

Two Genes Behind PLOSL:

Molecular and Pathological Characteristics of the Disease

by

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There is always a bigger fish
-unknown-

To Petra

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their roman numerals. In addition, unpublished data are presented.

- I. Paloneva J, Kestila M, Wu J, Salminen A, Bohling T, Ruotsalainen V, Hakola P, Bakker AB, Phillips JH, Pekkarinen P, Lanier LL, Timonen T, Peltonen L. Loss-of-function mutations in *TYROBP (DAP12)* result in a presenile dementia with bone cysts. *Nature Genetics* 25(3):357-361 (2000)
- II. Paloneva J, Manninen T, Christman G, Hovanes K, Mandelin J, Adolfsson R, Bianchin M, Bird T, Miranda R, Salmaggi A, Tranebjaerg L, Konttinen Y, Peltonen L. Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype. *American Journal of Human Genetics* 71(3):656-662 (2002)
- III. Paloneva J, Autti T, Raininko R, Partanen J, Salonen O, Puranen M, Hakola P, Haltia M. CNS manifestations of Nasu-Hakola disease: a frontal dementia with bone cysts. *Neurology* 56(11):1552-1558 (2001)
- IV. Paloneva J*, Mandelin J*, Kiialainen A, Böhling T, Prudlo J, Hakola P, Haltia M, Konttinen YT, Peltonen L. DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. Submitted.

*These authors contributed equally to this work.

ABBREVIATIONS

aa	amino-acid
AD	Alzheimer's disease
bp	base pair
cDNA	complementary DNA
chr	chromosome
cM	centiMorgan
CNS	central nervous system
CT	computerized tomography
DNA	deoxyribonucleic acid
DSM-IV	Diagnostic and statistical manual of mental disorders, 4 th ed.
EST	expressed sequence tag
Gb	Gigabase
ICD-10	international classification of diseases, 10 th edition
ITAM	intracellular tyrosine-based activation motif
KARAP	killer activating receptor-associated polypeptide (=DAP12)
kb	kilobase
kD	kiloDalton
Mb	Megabase
M-CSF	macrophage colony stimulating factor
MRI	magnetic resonance imaging
mRNA	messenger RNA
NK cell	Natural Killer cell
OMIM	Online Mendelian Inheritance in Man
PCR	polymerase chain reaction
PBMC	peripheral blood mononuclear cell
PLOSL	Polycystic Lipomembranous Osteodysplasia with Sclerosing Leukoencephalopathy
RANKL	receptor activator of nuclear factor- κ B ligand
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase PCR
TYROBP	tyrosine kinase binding protein (=DAP12)
UTR	untranslated region

In addition, the standard abbreviations of nucleotides and amino-acids are used. The gene names are italicized, the protein names are written in regular letters. Human gene/protein names are capitalized, mouse gene/protein names are written in lower case letters.

SUMMARY

Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), also known as Nasu-Hakola disease, is a monogenic model for dementia. It is characterized by loss of white matter in the brain, cystic bone lesions, and osteoporotic features, leading to severe dementia and spontaneous bone fractures. PLOSL has an autosomal recessive mode of inheritance. The incidence of the disease is highest in Finland and PLOSL therefore belongs to the Finnish disease heritage.

When this study was started in 1998, the locus for PLOSL in Finnish patients had been restricted to a 153 kb region in chromosome 19q13.1. In this study we have used the positional cloning approach to identify the first gene mutated in PLOSL. We identified a homozygous 5.3 kb genomic deletion in all Finnish PLOSL patients in the *DAP12* gene. *DAP12* is a widely expressed transmembrane adaptor molecule that transmits activating signals from several cell-surface receptors that activate immune responses in myeloid and natural killer cells.

We did not detect mutations in a group of patients outside Finland. This observation suggested genetic heterogeneity in PLOSL, and encouraged us to search for other PLOSL genes. Since *DAP12* encodes a cell-surface receptor element that interacts with many different proteins depending on the cell type, we used a genetic approach to search for genes involved in PLOSL. By analyzing families not linked to *DAP12* for segregation of marker haplotypes flanking genes coding for the *DAP12*-associated polypeptides, we identified a single chromosomal region that showed a complete cosegregation with PLOSL. Sequencing of the regional candidate genes revealed mutations in *TREM2*. On the cell surface *TREM2* is expressed as a complex with *DAP12* and mediates the differentiation of dendritic cells.

Since then we have detected mutations in all PLOSL patients in either *DAP12* or *TREM2*. A total of 73% (n=37) of the patients carry a mutation in *DAP12* and 27% (n=14) in *TREM2*. We have thereby developed DNA diagnostic testing for PLOSL to enable earlier and accurate diagnosis.

The clinical course and pathological manifestations of PLOSL had been insufficiently characterized. In this study we present a detailed long-term neurological and neuropathological analysis of PLOSL patients. We have found that mutations in *DAP12* and *TREM2* result in a clinically identical disease phenotype. Further, although PLOSL in most patients manifests by skeletal problems, up to 25% of patients do not show any osseous symptoms and signs before the onset of neurological manifestations.

Therefore, radiographic examination of ankles or wrists should be considered in all cases of a presenile dementia of unknown origin to ensure the correct diagnosis.

In order to initiate the characterization of the pathogenesis of PLOSL, we tested our hypothesis that osteoclasts, the bone remodeling cells, are responsible for the generation of the bone lesions. We studied the differentiation of osteoclasts in patients with loss-of-function mutations in *DAP12* or *TREM2*, and found that loss of DAP12/TREM2 signaling results in a severely delayed differentiation of osteoclasts. Further, DAP12/TREM2 deficiency leads to impaired bone resorption by osteoclasts *in vitro*.

DAP12 and TREM2 have been suggested to play crucial roles in immune responses. However, DAP12- or TREM2-deficient PLOSL patients do not seem to present with primary immunological symptoms. This observation suggests that DAP12/TREM2 signaling has a more important role in the normal function of the central nervous system and bone than in immune responses.

Taken together, the molecular genetic basis for PLOSL has been characterized in this study by the identification of the causative genes, *DAP12* and *TREM2*. The neurological and neuropathological course of PLOSL was characterized in detail by performing a long-term follow-up analysis of a series of PLOSL patients. Finally, DAP12 and TREM2 signaling was found to mediate the differentiation and bone resorption function of osteoclasts *in vitro*.

REVIEW OF THE LITERATURE

THE HUMAN GENOME

The haploid human genome contains 3 billion base pairs of DNA divided between 22 autosomal and one sex chromosomes, ranging in size from ~50-260 Mb, as well as the DNA present in mitochondria. Somatic cells are diploid, i.e., they contain 22 pairs of autosomes in addition to one pair of sex chromosomes. The germ cells (i.e. sperm and egg) are haploid and contain a single copy of each autosome and one sex chromosome.

