocytes. Microglial cells were obtained in high amounts and the cultures contained >95% F4/80 positive cells (Fig. 4A in I). These cells positively stained with Dap12 and Trem2 specific antibodies (Fig. 10A), but not with Trem2 0-serum (Fig. 4A in I). Oligodendrocytes were obtained in much lower numbers. They stained with the markers: O4, GalC and MBP (Fig. 4A in I). Oligodendrocytes too, showed expression of Dap12 and Trem2 proteins (Fig. 4B in I). Dap12 and Trem2 proteins were additionally shown to co-localize in the same cell in both microglia and oligodendrocytes (Fig. 4B in I). Also in line with the RT-PCR analysis, separate primary cultures of mouse neurons showed no staining with the Dap12 antibody, but weak staining with the Trem2 antiserum. No expression was detected with either antibody in separate cultures of astrocytes. Thus we concluded that microglia and oligodendrocytes are the main Dap12 and Trem2 expressing cell types of the CNS.

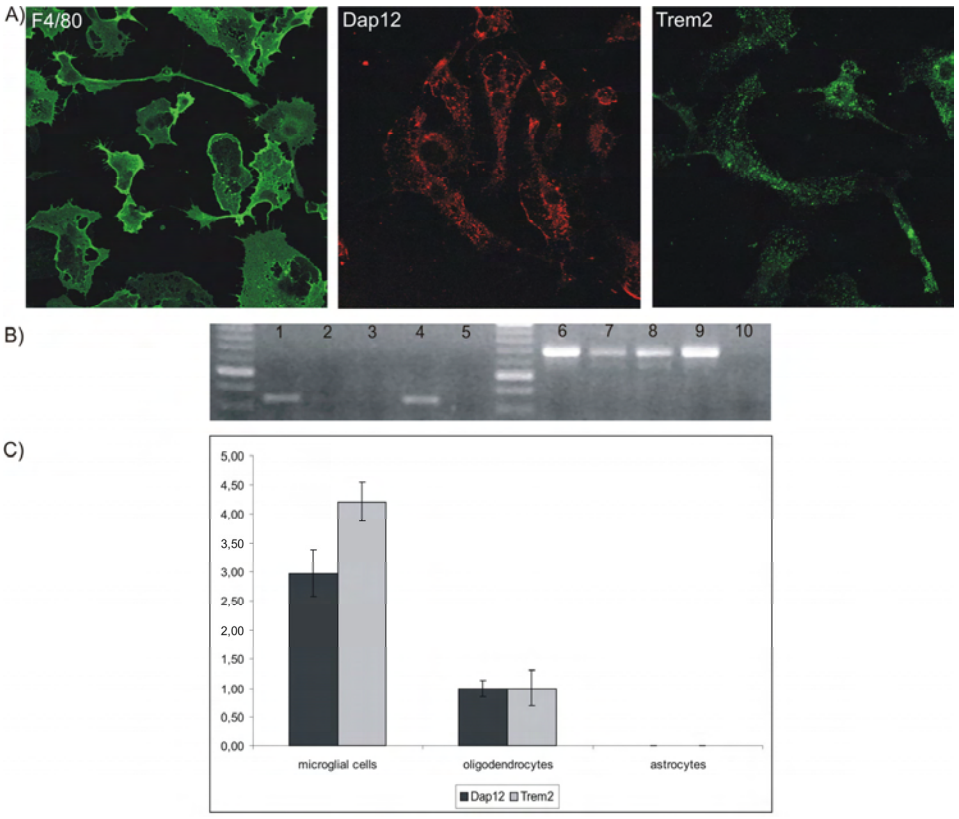

Figure 10. ***Dap12 and Trem2 expression in glial cells. Microglial cells stained with F4/80 and Dap12 and Trem2 antibodies (A). Dap12 (B, lanes 1-5) and Trem2 (B, lanes 6-10) expression is detected in cerebral cortex (lanes 1 and 6) and mixed glial cultures (lanes 4 and 9). Trem2 expression was also detected in neuronal cultures (lanes 7 and 8), but Dap12 was not (lanes 2 and 3). Lanes 5 and 10 are negative controls. Rat primary cultures of microglia and oligodendrocytes showed Dap12 and Trem2 expression in RT-PCR, whereas astrocytes did not (C).***

5.2 Transcript profiles of DAP12/TREM2 deficient dendritic cells point to defects in actin bundling and immune response

In the second part of the study we identified biological processes affected by lack of function of DAP12 and TREM2 in PLOSL patient dendritic cells (II).

5.2.1 *In vitro* differentiated DCs and macrophages express DAP12 and TREM2

We first isolated peripheral blood mononuclear cells (PBMC) of normal blood donors, enriched monocytes from them, and differentiated them into immature DCs and macrophages. Cells were collected after one, three, or seven days of differentiation with GM-CSF and IL-4 for DCs or GM-CSF for macrophages. Northern blot analysis revealed constitutive DAP12 mRNA expression in monocytes and throughout the differentiation process. TREM2 expression was detected at days three and seven, when the cells were already committed for DC or macrophage differentiation (Fig. 1 in II).

5.2.2 DAP12 and TREM2 deficient monocytes differentiate into DCs that respond to microbial stimuli

In order to identify biological processes involved in the pathogenesis of PLOSL, we studied PBMC of PLOSL patients lacking functional DAP12 or TREM2. Blood samples from five PLOSL patients were obtained. Three of the patients were homozygotes for the $PLOSL_{Fin}$ mutation (deletion of exons 1-4 of *TYROBP*; Paloneva et al. 2000), and two of the patients were homozygotes for point mutations in TREM2, one resulting in a premature stop codon (Q33Stp) and the other leading to an amino acid substitution (V126G) (Paloneva et al. 2002, Klunemann et al. 2005).