The chromosomes contain the genes that code for proteins, the building blocks of an organism. The number of human genes is estimated to be 30 000. The average human gene spans between 1 to 200 kb of genomic DNA. The size of the genes range from ~100 bp (e.g. the tRNA^{Tyr} gene) to over 2.3 Mb (e.g. the dystrophin gene). Associated with the genes are the regulatory elements (promoters, enhancers, inhibitory sequences among others) that determine where, when, and to what level gene expression occurs.

Coding sequences represent only 1-2% of the whole genome. The remaining noncoding DNA consists of repeat sequences and other sequences whose significance is poorly understood. Repetitive elements are likely to account for over 50% of the genome. Repetitive elements can be divided into 5 main categories: 1) transposon-derived repeats (such as *Alu* repeats), comprising approximately 45% of the genome; 2) processed pseudogenes (0.5% of the genome), that are non-functional genes that have been moved from their original location through an RNA intermediate; 3) simple sequence repeats that are typically repetitions of short n-mers (mono-, di- or trinucleotide repeats); 4) segmental duplications of blocks of around 10-300 kb that have been copied from one region of the genome to another region; and 5) blocks of tandemly repeated sequences, such as at centromeres and telomeres (Prak and Kazazian 2000; Lander et al. 2001; Scriver et al. 2001; Collins et al. 2003). At the ends of each chromosome there are telomeres, which stabilize the chromosome, prevent fusion with other DNA, and enable DNA replication without loss of chromosomal material.

In addition to chromosomal DNA, human cells also contain mitochondrial DNA. The mitochondrial genome is extremely compact. There are no introns, some genes even partially overlap each other, and almost every base pair of the 16569 bp of the human mitochondria can be assigned to a gene (Lewin 2000).

The complex structure and function of the human genome are only partially understood and several surprises are yet likely to appear. The Human Genome Project (HGP) was established to enable a better understanding of the human genome and to provide

fundamental tools for molecular genetic and biomedical research. HGP is an international research program whose primary goal was to obtain a finished sequence of the human genome and, in parallel, that of several model organisms apart from regions that cannot be cloned or sequenced with the currently available techniques. The HGP officially began in 1990. A milestone in the HGP was achieved in the year 2000, when the International Human Genome Sequencing Consortium published an approximately 90% complete draft sequence of the entire genome concurrently with a draft sequence produced by a private company Celera (Lander et al. 2001; Venter et al. 2001). The primary goal of the HGP was achieved in April 2003 when the International Human Genome Sequencing Consortium completed the sequencing of the human genome (NHGRI web pages), 50 years after the characterization of the structure of DNA (Watson and Crick 1953).

The comparative analyses of the genome of humans and model organisms have helped us to understand what makes human unique among other species. The size of the genome does not correlate well with the complexity of an organism. For example, the human genome is 200 times as large as that of budding yeast (*Saccharomyces cerevisiae*) but 200 times smaller than that of amoeba (*Amoeba dubia*), one of the lowest organisms (Gregory and Hebert 1999; Hartl 2000). A surprising finding is that the human genome contains only about twice the number of genes needed to make a fruitfly, worm, or plant. The unique complexity of humans compared to other organisms is partly based on a higher degree of alternative splicing, i.e., more proteins are encoded per gene in humans than in less complex species. In addition, complex posttranslational modifications and more complex use of protein domains create diversity in the gene products in human compared to other species (Rubin 2001; Rimoin et al. 2002).

Genetic Diversity in Humans

Genetic diversity and nongenetic factors together contribute to the differences between individuals. Due to the complexity of individuality, the following review is restricted only to the molecular genetic basis for diversity. The DNA of each individual contains millions of differences compared to the DNA of another individual, except in the case of monozygous twins. The DNA differences can be divided into four main categories: a) those with no phenotypic effect (e.g. most short tandem repeat polymorphisms), b) those causing phenotypic differences without affecting disease susceptibility (e.g. differences in height), c) those making a modest contribution to disease processes (e.g. complex diseases involving quantitative trait loci), and d) those playing a major role in causing a disease phenotype (e.g. monogenic disorders). Overall, 1 in 100-200 base pairs in the human genome are estimated to be polymorphic. Most human sequence variation is attributable to single-nucleotide polymorphisms (SNP), with the rest

attributable to tandemly repeated sequences, insertions or deletions of one or more nucleotides, and rearrangements. SNPs are estimated to occur every 1000-2000 bases when two human chromosomes are compared. Some 4% of SNPs fall within coding and untranslated regions, and 85% of exons are within 5 kb of the nearest SNP (Sachidanandam et al. 2001; Scriver et al. 2001).

Model organisms

Model organisms are key informational tools for understanding the contents of the human genome, and important experimental tools because these genomes help researchers characterize and interpret the human genome. To date, the genomes of numerous model organisms have been sequenced. Complete genomes have been obtained from nine eukaryotes including two widely used animal model organisms, namely fruit fly (*Drosophila melanogaster*) and worm (*Caenorhabditis elegans*), as well as baker's yeast (*Saccharomyces cerevisiae*). In addition, complete sequence have been obtained from over 1000 viral (viruses and viroids) genomes, over 100 microbial genomes (bacteria and archaea), and almost 500 organelles (mitochondria, plasmids, plastids, and nucleomorphs) (NCBI and NHGRI web pages; Lander et al. 2001).

The mouse (*Mus musculus*) is likely to be the most important model organism. In addition to being inexpensive to keep under well-controlled environmental conditions and reproducing efficiently, the mouse and humans have many common metabolic and developmental pathways. A draft sequence covering 96% of the euchromatic portion of the mouse genome was published at the end of year 2002. The haploid mouse genome consists of 20 chromosomes. Comparative analysis has revealed that the mouse genome is about 14% smaller than the human genome (2.5 Gb compared to 2.9 Gb). At the nucleotide level, approximately 40% of the mouse genome can be aligned to the human genome. Interestingly, the mouse and human genomes contain the same number of protein-coding genes. The proportion of mouse genes with a single identifiable orthologue in the human genome seems to be approximately 80%. Less than 1% of human genes do not have any homologue in the mouse (Waterston et al. 2002).

GENETIC FACTORS AND DISEASE

The contribution of genetic factors in the etiology of disorders varies from completely genetically determined (e.g. phenylketonuria) to completely environmentally induced (e.g. accidents). The role of genetic factors in diseases can be divided into five categories: a) Mendelian (monogenic) disorders, b) chromosomal aberrations, c)

mitochondrial disorders, d) multifactorial disorders, and e) somatic cell genetic disorders.

For simplicity, genetic disorders are classically divided into single-gene disorders or complex disease traits. In reality, there is a complex continuum involving a) the number of loci, b) the relative influence of each allele, c) nongenetic factors, such as environmental differences (diet, smoking), stochastic factors (immunoglobulin rearrangements), somatic mutations, and d) variable contributions in individuals with a similar or virtually identical phenotype (Scriver et al. 2001).

Thousands of human disorders are caused by single-gene defects and are therefore called monogenic or Mendelian diseases. In March 2003 the Human Gene Mutation Database (HGMD) contained 33252 mutations found in 1338 genes (Table 1). The true number of identified disease mutations is even higher due to incompletely updated databases.

Table 1. Number of Mutations by Mutation Type in 1338 Identified Disease Genes in the HGMD Database

<u>Mutation type</u>	<u>No. of mutations</u>
Micro-lesions	
Missense/nonsense	19368
Splicing	3207
Regulatory	266
Small deletions	5498
Small insertions	2093
Small indels*	322
Gross lesions	
Repeat variations	57
Gross insertions & duplications	274
Complex rearrangements (including inversions)	340
Gross deletions	1827
Total	33252

*a deletion followed by an insertion after the nucleotides affected.
Updated March 26, 2003. For HGMD URL, see Electronic Database Information.

Monogenic diseases become manifest when the patient carries one or two disease alleles, depending on whether the mode of inheritance is autosomal recessive, autosomal dominant, or X-linked. Environmental factors have little or no influence on the onset or severity of monogenic diseases. The overall population frequency of monogenic disorders is about 1 per 100 live births, consisting of 7 in 1000 dominant, 2.5 in 1000 recessive, and 0.4 in 1000 X-linked disorders (excluding color blindness present in 1 in 12 males) (Scriver et al. 2001).

Although the rules of Mendelian inheritance in humans are generally correct, there are some exceptions: unstable mutations, uniparental disomy and imprinted genes. Unstable mutations involve the expanding triplet diseases such as Huntington disease and spinocerebellar ataxias, in which the disease phenotype worsens over the generations (anticipation) due to increasing number of repeats. Uniparental disomy exists when an individual inherits two copies of part or all of a chromosome from one parent. The significance of uniparental disomy is mostly caused by genetic imprinting, in which the maternal and paternal copy of a gene are differentially expressed. For example, the genes causing Prader-Willi syndrome are inherited from the father, while the same genes cause Angelman syndrome when expressed from the maternal copy. Imprinting may also occur without uniparental disomy when a heterozygous mutation causes a disease phenotype or not depending on the parent of origin of the mutation. This is the situation with hereditary paragangliomas (inherited tumor syndrome) in which the phenotype is expressed only when the mutated allele is inherited from the father (Scriver et al. 2001).

The clinical risk for X-linked disorders are different for the two sexes. In males, the disorder, such as red-green color blindness, becomes manifest when an individual inherits one mutant allele, whereas females require two mutated alleles to develop the disorder.

The mitochondrial genome is inherited from the mother. Therefore, males do not transmit mutations in the mitochondrial genome to their offspring. The proportion of mutated to normal mitochondria varies among individuals in a pedigree, often leading to a phenotypic heterogeneity (Scriver et al. 2001).

Multifactorial or complex diseases are caused by interactions of multiple genes and environmental factors. These disorders become manifest only when an individual inherits a certain combination of disease-predisposing alleles, and triggering environmental influences are present. Many of the common chronic illnesses of adulthood (e.g. bipolar disorder, Alzheimer's disease, essential hypertension, and schizophrenia), as well as the common birth defects (such as cleft lip palate, congenital heart disease) fall into this category (Scriver et al. 2001).

IDENTIFICATION OF HUMAN DISEASE GENES

There are several strategies that can be utilized to identify the causative genes for human diseases. The strategies can be divided into four main categories: functional cloning, the candidate gene approach, positional cloning and the positional candidate

gene approach. Often the gene identification process is a combination of these strategies.

Functional cloning was the only way of finding a gene a couple decades ago when no information on human genome mapping was available. A prerequisite for this method is knowledge of the basic biochemical defect underlying the disease, such as a purified defective protein that can be used to determine the amino-acid and cDNA sequence of the mutated protein and gene. Therefore, the possibilities to utilize this strategy are rather limited. Classical examples of the usefulness of having a purified protein available include studies on the mutations underlying sickle cell anemia, and hemophilia caused by mutations in coagulation factor III (Ingram 1957; Gitschier et al. 1984).

The candidate gene approach also requires some knowledge of the pathogenesis or molecular basis of the disease. The candidate genes, selected according to their known or supposed function, are directly analyzed for mutations or linkage in families. The candidate gene approach is perhaps the most elegant and least laborious way of identifying disease genes when sufficient knowledge on the basic biochemical defect underlying the disease is available. As the knowledge on the function of human genes and biochemical pathways increase, this method is likely to become more widely used. For example, the candidate gene approach was successfully utilized in this study (II).

Positional cloning means the isolation of a disease gene based solely on its location in the genome; no knowledge of its function is required. This strategy depends on the analysis of affected families with genetic mapping to find a chromosomal region linked to the disease. Having localized the defective gene in a chromosomal locus, the critical region is further restricted with linkage disequilibrium and shared haplotype analyses. With increasing knowledge of the human genome, the principal disease gene identification method nowadays is a mixture of positional cloning and candidate gene methods called the positional candidate gene approach. After assignment of the disease locus, the regional candidate genes are selected and analyzed based on their known function (Scriver et al. 2001). When sufficient knowledge of the molecular mechanisms of the disease are available this method may be very efficient and fast. However, the pathogenic mechanisms of the disease or the function of the regional candidate genes are often not sufficiently understood. Therefore, in many cases several genes in the critical region have to be analyzed.

THE FINNISH DISEASE HERITAGE

The Finnish disease heritage, initially described in 1973, is a widely known concept in genetic research (Norio et al. 1973). To date, this group includes almost 40 monogenic

disorders that are more prevalent in Finland than elsewhere in the world (Table 2). The majority of these diseases are recessively inherited; only two dominant (amyloidosis V and tibial muscular dystrophy) and two X-chromosomal (retinoschisis and choroidemia) disorders have been characterized.