We isolated PBMC from the PLOSL patients as well as from healthy donors, enriched monocytes by adherence, and differentiated them into DCs. In order to determine whether DAP12 and TREM2 deficient cells differentiate into immature DCs, the cells were stained with antibodies specific for surface marker proteins of immature DCs and the expression was monitored by flow cytometry. DAP12 and TREM2 deficient cells expressed the immature DC surface markers CD1a, CD86, and HLA class II molecules. Although there was slight variation in CD1a and CD86, the expression levels did not differ significantly from the levels detected in control cells (Fig. 2 in II). The immature DC character was further proven by the absence of the monocyte marker CD14 and the mature DC marker CD83 on both, DAP12 and TREM2 deficient cells. Thus we concluded that DAP12 and TREM2 deficient cells have the capacity to differentiate into immature DCs *in vitro*.

DCs are the sentinels for foreign antigens in peripheral tissues. When immature DCs come in contact with microbes or their components, they mature into professional antigen presenting cells (APCs) that produce multiple cytokines and express cell surface molecules important for their proper function (Cella et al. 1997). We tested whether DAP12 and TREM2 deficient DCs can respond to microbial stimuli using lipopolysaccharide (LPS) and Sendai virus. It has been demonstrated that in human DCs TREM2 is effectively downregulated within 24 hours after LPS stimulation (Bouchon et al. 2001b). Consistent with this, our *in vitro* differentiated DCs from normal blood donors showed a strong downregulation of TREM2 transcript within 12 hours after LPS stimulation (Fig. 4 in II). DAP12 expression was not affected for up to 24 hours after LPS treatment.

We stimulated the DAP12 and TREM2 deficient DCs as well as control DCs with LPS or Sendai virus for 24 hours. CD86 and HLA class II expression were determined by flow cytometry before and after LPS and Sendai virus stimulation. We also monitored the ability of DAP12 and TREM2 deficient DCs to produce different cytokines in response to LPS and Sendai virus. The production of the pro-

inflammatory cytokines TNF-α and IL-12, the anti-inflammatory cytokine IL-10, and the chemotactic molecules CCL2 (MCP-1), CCL19 and CXCL10 (IP-10) were determined in cell culture supernatants collected at 24 hours after LPS stimulation or Sendai virus infection. Altogether DAP12 and TREM2 deficient cells responded to LPS and Sendai virus stimulation very similarly to control cells (Fig. 5 in II). The major differences observed were that LPS-induced expression of CD86 was reduced in DAP12 and TREM2 deficient DCs and the DAP12 deficient DCs produced no TNF-α in response to LPS.

5.2.3 DAP12 and TREM2 deficient DCs show morphological changes

We have previously shown that actin ring formation is disturbed in *in vitro* differentiated osteoclasts from DAP12 and TREM2 deficient patients (Paloneva et al. 2003). In order to visualize the actin cytoskeleton of the DAP12 and TREM2 deficient DCs, the DCs were centrifuged on microscope slides and stained with fluorescent phalloidin, which binds to F-actin in the cells. Confocal microscopy revealed that the DAP12 and TREM2 deficient DCs have more processes than control DCs (Fig. 11).

We measured the surface areas (μm^2) of DCs of two control individuals (number of cells, n=30 and 35), one DAP12 deficient individual (n=30), and one TREM2 deficient individual (n=46). Phalloidin stained cells were scanned with confocal microscope and the surface areas determined with the microscope's software. Statistical analysis showed that the mean surface areas of both DAP12 deficient DCs and TREM2 deficient DCs were larger than those of control DCs (Mann-Whitney U-test, $p<0.0001$). The difference in surface area is likely to result from the increased number of processes of the DAP12 and TREM2 deficient cells.

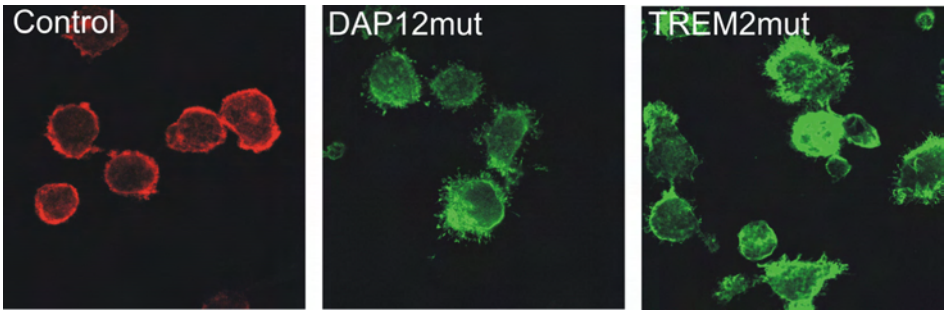

Figure 11. ***DAP12 and TREM2 deficient DCs show morphological changes when stained with fluorescent phalloidin for F-actin.***

5.2.4 Transcript profiles of PLOSL DCs versus control DCs reveal differential gene expression

We analyzed the transcript profiles of DCs of PLOSL patients and control individuals with Affymetrix microarrays. DCs were collected at day seven of GM-CSF and IL-4 stimulated differentiation. Total cellular RNAs of DCs of three controls, three DAP12 deficient individuals, and two TREM2 deficient individuals were hybridized to the Affymetrix HG-U133A GeneChip probe arrays individually. The data was analyzed with the Genespring 7.1 software. After data processing and quality control, the initial 22 283 probe sets were reduced to 7 606. Due to the limited number of patient samples we analyzed the DAP12 and TREM2 deficient samples as one group to look for differences between PLOSL and controls. We performed iterative pathway analysis on the 7 606 probe sets, separately for the down- and upregulated genes. The downregulated transcripts showed enrichment for four Gene Ontology (GO) biological process classes (Fig. 12, Table 1 in II): inflammatory response, innate immune response, chemotaxis, and actin filament

bundle formation. The upregulated transcripts were enriched for ten GO biological processes: inflammatory response, innate immune response, chemotaxis, complement activation (classical pathway), lymphocyte activation, cell-cell signaling, calcium ion homeostasis, negative regulation of cell proliferation, cytoplasmic sequestering of NF-κB, and pyridine nucleotide metabolism.