The Finnish disease heritage has its origin in the special population history of Finland. The population of Finland has evolved in isolation without significant genetic influence from the outside. The reason for the genetic isolation have mainly been geographical, but also language and religion have had an effect on the isolation. Based on Y-chromosomal haplotype studies, Finland has been inhabited in two waves (Kittles et al. 1998). The first migratory wave of Uralic speakers from the east occurred some 4000 years ago and has had a distinct effect on the Finnish gene pool. The majority of the Finnish gene pool is thought to originate from later small founder populations which immigrated from the south approximately 2000 years ago. However, since there is no unequivocal evidence for two distinct immigration waves, it is actually more probable that small immigration groups arrived in Finland continuously after the glacial period (Peltonen et al. 1999). The founder populations first spread along a narrow strip of the southern and western coastline of Finland (early settlement). For a long time Finland remained very sparsely populated. In the 12th century the population was only ~50 000. By the 16th century the population had expanded to ~250 000, but was still concentrated on the coastal areas. An internal migration began in the 16th century from a small southeastern area (South Savo) to other regions of Finland (late settlement) (Peltonen et al. 1999) (Fig. 1). The expansion of the population has not been continuous; several bottle necks have been met. The most severe was the great famine at the end of the 17th century. This and several smaller epidemics killed one third of the population of 400 000 during the years 1690-1730. Since then, the population of Finland has grown rapidly to today's 5.2 million inhabitants over three centuries (Peltonen et al. 1999).

The Finnish gene pool is very homogeneous. Analyses of the genetic diversity of the Y chromosome and mitochondrial DNA show that Finns differ from other European populations in having an exceptionally reduced amount of Y-chromosomal and mitochondrial diversity. This indicates that relatively few people have contributed to the genetic lineage of today's Finns (Sajantila et al. 1996).

The small number of original founders, isolation, rapid expansion of the population and the creation of small immigrant groups from the main population allowed the founder effect (i.e., a mutation enriched in a specific population due to the presence of that mutation in a single ancestor or small number of ancestors) and genetic drift (i.e., random transmission of alleles from parent to offspring resulting in an enrichment or

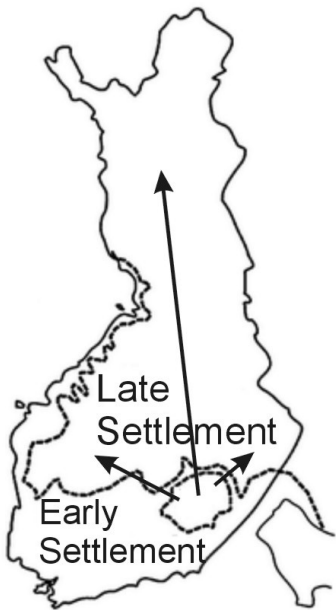


Figure 1. The first founder populations spread along the southern and western coastline of Finland (early settlement). The internal migration began in the 16th century from the southeastern area of Finland (late settlement) (Modified from Peltonen et al. 1999; Norio 2003a).

disappearance of alleles) to shape the gene pool, especially in the regional subisolates (Norio et al. 1973; de la Chapelle 1993; Peltonen et al. 1999). These factors have led to the enrichment of some Mendelian diseases in Finland, creating the Finnish disease heritage. On the other hand, some other disorders relatively common in other white populations (e.g. cystic fibrosis, phenylketonuria, galactosemia and maple syrup disease) are very rare in Finland since the respective genes were either lacking or lost from the gene collection of the original settlers (Palo et al. 1987; Kere et al. 1989).

Most diseases belonging to the Finnish disease heritage present a regional clustering. The ancestors of the patients originate from the late settlement region in half of the cases. In six diseases of the Finnish disease heritage, the birthplaces of the ancestors have been distributed relatively evenly throughout the country, however, the late settlement area is overrepresented (AGU, Batten disease, cartilage-hair hypoplasia, congenital nephrosis, INCL and progressive myoclonus epilepsy). Only diastrophic dysplasia and Meckel syndrome show distributions equal to the population density; most patients are found in southern and western Finland (Norio 2000).

The distribution of the birthplaces of great-grandparents of the patients can be used to estimate the age of the mutation. Tight clustering, such as in the case of variant infantile neuronal ceroid lipofuscinosis and progressive epilepsy with mental retardation, suggests that the mutation is young. A distribution equal to the population density, as described above, indicates that the mutation is old.

Table 2. Diseases of the Finnish Disease Heritage. Updated from (Norio 2000; Norio 2003b)**A) IDENTIFIED MUTATIONS**

<u>Disease</u>	<u>Defective protein</u>	<u>Reference (Finnish Mutations)</u>
Gyrate atrophy (GA)	Ornithine aminotransferase (OAT)	Mitchell et al. 1989
Amyloidosis V	Gelsolin (GSN)	Levy et al. 1990b, Maury et al. 1990
Aspartylglucosaminuria (AGU)	Aspartylglucosaminidase (AGA)	Ikonen et al. 1991
Choroideremia	Rab escort protein 1 (REP1)	Sankila et al. 1992
Nonketotic hyperglycinemia (NKH)	Glycine decarboxylase (GLDC)	Kure et al. 1992
Diastrophic dysplasia (DTD)	Solute carrier family 26, member 2 (SLC26A2)	Hästbacka et al. 1994
Infantile neuronal ceroid-lipofuscinosis (INCL)	Palmitoyl protein thioesterase 1	Vesa et al. 1995
Batten disease (CLN3)	CLN3 protein	The international Batten disease Consortium (Consortium 1995)
Hypergonadotrophic ovarian dysgenesis (ODG1)	FSH receptor (FSHR)	Aittomäki et al. 1995
Congenital chloride diarrhea (CCD)	Solute carrier family 26, member 3 (SLC26A3)	Höglund et al. 1996
Progressive myoclonus epilepsy (EPM1)	Cystatin B (CSTB)	Pennacchio et al. 1996, Virtaneva et al. 1997
Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)	Autoimmune regulator (AIRE)	Nagamine et al. 1997, The Finnish German APECED Consortium (Consortium 1997)
Congenital nephrosis (NPHS1)	Nephrin	Kestilä et al. 1998
Variant late infantile neuronal ceroid-lipofuscinosis (vLINCL)	CLN5 protein	Savukoski et al. 1998
Retinoschisis	Retinoschisin	The retinoschisis Consortium (Consortium 1998)
Lysinuric protein intolerance (LPI)	Solute carrier family 7, member 7 (SLC7A7)	Torrents et al. 1999, Borsani et al. 1999
Megaloblastic anemia 1	Cubilin (CUBN)	Aminoff et al. 1999
Progressive epilepsy with mental retardation (EPMR)	CLN8 protein	Ranta et al. 1999
Free sialic acid storage disease (Salla disease)	Solute carrier family 17, member 5 (SLC17A5)	Verheijen et al. 1999