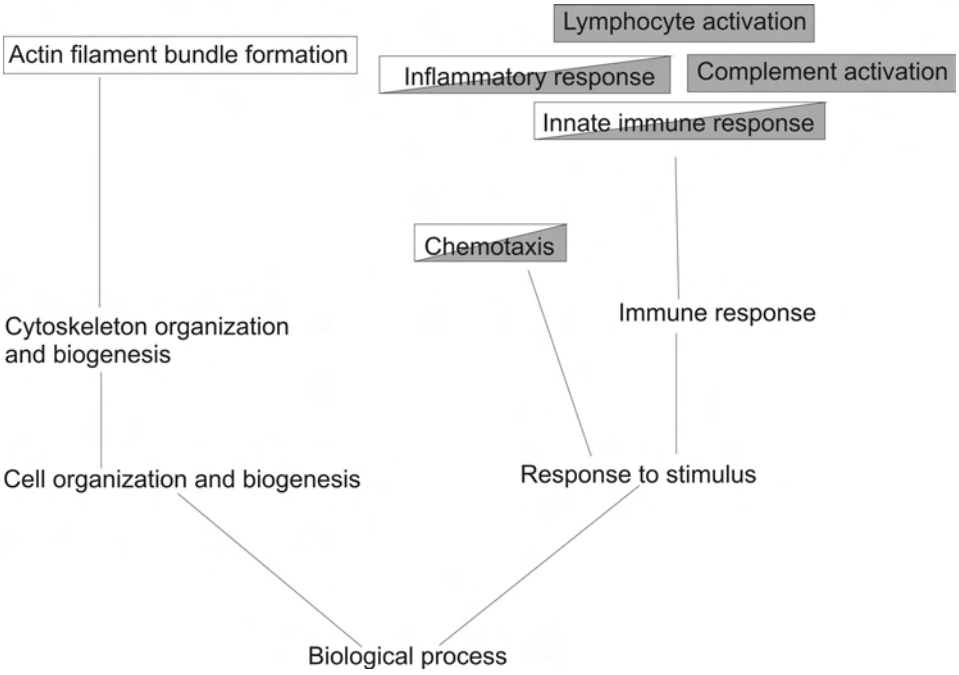

Figure 12. ***Pathways of actin bundling and immune response are regulated in PLOSL DCs. Downregulated pathways are shown in white, upregulated in grey.***

We further analyzed a subset of the differentially expressed genes by quantitative real-time PCR to validate the results obtained by the microarray analysis. The expression levels of three downregulated genes (SPP1, NEDD9, and FSCN1) and three upregulated genes (CCL2, BCL3, and NFKBIA) were analyzed with RT-PCR

from three controls, three DAP12 deficient samples and two TREM2 deficient samples (Table 3 in II). Changes in FSCN1 and NFKBIA transcript levels were seen between DAP12 mutants and controls, but not between TREM2 mutants and controls. The fold changes of the upregulated genes, CCL2 and BCL3, were lower and the fold changes of the downregulated genes, SPP1 and NEDD9, were higher when analyzed with RT-PCR as compared to the array hybridization.

5.3 Dap12 deficient mice show altered synaptic transcripts and defects in microglial function

In the third part of the study (III) we identified changes in expression of synaptic and myelin transcripts in the brain of Dap12 deficient mice and analyzed the functionality of Dap12 knockout microglia.

5.3.1 Dap12 knockout mice show increased expression of synaptic and myelin associated transcripts

The Dap12 knockout mice used in this study were originally produced at the laboratory of Dr. Lewis Lanier in University of California San Francisco (Bakker et al. 2000). The mice lack the exons three and four of the *Tyrobp* gene. In order to analyze the effect of the Dap12 mutation on the central nervous system of the mice, we performed genome wide expression analysis of the diencephalon and basal ganglia, the brain areas where most dramatic PLOSL pathology has been described, of a month old Dap12 mutant mice. RNA from the brain of three heterozygous control and three homozygous male mice were hybridised to the Affymetrix Mouse430_2 probe arrays individually. The data was analyzed with the GeneSpring GX7.3 software. Genes that passed the quality control were then ranked according to their expression fold change, separately for up- and downregulated genes, and subjected to the iterative pathway analysis. Several pathways involved in synaptic

function were significantly up-regulated in Dap12 knockout mice (Fig. 1 and Table 1 in III). Among the up-regulated genes were also five myelin related genes: myelin-associated oligodendrocytic basic protein (Mobp), myelin basic protein (Mbp), myelin proteolipid protein (Plp1), myelin and lymphocyte protein (Mal), and cyclic nucleotide phosphodiesterase 1 (Cnp1). The two down-regulated pathways were involved in regulation of development.

5.3.2 Differential gene expression in Dap12 knockout microglia

In the first part of the study we showed that microglia express both Dap12 and Trem2 (I). As the CNS is a very heterogeneous tissue we were looking to reduce the complexity and in addition to brain tissue analyzed the transcript profiles of primary microglia. RNA isolated from three Dap12 deficient and three wild type microglial cell cultures were hybridised to the Affymetrix Mouse430_2 probe arrays individually. The data were analyzed with GeneSpring and biological associations were assigned to differentially expressed genes with the Webgestalt tool. Several pathways were differentially regulated between Dap12 knockout and wild type microglia (Fig. 3 and Table 2 in III).

5.3.3 Dap12 knockout microglia show decreased survival and migration as well as morphological changes *in vitro*

It has been shown that the DAP12/TREM2 receptor complex promotes survival of human *in vitro* induced dendritic cells (Bouchon et al. 2001b). We studied the survival of Dap12 deficient microglia. We plated 15 000 Dap12 knockout and wild type cells per well and assayed them at day one, three, and five after plating with the CellTiter 96® Aqueous One Solution reagent that measures the amount of living cells in a well. At day one, the measured absorbance at 490 nm was about the same for both genotypes. At days three and five the absorbance was considerably higher for the wild type cells than Dap12 knockouts indicating greater amount of living

cells. The absorbance remained constant throughout the course of the experiment for the wild type cells, but decreased considerably from day one to day three for the Dap12 knockout cells (Fig. 4 in III). This indicates that the Dap12 knockout cells start dying in culture before day three and Dap12 is needed for the survival of microglia.

To study the migratory capacity of the cells, we plated Dap12 knockout and wild type microglia on polyester membrane inserts in Transwell 96 well plates at 25 000 cells per well. Migration of microglia through the membranes was assessed 24 hours after plating. The number of cells that had migrated through the membrane and attached to the bottom of the wells was counted. The number of wild type microglia that had passed through the membrane was 1.4 fold greater ($n=10$, $p<0.05$, Fig. 7 in III) than that of the Dap12 knockout microglia indicating a defect in the migratory capacity of the knockout microglia.

We have shown that DAP12 deficient *in vitro* induced human dendritic cells and osteoclasts show morphological changes and abnormal F-actin distribution compared to control cells (II, (Paloneva et al. 2003)). To look at the morphology of the Dap12 knockout microglia in vitro, we fixed them at day one after shaking and stained them with the myeloid marker F4/80 as well as fluorescently labelled phalloidin for F-actin. Dap12 knockout primary microglia showed different morphology and altered F-actin distribution compared to the wild type microglia (Fig. 13 and Fig.5 in III). In culture, the wild type microglia (Fig.5 A&C in III) have an elongated morphology and a number of processes whereas the Dap12 knockout microglia (Fig.5 B&D in III) are more round with less processes. Activated microglia retract their processes and show ameboid morphology (Haynes et al. 2006). We also performed immunohistochemical staining of Dap12 knockout and heterozygous mice brain with the microglial marker Iba1. We did not observe gross abnormalities in the morphology of the Dap12 knockout microglia *in vivo*

(Fig. 13). Dap12 thus seems to influence the activation status of the microglia, at least *in vitro*.

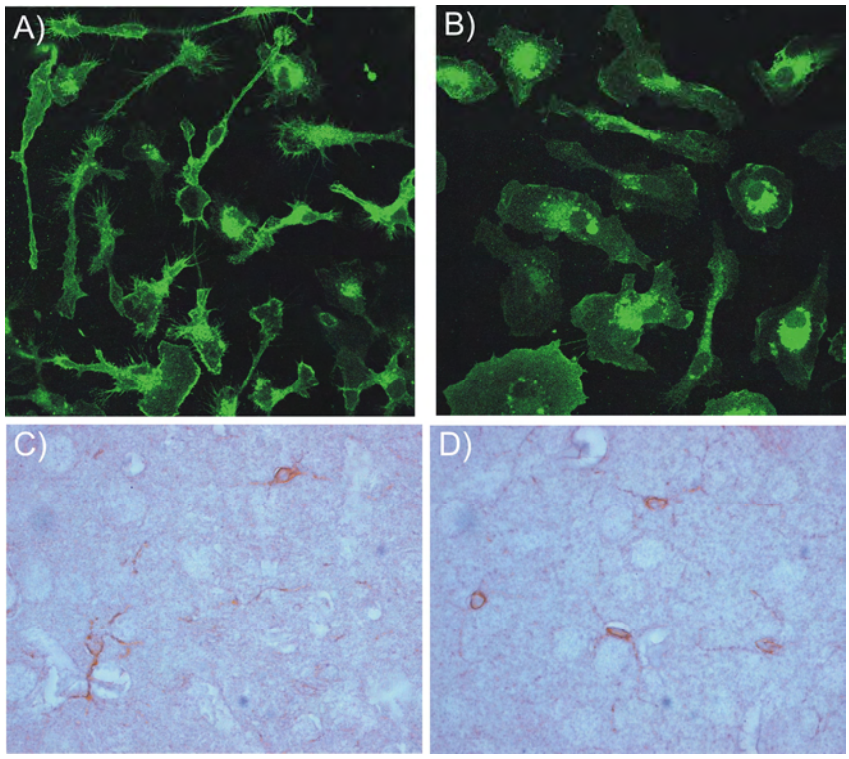

Figure 13. ***Morphology of microglia in vitro and in vivo. Wild type (A) and Dap12 knockout (B) primary microglia stained with the F4/80 antibody. Dap12 heterozygote (C) and knockout (D) brain slices stained with Iba-1. Pictures are from caudate-putamen of one month old mice.***

5.3.4 Primary oligodendrocytes can be cultured from Dap12 deficient mice

It has been suggested that oligodendrocyte differentiation is impaired in the Dap12 knockout mice (Kaifu et al. 2003; Nataf et al. 2005). We cultured oligodendrocytes from Dap12 knockout as well as wild type mice. Mixed glial cell cultures were first grown for two weeks and oligodendrocyte progenitors then separated by shaking. Oligodendrocyte progenitors were proliferated and differentiated into mature oligodendrocytes. We did not observe differences in the differentiation process of wild type and Dap12 knockout oligodendrocytes (Fig. 14).

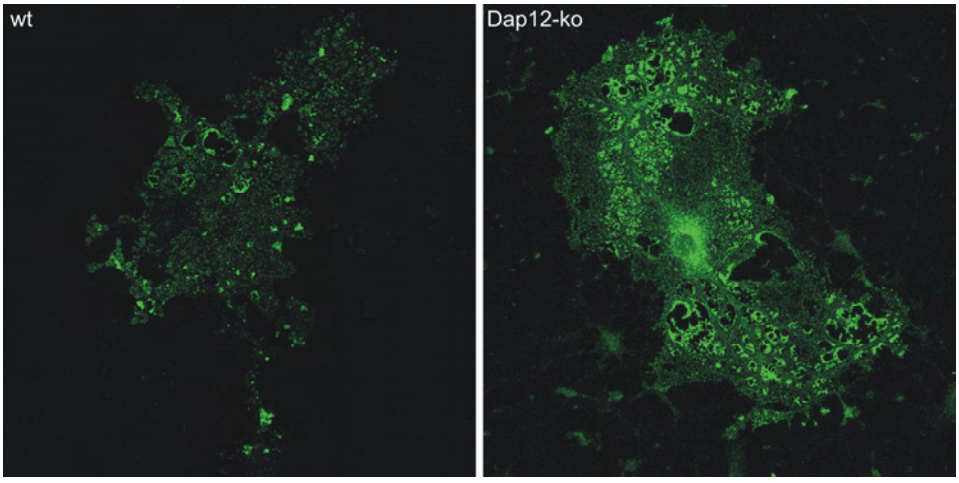

Figure 14. ***Oligodendrocytes cultured from wild type and Dap12 knockout mice. The cells were stained with the oligodendrocyte marker O4 antibodies.***