Cornea plana congenita (CNA2)	Keratocan (KERA)	Pellegata et al. 2000
Mulibrey nanism	TRIM37	Avela et al. 2000
Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL)	DAP12 (TYROBP, KARAP)	This study (I)
Cartilage-hair hypoplasia (CHH)	RNA component of RNase MRP (RMRP)	Ridanpää et al. 2001
Usher syndrome, type III	USH3A	Joensuu et al. 2001
Tibial muscle dystrophy (TMD)	Titin (TTN)	Hackman et al. 2002
GRACILE syndrome	BCS1-like (BCS1L)	Visapää et al. 2002
Hydrolethalus syndrome	11q, novel protein	Visapää et al. 1999; Lanyi et al. 2002
Cohen syndrome	COH1 protein	Kolehmainen et al. 1997; Kolehmainen et al. 2003
RAPADILINO syndrome	unpublished	Kestilä et al., personal comm.
Infantile onset spinocerebellar ataxia (IOSCA)	10q, unpublished	Nikali et al. 1995 and personal comm.
Muscle-eye-brain disease (MEB)	1p, unpublished	Cormand et al. 1999; Lehesjoki personal comm.

B) MAPPED LOCUS or LOCUS UNIDENTIFIED

<u>Disease</u>	<u>Locus</u>	<u>Reference</u>
Meckel syndrome	17q	Paavola et al. 1995
Lethal congenital contracture syndrome (LCCS)	9q	Mäkelä-Bengs et al. 1998, Järvinen et al. 1998
Congenital lactase deficiency (CLD)	2q	Järvelä et al. 1998
PEHO syndrome	unknown	Salonen et al. 1991
Lethal arthrogryposis with anterior horn cell disease	unknown	Vuopala et al. 1995

PLOSL (NASU-HAKOLA DISEASE)

The PLOSL syndrome (ICD-10: G31.8, MIM221770) is characterized by spontaneous bone fractures and presenile dementia leading to death in the early 40's. The acronym is composed of the first letters of the characteristic pathologic findings: Polycystic Lipomembranous Osteodysplasia with Sclerosing Leukoencephalopathy. PLOSL is also known as Nasu-Hakola disease, or membranous lipodystrophy in the early literature. PLOSL was first described in the early 1960s concurrently in Finland (Järvi et al. 1964; Järvi et al. 1968; Hakola 1972) and Japan (Terayama 1961; Nasu et al. 1973).

Approximately 200 patients have been reported worldwide (Pekkarinen et al. 1998a and unpublished). Most of the patients have been diagnosed in Japan (~100) and Finland (~40). Sporadic patients have been diagnosed worldwide, most of them in Europe. The reported incidence is highest in Finland, 2×10^{-7} /year (Hakola 1990b), and PLOSL therefore belongs to the Finnish disease heritage. The incidence in Japan is unknown.



Figure 2. Regional clustering of the birthplaces of grandparents of PLOSL patients (dots) in the eastern Finland (Kuopio district) indicates that the PLOSL mutation is relatively young. According to (Norio 2000; Norio 2003b).

Symptoms of PLOSL

The natural course of PLOSL can be divided into four stages: 1) latent, 2) osseous, 3) early neurological, and 4) late neurological stages (Hakola 1972). The latent period of PLOSL extends to adolescence. 2) The disease usually manifests itself approximately at the age of 20 years with pain and swelling in ankles and feet following strain. The first fractures in the bones of the extremities after minimal trauma typically occur shortly

before age 30 years. Radiographs show symmetric cyst-like bone cavities especially in the bones of wrists, hands, ankles and feet, in addition to trabecular bone loss in the distal end of the long tubular bones. The fractures usually heal well. 3) At the early neurological stage, a change of personality begins to develop insidiously in the early fourth decade. The patients gradually develop the full-blown picture of the frontal lobe syndrome: loss of judgment, euphoria, disinhibition (including moria, i.e. a tendency to joke), disturbance of concentration, as well as a lack of insight. Urinary incontinence and impotence may be among the first symptoms. Progressive signs of upper motor neuron involvement (spasticity, Babinski's sign) also become noticeable. Memory disturbances set in at the same age as the personality change. The memory disturbance is less severe than the personality change at the initial neurological stage but it is progressive. Epileptic seizures and involuntary slow choreiform movements or myoclonic twitches are common. 4) In the late neurological stage the disease culminates in profound dementia. Finally, the patients lay without any active movement in an apallic state. Primitive reflexes, such as the visual and tactile grasp and mouth opening reflexes, as well as the sucking reflex, become noticeable. Death usually occurs by 50 years of age (Table 3).

Table 3. Natural Course of PLOSL		
<u>Stage</u>	<u>Age (years)</u>	<u>Symptoms</u>
I) Latent	-20	
II) Osseous	20-30	Pain in ankles and wrists Pathological fractures
III) Early Neurological	30-40	Frontal lobe syndrome Progressive dementia Memory disturbance Other disturbances of higher cortical functions ^a Epileptic seizures
IV) Late neurological	40-	Profound dementia Primitive reflexes Apallic state Premature death

^aAgnostic-aphasic-apraxic symptoms

Identification of the First PLOSL Locus on Chr. 19q13.1

The first PLOSL locus was identified using a genome-wide scan. Initially, five affected individuals from two families were analyzed for genomic regions that were shared within families. In the second stage, the six most promising regions were characterized in the whole family series. Using this approach, the PLOSL locus was assigned to chr. 19q13.1. Using haplotype analysis, the critical PLOSL region was restricted to 153 kb between markers D19S1175 and D19S608. Sequencing of the coding region of a potential candidate gene *APLP1*, encoding amyloid precursor-like protein, revealed no mutations (Pekkarinen et al. 1998a; Pekkarinen et al. 1998b).

DEMENTIA

Dementia is defined as a wide-spread decline of cognitive functions (DSM-IV). The most prominent symptom is memory impairment. The diagnosis of dementia also requires the presence of other disturbances of higher cortical functions including aphasia, apraxia, agnosia or a disturbance of executive functioning. A wide variety of disorders are associated with dementia (Table 4). Dementia is usually progressive (e.g. Alzheimer's disease) but may also remain unchanged (e.g. sequel to brain contusion) or in some instances it is even curable (e.g. Pellagra, dementia caused by deficiency of niacin). As the population ages, the incidence of dementia rises exponentially until the ninth decade of life. The prevalence of dementia is about 1.5% at age 65 years, but doubles every 4-5 years to reach about 30% at 80 years. The most common causes for dementia are Alzheimer's disease (AD), vascular dementia and dementia with Lewy bodies. In Europe and North America, AD is estimated to be the most common cause of dementia, whereas vascular dementia has been reported to be more prevalent than AD in several Asian countries such as China and Japan (Mirra and Hyman 2002; Ritchie and Lovestone 2002).