6 DISCUSSION

6.1 Dap12 and Trem2 expression in the CNS

In this study, Dap12 and Trem2 expression was detected in the mouse CNS already at the embryonic stage and the steady state expression levels did not change significantly during development. Dap12 and Trem2 transcripts were expressed in the same brain regions with similar distributions. Furthermore, Dap12 and Trem2 transcripts and proteins were detected in primary cultures of microglial cells and oligodendrocytes. Astrocytes did not show any Dap12 or Trem2 expression. Neurons showed no Dap12 and only little Trem2 expression *in vitro* (I). Other studies have also been conducted on Dap12 and Trem2 expression in the CNS. Dap12 mRNA and protein expression have been detected in cultured microglia and oligodendrocytes (Kaifu et al. 2003). Trem2 transcripts have been detected in mouse microglia both *in vitro* and *in vivo* (Schmid et al. 2002). Roumier et al detected Dap12 protein expression only in mouse microglia and not in neurons, astrocytes, or oligodendrocytes in mixed glial cultures and *in vivo* (Roumier et al. 2004). Similarly, Trem2 protein expression was detected in primary microglia, but not in astrocytes, oligodendrocytes, or neurons (Takahashi et al. 2005). In contrast, Sessa et al. detected Dap12 and Trem2 expression in human and mouse neurons in addition to microglia *in vivo* (Sessa et al. 2004). All of these studies have found Dap12 and Trem2 expression in microglia. Thus the expression of Dap12/Trem2 complex in microglial cells is well established, but there are contradictory results on whether other CNS cell types also express them. One of the possible explanations is that Dap12 and Trem2 expression might be induced in culture even in cells that do not express them *in vivo*. Another explanation could be that Dap12 and Trem2 are expressed at a specific time point during oligodendrocyte differentiation or induced in response to some specific environmental cues.

6.2 DAP12/TREM2 deficient DCs

TREM2 was originally described as a receptor that forms an activating complex with DAP12 in human *in vitro* differentiated immature DCs (Bouchon et al. 2000; Bouchon et al. 2001b). Our genome wide expression analysis of DAP12 and TREM2 deficient DCs showed differential expression of transcripts with biological functions well in agreement with previously reported functions of the DAP12/TREM2 receptor complex (II). DAP12 and TREM2 have been implicated in triggering and controlling inflammatory responses (Lanier et al. 1998; Bouchon et al. 2001b) as well as regulating chemotaxis (Takahashi et al. 2005). Accordingly, our analysis showed up- and down regulation of transcripts involved in inflammatory response, innate immune response, and chemotaxis. Ligation of TREM2 on DCs has been shown to result in an increase in intracellular calcium concentration, increased survival, and decreased proliferation of DCs (Bouchon et al. 2001b). The genes for cell-cell signaling, calcium ion homeostasis, and negative regulation of cell proliferation were upregulated in our expression analysis of DAP12/TREM2 deficient patient DCs. It is likely that other proteins, involved in signal transduction and calcium ion homeostasis are able to compensate for the missing DAP12/TREM2 signalling, since the DCs obtained from PLOSL patients can respond to microbial stimuli and the patients do not manifest major immunological problems.

We observed certain interesting differences in the response of the DAP12/TREM2 deficient DCs to microbial stimuli compared to control DCs. First, LPS-induced expression of CD86 in DAP12/TREM2 deficient cells was reduced. In agreement with our data, LPS-induced expression of CD86 in myeloid DCs was also found deficient in DAP12 knockout mice (Terme et al. 2004). Similarly, like our DAP12 deficient patient cells, DAP12 deficient mouse DCs produced reduced amounts of TNF-α in response to LPS stimulation (Hamerman et al. 2005). It was thus proposed that DAP12 mediated signals regulate signaling via Toll-like receptors (TLRs)

(Hamerman et al. 2005; Turnbull et al. 2005). TLR signalling results in NF-κB activation (Akira and Takeda 2004). Our microarray data showed upregulation of transcripts for cytoplasmic sequestering of NF-κB in DAP12/TREM2 deficient cells. The upregulated genes: BCL3 and NFKBIA encode inhibitors of NF-κB (Wulczyn et al. 1992; Hoffmann et al. 2002). Thus, our findings support the hypothesis that DAP12/TREM2-mediated signaling may influence on TLR signaling and suggests that the effect may be mediated via inhibition of NF-κB.

6.3 Microglia in neurodegeneration

Others and we have shown that microglia express Dap12 and Trem2 (I, (Takahashi et al. 2005)). Since DAP12 and TREM2 were initially identified in cells of the immune system, it is not surprising to find them in these cells that share the same lineage of origin. Microglial cells are the resident immune cells of the CNS (Aloisi 2001). They are found in all brain regions and comprise between 5 and 15% of cells in the CNS (Schmid et al. 2002). Their activation is characteristic to most neurodegenerative diseases (Woodroofe et al. 1986; Dickson et al. 1993; Morioka et al. 1993; Lenzlinger et al. 2001; Vila et al. 2001; Carson 2002). Activation of microglia has also been demonstrated in autopsied PLOSL brain (Paloneva et al. 2001). Still, the role of microglial activation in neurodegeneration is complicated and unclear. Release of proinflammatory and cytotoxic factors by microglia have been suggested to contribute to neurodegeneration (Chao et al. 1992; Lee et al. 1993). On the other hand, microglia can have proregenerative and neuroprotective functions as injured neurons recover from injury when activated microglial cells are present (Streit 2002). Microglial cells also produce neuroprotective agents and scavenge for cellular debris resulting from injury or disease (Imai and Kohsaka 2002). Consequently, it has been suggested that neurodegeneration might not result primarily from microglial aggression, but rather from neglect by these cells (Streit 2002). The type of signals mediated by the Dap12/Trem2 complex in microglia is

currently unknown. The DAP12/TREM2 complex was initially found to transduce activating signals in DCs (Bouchon et al. 2001b). By contrast, it was recently shown that the Dap12/Trem2 complex transduces inhibitory signals in macrophages (Hamerman et al. 2006; Turnbull et al. 2006). There is some evidence that the Dap12/Trem2 complex would have an inhibitory role also in microglia. Our Dap12 deficient microglia had more activated morphology than wild type microglia (III). Trem2 signalling in microglia downregulates inflammation, since Trem2 knockdown microglial cells have been shown to express increased amounts of the proinflammatory transcripts TNF-α, IL-1β and NOS2 (Takahashi et al. 2005). Further characterization of the downstream effects of Dap12/Trem2 mediated signal transduction in microglia will be needed to solve this issue.

In response to demyelination, microglia are able to proliferate, migrate into demyelinated areas and present antigen (Remington et al. 2007). Following injury, microglia migrate or extend processes toward sites of tissue damage (Nimmerjahn et al. 2005; Haynes et al. 2006). One of the pre-requisites for cell migration or process extension is reorganization of the actin cytoskeleton (Vicente-Manzanares and Sanchez-Madrid 2004). The *in vitro* induced DAP12/TREM2 deficient dendritic cells in this study showed abnormal actin staining pattern and more cellular processes than control cells as well as downregulation of transcripts involved in actin filament bundle formation (II). This observation is well in line with previous findings of abnormal actin staining pattern in DAP12/TREM2 deficient osteoclasts, also induced *in vitro* (Cella et al. 2003; Paloneva et al. 2003). We observed that F-actin distribution in Dap12 deficient microglia differed from that in wild type microglia. It has earlier been demonstrated that crosslinking of Trem2 on microglia leads to polarization and reorganization of F-actin (Takahashi et al. 2005). We saw downregulation of transcripts involved in cell migration in the expression analysis and decreased migration through the membranes in our migration assay by Dap12 deficient microglia. In line with our results, it has been shown that Trem2 stimulation in microglia increased migratory activity of microglia (Takahashi et al.

2005). We also noted that Dap12 microglia do not survive in culture for as long as wild type microglia. It has earlier been shown that the DAP12/TREM2 receptor complex promotes survival of human dendritic cells (Bouchon et al. 2001b). Also, genes involved in negative regulation of cell proliferation were upregulated in our expression analysis of the patient DCs (II). Our results add to the evidence that Dap12 plays a role in microglial migration and survival/proliferation, both of which are important properties for the response of these cells to CNS damage such as demyelination.

6.4 Loss-of-myelin in PLOSL

The most prominent feature of PLOSL is the loss-of-myelin in the brain. We saw differential expression of myelin components (Mobp, Mbp, Plp, Mal, and Cnp1) in our expression analysis of Dap12 knockout mouse brain. It has been demonstrated that Dap12 deficient mice develop thalamus accentuated hypomyelinosis and show reduction in the number of myelinated axons in electron microscopy of the thalami (Kaifu et al. 2003). In histological analysis, Nataf et al. also observed diffuse hypomyelination predominating in anterior brain regions not accompanied with oligodendrocyte degeneration or microglial activation in Dap12 mutant mice (Nataf et al. 2005). Although we did not observe changes in Mbp staining in immunohistochemistry, electron microscopy might reveal similar changes also in the mice used in this study. Upregulation of the transcripts for myelin components might reflect a trial to compensate for the loss-of-myelin. It has been suggested that the hypomyelination is due to developmental arrest of oligodendrocytes (Kaifu et al. 2003) or developmental defect of myelin formation (Nataf et al. 2005). Our results do not support this hypothesis, since we did not notice differences in primary oligodendrocyte differentiation from Dap12 deficient and wild type mice (III).

Myelin loss also occurs in the human disease multiple sclerosis (MS). Experimental autoimmune encephalomyelitis (EAE) is studied as an animal model of MS. It has

recently been demonstrated that blocking Trem2 with an antibody at the onset of MOG35-55 induced EAE worsens the disease outcome (Piccio et al. 2007). It was also shown that Trem2-transduced bone marrow derived myeloid cells (BM-MC) that were injected (i.v.) into mice at the peak of EAE, migrated into the CNS lesions, induced clearance of myelin debris, and created an anti-inflammatory cytokine environment. Mice injected with the Trem2 transduced BM-MC showed less axonal injury and demyelination than mice injected with GFP transduced BM-MC (Takahashi et al. 2007). The myelin loss observed in PLOSL could also be due to an impaired support function of the Dap12/Trem2 deficient myeloid cells.

6.5 Synaptic defects in PLOSL

The Dap12 deficient mice used in this study do not show any gross abnormalities in their CNS. We were interested to find out whether they show changes in the molecular level that would give us clues to the CNS pathogenesis of PLOSL. In the genome wide expression analysis we observed changes in expression of many transcripts and saw regulation of specific biological pathways. Most of the differentially expressed pathways in Dap12 deficient mouse brain were involved in synaptic function. Synaptic function has been shown to be impaired in the other two Dap12 mutant mice (Kaifu et al. 2003; Roumier et al. 2004). Dap12 deficient mice described by Kaifu et al. (Kaifu et al. 2003) showed synaptic degeneration, reduced startle response, impaired prepulse inhibition to acoustic stimuli, and aberrant γ-aminobutyric acid (GABA) receptor mediated miniature inhibitory postsynaptic currents (mIPSCs) in the thalamus. Roumier et al. (Roumier et al. 2004) noted that in the Dap12 loss-of-function mutant mice long-term potentiation (LTP) was enhanced. They concluded that by affecting LTP Dap12 deficiency impacts on hippocampal synaptic plasticity. They also observed changes in synaptic receptor content by electrophysiology and biochemical analysis. The expression levels of the glutamate receptor GluR2 and the brain derived neurotrophic factor (BDNF)