Inherited Dementias

A significant advance in our understanding of the genetic basis of dementias has been made in the past decade. However, the genetic background of most of the common forms of dementia is still poorly understood. The genetics of dementia is a very challenging topic to review since several dementing conditions are extremely complex disorders. The following review covers some of the most illustrative examples of adult-onset dementing diseases for which genes have been identified.

Table 4. Classification of Dementia

Neurodegenerative disorders	Primary dementias Alzheimer's disease Dementia with Lewy Bodies Frontotemporal Dementia with parkinsonism linked to chr. 17 (FTDP-17) Secondly dementing disorders Huntington disease Parkinson disease
Vascular disease	Multi-infarct dementia CADASIL Hereditary cerebral hemorrhage with amyloidosis, Finnish, Dutch and Icelandic types
Infectious, inflammatory and immune-mediated disorders	Prion disorders AIDS
Toxic and metabolic disorders	Alcoholism Vitamin B ₁₂ deficiency
Other conditions	Traumatic brain injury
NOTE: The list is not complete, only some illustrative examples of each category are presented. Modified from Mirra & Hyman (2002).	

Alzheimer's disease (AD)

Alzheimer's disease is characterized by slowly progressive adult-onset dementia associated with cerebral atrophy most prominent in the frontal, temporal and parietal cortical areas. The typical clinical duration of the disease is eight to ten years, with a range of one to 25 years. The histopathological hallmarks of AD are neuritic plaques and neurofibrillary tangles. The cores of the neuritic plaques consist mainly of aggregated β -amyloid. Neurofibrillary tangles are intraneuronal inclusions composed of abnormally phosphorylated tau-protein. The exact pathogenic mechanisms of AD are unknown but it is believed that plaques and tangles are involved in neuronal cell death (Mirra and Hyman 2002; Cummings et al. 1998; Hardy and Selkoe 2002).

Alzheimer's disease can be divided into three main categories: sporadic AD, familial AD, and AD associated with Down syndrome. Sporadic AD accounts for ~75%, familial AD for ~25% and AD associated with Down syndrome for <1%. It should be noted that sporadic and familial forms of AD cannot be distinguished from each other clinically or at the histopathological level. In practice, positive family history or age of onset before 65

years are the most important signs of familial AD (Cummings et al. 1998; GeneReviews web pages).

Familial AD can be subdivided by the mode of inheritance and gene to late-onset (15-25%) and early-onset AD (<2%). Late-onset familial AD (AD type 2) is considered as a complex disease with the most important risk factor being *ApoE-ε4* allele. Most of the patients with AD before age 65 suffer from early-onset familial AD. Early-onset forms of familial AD are autosomally dominantly inherited and caused by mutations in genes coding for amyloid precursor protein (*APP*; AD type 1) (Cummings et al. 1998), presenilin 1 (*PSEN1*; AD3) (Sherrington et al. 1995; Crook et al. 1998), or presenilin 2 (*PSEN2*; AD4) (Cruts and Van Broeckhoven 1998). Mutations in either one of these three genes lead to an increased production of the amyloidogenic peptide Aβ42 (Hardy and Gwinn-Hardy 1998).

By the age of 40 years, virtually all individuals with Down syndrome (trisomy 21) develop manifestations of AD. This is thought to be caused by the lifelong over-expression of the amyloid precursor protein (*APP*) gene on chr. 21 and the resultant overproduction of β-amyloid in persons who are trisomic for this gene (Brugge et al. 1994).

CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a microangiopathy primarily affecting the brain. Ischemic attacks (TIAs or stroke) at the mean age of onset of 45 years are the most frequent findings manifesting in ~85% of the patients. Cognitive decline, including dementia, begins to develop at the same time. By age 65, two-thirds of the patients suffer from dementia. Migraine with aura is among the first symptoms of CADASIL and manifests in about 30% of the patients (Kalimo et al. 1999). CADASIL is caused by mutations in *NOTCH3* gene (Dichgans 2002). *NOTCH3* encode a cell-surface receptor that is involved in cell fate specification during development; the exact role for *NOTCH3* is unknown (Weinmaster 1997).

Frontotemporal Dementia with Parkinsonism-17

FTDP-17 is a major class of autosomally dominantly inherited dementing disorders linked to chr. 17. FTDP-17 affects the frontal and temporal cortex and some subcortical nuclei. Although the manifestations of different subtypes of FTDP-17 vary considerably depending on the mutation, several common factors exist. The age of onset of FTDP-17 is typically 40-60 years. The patients present with slowly progressive behavioral changes, such as disinhibition and loss of initiative in addition to language disturbances and extrapyramidal signs. Some patients present with rigidity, bradykinesia, supranuclear palsy, and saccadic eye movement disorders. The disease progresses

over a few years into a profound dementia. Neuropathologically, the patients have a pronounced frontotemporal atrophy with loss of neurons. Most patients show neuronal and glial aggregations of the microtubule-associated protein tau. FTDP-17 is caused by mutations in microtubule-associated protein tau gene (*MAPT*) in majority of the patients. (Hutton et al. 1998; Spillantini et al. 1998; GeneReviews web pages).

Familial Cerebral Amyloid Angiopathies (CAA)

CAA is associated with dementia in a number of familial conditions, such as hereditary cerebral hemorrhage with amyloidosis (HCHWA), Finnish, Dutch and Icelandic types. The Finnish type HCHWA (gelsolin-related amyloidosis) is an autosomal dominant disorder and is caused by mutations in the gelsolin gene (*GSM*) (Levy et al. 1990b; Paunio et al. 1992). Gelsolin-related amyloidosis is characterized by facial palsy, peripheral neuropathy and corneal lattice dystrophy. Some patients present with cognitive decline (Kiuru et al. 1999). The Dutch type (-D) of HCHWA is characterized by multiple hemorrhages and infarcts associated with amyloid deposits in the meningeal and cortical blood vessels of the cerebrum and cerebellum. Dementia manifests in most patients that survive their initial stroke (Mirra and Hyman 2002). HCHWA-D is caused by a point mutation at codon 693 of the *APP* gene (Levy et al. 1990a). The Icelandic type (-I) of HCHWA is caused by a mutation in the gene coding for cysteine protease inhibitor cystatin C (*CST3*) (Levy et al. 1989). HCHWA-I usually manifests with cerebral hemorrhages associated with cerebral and meningeal amyloid deposits. Dementia occurs in some, but not all patients (Mirra and Hyman 2002). Although these three types of cerebral amyloid angiopathies are caused by mutations in different genes, there is a common neuropathological feature: the pathological accumulation of amyloid.