tyrosine kinase receptor B (TrkB) in the whole cell level were not affected, but there was impairment of the synaptic accumulation of these receptors in the Dap12 deficient mice (Roumier et al. 2004). In line with these previous findings, our genome wide expression analysis of Dap12 deficient thalami showed up-regulation of transcripts involved in protein trafficking into synapses and GABA mediated synaptic transmission. In addition to GABA receptor transcripts, transcript for Kcnq2 was highly up-regulated (14 fold). Kcnq2 is a potassium channel protein that modulates GABA release (Martire et al. 2004). In humans, Kcnq2 mutations are associated with an inherited epilepsy syndrome (Singh et al. 1998), which is of interest since PLOSL patients also suffer from epileptic seizures.

Microglia are able to control developmental apoptosis and synaptogenesis and affect synaptic function (Bessis et al. 2007). Overt nitric oxide (NO) production by activated microglia has been shown to block transport of synaptic vesicle precursors, influence axonal transport of synaptotagmin and synaptic properties (Stagi et al. 2005). We saw up-regulation of transcripts involved in NO metabolism and biosynthesis in Dap12 knockout microglia. We also saw upregulation of transcripts involved in vesicle-mediated transport and synaptic vesicle membrane in Dap12 knockout brain. One of the transcripts belonging to these categories was synaptotagmin (8 fold up). It has been suggested that microglial Dap12 affects synaptic function and plasticity through a microglia-neuron interaction, where microlial cells regulate synapse maturation and plasticity by secreting soluble factors (Roumier et al. 2004). In our study, we also observed changes in synaptic transcripts without clear CNS damage, but with functional defects of microglia in Dap12 knockout mice (III).

6.6 Hypothesis of PLOSL pathogenesis

The spatial expression patterns of Dap12 and Trem2 in the mouse CNS are in line with the pathological findings in PLOSL patients. Patients with PLOSL, who lack functional DAP12 or TREM2, suffer from a dramatic loss of CNS white matter. Loss-of-myelin is most prominent in the deep frontal and temporal white matter (Paloneva et al. 2001) and is of late onset. In addition, Dap12 knockout mice have been shown to suffer from hypomyelinosis (Kaifu et al. 2003; Nataf et al. 2005). Still, no developmental defects have been described in PLOSL patients or Dap12 knockout mice. So, it seems that even though Dap12 and Trem2 are already expressed at the embryonic stage, they are not essential for normal CNS development, but rather for homeostasis of the adult CNS. Since myelin development seems not to be impaired in PLOSL patients, DAP12/TREM2 signaling could be important in maintenance of the myelin sheath or remyelination. The capacity of CNS to repair demyelinating damage decreases with age (Ibanez et al. 2003). This might partially explain the late onset of PLOSL. In line with this, the skeletal system in PLOSL patients develops normally and the pathological changes manifest only at early adulthood (Hakola 1972).

It has been shown that expression of Trem2 by microglia in healthy CNS varies between and within brain regions. So, not all microglia express Trem2. It has been suggested that different subsets of microglia are specialized to respond to defined extracellular signals, and regional variation in Trem2 expression may contribute to the varying sensitivities of different brain regions to similar pathological signals (Schmid et al. 2002). This might contribute to the different degrees of damage in different parts of the CNS in PLOSL.

Concerning the clinical symptoms in PLOSL, it is also interesting, that we saw differential expression of SPP1 (secreted phosphoprotein 1) and CCL2 (c-c chemokine ligand 2/monocyte chemotactic protein 1 (MCP-1)) in PLOSL patients' DCs. The donwregulated SPP1, also known as osteopontin, functions in the immune

system, but is also important for normal development and function of osteoclasts. It has been demonstrated that in human cells, reduced expression of osteopontin at early times of differentiation leads to a defect in osteoclastogenesis *in vitro* (Aitken et al. 2004). Further, osteopontin transcripts are increased in brain samples of multiple sclerosis (MS) patients (Chabas et al. 2001). The mRNA level of CCL2 was highly upregulated in patients' DCs. Increased CNS or cerebrospinal fluid (CSF) levels of CCL2 have been reported in chronic neurological disorders such as Alzheimer's disease (Ishizuka et al. 1997), HIV-associated dementia (Kelder et al. 1998), amyotrophic lateral sclerosis (ALS) (Wilms et al. 2003) and MS (Simpson et al. 1998). Further, transgenic mice overexpressing CCL2 develop late (>6 months) onset encephalopathy with mild perivascular leukocyte infiltration, impaired blood brain barrier function, and increased numbers of microglia with morphological features of activation (Huang et al. 2005). These findings of altered steady state levels of these two transcripts provide biological links with the pathology of both, brain and bone, characteristic to PLOSL.

Loss of function of DAP12 or TREM2 affects specific biological pathways, especially those involved in actin remodelling, cell migration, cytokine production, and synaptic function. DAP12/TREM2 mediated signalling obviously plays an important role in the CNS, since its failure leads to PLOSL with severe CNS manifestations. Synaptic defects have been proposed to account for the early onset of some dementias (Terry and Katzman 2001; Selkoe 2002). It is not known whether the myelin loss in PLOSL causes the degeneration of the synapses or whether the synapses degenerate even before the loss-of-myelin. Microglia can affect both of these processes: myelin stability and synaptic function. In light of this study, it seems likely that, the primary defect in PLOSL would be in the communication of the microglia with their surroundings, in such a way that loss of the inhibitory Dap12/Trem2 signaling would lead to increased cytokine production and decreased migration by the microglia (Fig. 15). This would then disturb the CNS homeostasis and lead to the synaptic dysfunction and loss-of-myelin observed in PLOSL.

It would also be interesting to know if PLOSL patients would benefit from bone marrow transplantation. It has been shown that, at least in mice, bone marrow stem cells can migrate into the CNS and differentiate into microglia also in adulthood (Simard and Rivest 2004). It has also been shown that blood-derived microglia have the ability to eliminate amyloid deposits in a mouse model of Alzheimer's disease (Simard et al. 2006). Since the Dap12 deficient mice do not reproduce the drastic CNS damage observed in PLOSL and the bone phenotype is quite the opposite, better animal models would be needed to test this hypothesis. Hopefully the Trem2 knockout mice currently being generated in our laboratory will further contribute into better understanding of the CNS damage behind PLOSL.

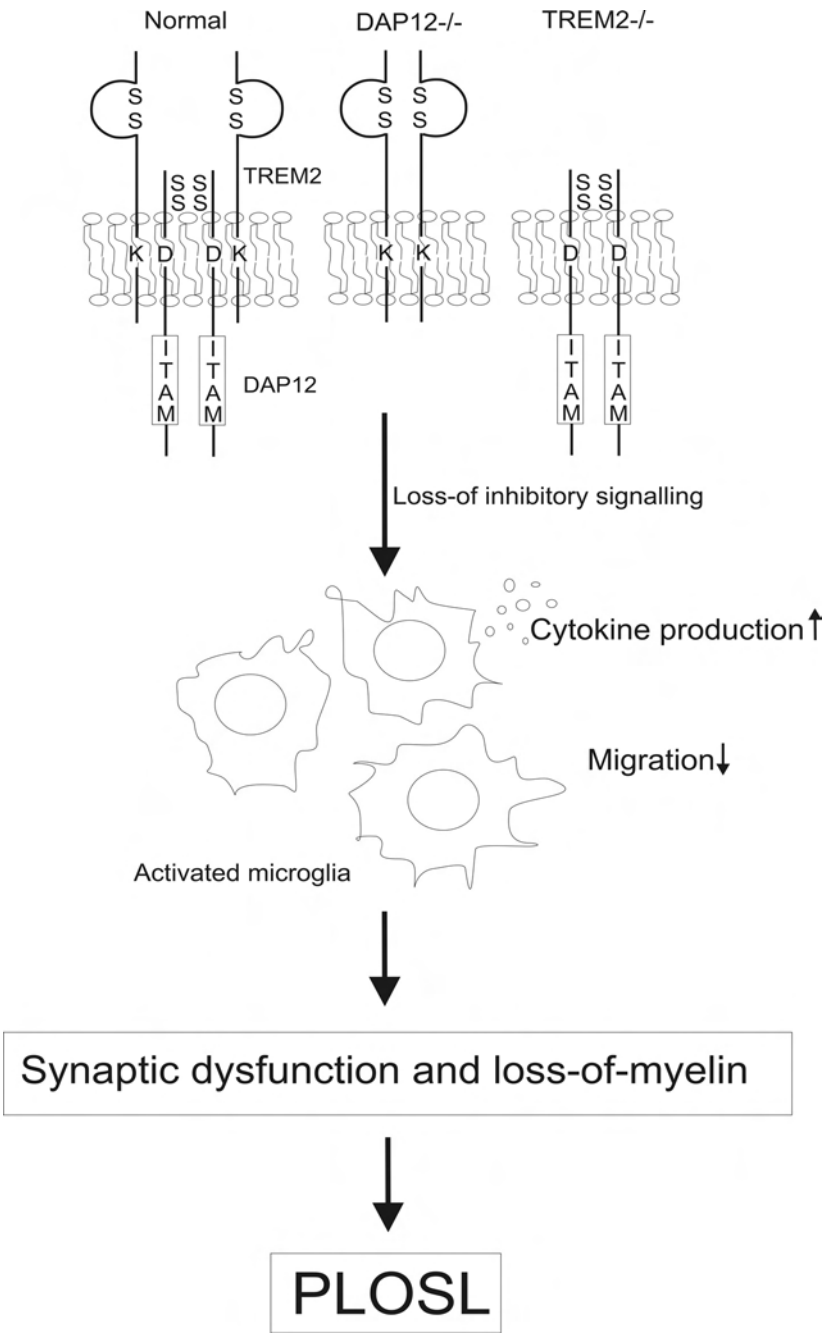

Figure 15. ***Hypothesis of molecular events resulting in PLOSL.***

7 CONCLUSIONS

In this thesis work microglia and oligodendrocytes were identified as the Dap12 and Trem2 expressing cells of the CNS. In light of other studies on Dap12 and Trem2 expression in the CNS published during this thesis work, there is increasing evidence that microglia are the cell type responsible for the CNS manifestations of PLOSL. When this study was undertaken, the DAP12/TREM2 complex was generally thought to transduce activating signals into cells (Bouchon et al. 2001b). During this work it has been shown that the DAP12/TREM2 complex can also transduce inhibitory signals (Hamerman et al. 2006; Turnbull et al. 2006). It now seems that the outcome of DAP12/TREM2 mediated signalling is dependent on the stimulating agent and the cell type in which the signalling occurs, which makes it important to further characterize the nature of the DAP12/TREM2 mediated signals in microglial cells.

Specific biological processes are affected by lack of DAP12 and TREM2 in dendritic cells (DCs). Loss of Dap12 in mouse brain leads to changes in synaptic transcripts and transcripts of myelin components as well as functional defects in microglia. In both DCs and microglia, the DAP12/TREM2 signalling affects actin remodelling and survival/proliferation. In microglia it also affects cell migration. In addition to their role as immune cells, microglia can influence myelin stability and synaptic function. It is not known whether the myelin loss in PLOSL causes the degeneration of the synapses or whether the synapses degenerate even before the loss-of-myelin. Thus the exact role of DAP12/TREM2 mediated signalling in microglial communication with their surroundings warrants further study. Understanding how that works as well as identification of the TREM2 ligand and other DAP12/TREM2 interacting molecules will provide new insight into the pathogenesis of PLOSL and into the molecular pathogenesis of dementias more generally.

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