Prion disorders

Prion diseases, also known as transmissible spongiform encephalopathies, are unique in that they can be at the same time inherited and infectious. The prion protein is encoded by the *PRNP* gene. The pathogenicity of abnormal prion polypeptides (PrP^{Sc}) is mediated by their abnormal conformation. PrP^{Sc} molecules are able to cause a conversion of normal isoform of the prion protein (PrP^{C}) to pathogenic PrP^{Sc} that is insoluble and tends to aggregate. Six different forms of human prion diseases have been described, including Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS), fatal familial insomnia (FFI), new variant CJD, kuru, and atypical prion disease. Autosomal dominant mutations in *PRNP* gene either result in CJD, FFI, GSS, phenotype intermediate between CJD and GSS, or a phenotype lacking distinctive histopathological features. The predominant neuropathological change in prion disorders is spongiform encephalopathy but different subtypes have specific histological characteristics (Haltia 2000).

Approximately 5-15% of CJD cases are familial. *PRNP* mutations known to associate with CJD are E200K and D178N. Interestingly, in families with the D178N mutation, the phenotype depends on the genotype at a different codon (position 129). Patients carrying the D178N mutation and valine at position 129 present with typical CJD, whereas patients with D178N and methionine at position 129 develop FFI. The typical symptoms of CJD are progressive dementia, gait disturbance and behavioral disturbances whereas the symptoms of FFI, e.g. complete insomnia, dementia, rigidity, and mutism, markedly differ from CJD. Similarly, the neuropathologic manifestations of CJD are distinct from FFI. The GSS syndrome with dementia, ataxia, dysphagia, dysarthria, and hyporeflexia are caused by several mutations in the *PRNP* gene (Haltia 2000; Tsuang and Bird 2002).

Huntington's disease (HD)

The typical symptoms of HD include chorea, dementia, and behavioral disturbances. The mode of inheritance of HD is autosomal dominant. Huntington's disease is a trinucleotide repeat disorder and caused by abnormally high number CAG repeats in the 5' region of the *HD* gene. The age of onset of HD correlates reciprocally with the number of CAG repeats. Anticipation, i.e. worsening and earlier age of onset of the disease from generation to generation, is observed in HD. Further, HD alleles inherited from the father tend to undergo more significant expansion than maternal alleles. The primary histopathological finding in HD is neuronal loss in the basal ganglia (caudate nucleus, putamen and globus pallidus) (Tsuang and Bird 2002).

DISORDERS OF THE BONE

The skeleton is a dynamic tissue that is continuously under remodeling throughout the whole life. A strict balance is maintained between bone formation by osteoblasts and bone resorption by osteoclasts. The disorders of bone can be divided into the following categories: hereditary, congenital (e.g. anomalies), acquired metabolic (e.g. rickets and osteomalacia), and infectious diseases (e.g. osteomyelitis) as well as traumas (fractures) and tumors (Kumar et al. 1997). Although the skeleton houses the bone marrow, the hematopoietic element, it is categorized as a distinct organ from bone. Since the scope of this thesis are inherited disorders of bone and osteoporosis, these entities are reviewed in more detail below.

Numerous inherited disorders of the skeleton have been characterized. Monogenic bone disorders can be classified into two major categories: impaired development and defective maintenance of bone. Achondroplasia is a disorder of bone development and the most common human disease that results in dwarfism. The disorder is caused by autosomal dominant mutations in gene coding for fibroblast growth factor receptor 3

(*FGFR3*). *FGFR3* mutations result in an impaired maturation of cartilage in the developing growth plate of bone leading to short bones (Vajo et al. 2000). Osteogenesis imperfecta (OI) is an important group of monogenic disorders of bone maintenance. OI is usually caused by autosomal dominant mutations in *COL1A1* or *COL1A2* genes that code for collagen type I, a crucial bone matrix component. This spectrum of disorders is characterized by the occurrence of multiple spontaneous fractures due to abnormal synthesis of collagen type I leading to bone fragility (Kumar et al. 1997). The clinical phenotype of OI varies considerably ranging from early death to normal life span with a mild decrease in bone mass (Scriver et al. 2001).

Osteoporosis is an extremely common disease of the elderly. The World Health Organization defines osteoporosis as bone mineral density in the spine over 2.5 standard deviations below the "young normal" mean value. Osteoporotic bones are brittle and prone to pathological fractures. The complications of osteoporosis (e.g. hip fractures) are a significant cause of death in Western countries. The pathogenic mechanism of osteoporosis is a long-term imbalance between bone formation and resorption that results in a decrease in total bone mass and density. Metabolic and genetic, in addition to nutritional and several other lifestyle factors together have their own influence in the pathogenesis of this complex disorder. The genetic factors influencing the pathogenesis of osteoporosis are poorly understood (Kumar et al. 1997; Lazner et al. 1999).

AIMS OF THE STUDY

This study was undertaken when the autosomal recessive mode of inheritance of PLOSL was established and the locus for PLOSL in Finnish patients mapped to chromosome 19q13.1. The clinical features of PLOSL were partially characterized. The molecular pathogenesis of the disease was completely unknown.

The initial aim of this study was to identify the PLOSL gene in Finnish families. During the research project, the following additional goals were formulated:

- The identification of the causative gene in PLOSL patients not linked to 19q13.1
- The development of DNA diagnostics for earlier and more accurate diagnosis of PLOSL
- More accurate characterization of the natural course and pathological features of PLOSL
- The initiation of the characterization of the pathogenic mechanisms underlying PLOSL

MATERIALS AND METHODS

PATIENTS AND ETHICAL ASPECTS (I-IV)

The family material was obtained through a worldwide collaboration with clinicians. This study has been approved by the ethical committees of National Public Health Institute and Helsinki University Central Hospital. Informed written consent was obtained from the patients, or their statutory guardians.

For the characterization of the natural course of PLOSL, clinical, neurophysiological, and imaging data on eight patients were available (III). Five patients were examined at least twice at 2- to 10-year intervals. Autopsy specimens were available from three patients.

SEQUENCE AND MUTATION ANALYSIS (I, II, IV)

The known genes in the critical DNA region were identified by searching NCBI databases using the BLASTN algorithm. Putative transcripts in the critical region were identified by searching EST databases using the BLASTN algorithm at NCBI. Further, several different gene prediction computer programs were used: Fgenes (URL provided in the separate section), Genotator (Harris 1997), Genscan (Burge and Karlin 1997), Grail (Xu et al. 1994) and MZEF (Zhang 1997).

The primers were designed to flank the coding regions of the genes and predicted transcripts. The primer sequences are provided in the original publications. The PCR-amplified coding regions were sequenced by automated sequencing (ABI377; Perkin-Elmer -Applied Biosystems) using the PCR primers as sequencing primers.

RNA ANALYSES (I, II, IV)

Northern-blot analysis of human cells and tissues were performed using [³²P]-radiolabeled human *DAP12* and *TREM2* cDNAs as probes. Poly(A)⁺ RNA from the Finnish PLOSL and control lymphoblast cell lines for northern-blot analysis was isolated using a Fast Track 2.0 mRNA isolation kit (Invitrogen). Poly(A)⁺ RNA was separated by electrophoresis and transferred to a nylon membrane as previously described (Sambrook 1989). Human multiple-tissue northern blots (Clontech) and a multiple-tissue mRNA expression array (Clontech) were hybridized according to the manufacturer's instructions. The probes, corresponding to the transcribed region of *DAP12* and *TREM2*, were generated by PCR from cDNA clones (GenBank accession numbers are found below in a separate section) obtained from the IMAGE Consortium. The probes were

labeled and purified using the Rediprime II Random Prime Labelling System (Amersham Pharmacia Biotech) and the QIAquick Nucleotide Removal Kit (Qiagen) and hybridized using the ExpressHyb hybridization solution, according to the manufacturer's (Clontech) protocol.

For quantitative RT-PCR analyses monocytes were stimulated with M-CSF and RANKL for 1, 3, 7 and 21 days as described below. Total RNA was isolated using TRIzol reagent (GibcoBRL). Quantitative RT-PCR experiments were performed using a LightCycler PCR machine (Roche, Molecular Biochemicals) as described previously (Paloneva et al. 2002; Konttinen et al. 2001). Serial dilutions of human *DAP12*, *TREM2*, *TRAP*, and *CALCR* cDNA cloned in a plasmid vector were used to determine the copy number of the amplicon per housekeeping gene cDNA copies (porphobilinogen deaminase, *PBGD*). Probes and human genomic DNA were used to determine the copy number of *β-actin* (*ACTB*), *Cathepsin K*, *PBGD* and *RANK*. The primer sequences used in quantitative RT-PCR analyses are provided in the original publications (II, IV).

PHENOTYPIC ANALYSIS OF PBMC (I)

Samples of 1×10^5 PBMC were labelled with FITC- or phycoerythrin-conjugated monoclonal antibodies against CD3, CD4, CD8, CD16 and CD56 (Becton Dickinson) and immediately analysed by flow cytometry using a FACScan (Becton Dickinson).

CYTOTOXICITY ASSAY (I)

PBMC were tested for cytotoxicity against K562 erythroleukaemia cells. Before the cytotoxicity assay, PBMC were incubated overnight at 37 °C in the presence or absence of interleukin-2 (500 U/ml; IL-2, R&D Systems). The target cells were labelled with sodium⁵¹chromate (Pharmacia Biotech), washed 3 times, and exposed to PBMC at effector to target cell ratios of 50:1, 25:1 and 12.5:1 in 96-well plates. Supernatant were collected after 4 h incubation and the release of ⁵¹Cr was measured with a γ-counter (Wallac). Triplicate wells were prepared for each effector to target cell combination. Percent cytotoxicity (Cx) was calculated using the formula % Cx=(test release–spontaneous release (without effector cells)/maximal release (with 10% Triton X)–spontaneous release)100.

IMMUNOPRECIPITATION AND WESTERN-BLOT ANALYSIS (I)

PBMC were incubated overnight at 37 °C in RPMI 1640 media supplemented with 10% fetal bovine serum (Life Technologies), antibiotics and L-glutamine. A total of $2-3 \times 10^7$

cells were washed with PBS and lysed with NP40 lysis buffer. The proteins were immunoprecipitated from lysates with the anti-human DAP12 monoclonal antibody DX37 (a mouse IgG1 generated by immunizing Balb/c mouse with a polypeptide corresponding to the cytoplasmic domain of human DAP12). Immunoprecipitates were collected with protein A/G–Sepharose (Pharmacia Biotech) and washed with NP40 lysis buffer. The beads were boiled in sample buffer (Laemmli), and aliquots were separated on 4–20% gradient SDS–PAGE gels (Readygel, BioRad) for western-blot analysis using the same monoclonal antibody. DAP12 was visualized using HRP conjugated anti-mouse Ig (Pharmacia Biotech) and ECL (SuperSignal, Pierce Chemical Company). DAP10 lysates were immunoprecipitated from lymphoblasts obtained from the Finnish PLOSL patient and a control subject using an affinity-purified (with the immunizing peptide) polyclonal rabbit antiserum against a polypeptide, which corresponds to the human DAP10 cytoplasmic domain, or with a control rabbit IgG. Immunoprecipitates were analyzed by western blot using the same rabbit anti-DAP10 antiserum (Wu et al. 1999).

NEUROIMAGING (III)

Five CT and 11 MRI of eight patients were re-evaluated, and the bicaudate ratio was calculated (Aylward et al. 1991). In addition, the brains of three patients were examined with a 1.5 T imager post mortem, with a technique described previously (Autti et al. 1997).

NEUROPHYSIOLOGY (III)

Conventional EEG was recorded on all eight patients (altogether 12 recordings) with Ag/AgCl electrodes, using the international 10/20 system.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY (III, IV)

The brains, spinal cords and bones were fixed in 4% phosphate-buffered formaldehyde. Representative brain samples were embedded in paraffin and stained with hematoxylin-eosin, van Gieson, Luxol Fast Blue-cresyl violet, modified Bielschowsky silver stain, periodic acid-Schiff (PAS), elastin, and Congo red methods, according to standard protocols. The bones were decalcified before cutting them into sections, and stained with H&E and for TRAP.

Immunostaining was performed by using the avidin-biotin complex (ABC) technique (Vectastain ABC-kit, Vector Laboratories, Burlingame, CA). Monoclonal antibodies

against amyloid beta-protein (clone 4G8, Senetek, MD Heights, MO; Clone 6F3D, Novocastra Laboratories, Newcastle, UK), α -synuclein (clone LB509, Zymed Laboratories, South Francisco, CA), phosphorylated tau-protein (Clone AT8, Innogenetics, Zwijndrecht, Belgium), phosphorylated neurofilaments (SMI-34, Sternberger-Meyer, Jarrettsville, MD), ubiquitin (Dako, Glostrup, Denmark), CD68 (clone PG-M1, Dako), and collagen IV (clone CIV22, Dako), or rabbit antiserum against glial fibrillary acidic protein (GFAP; Dako) and α -B-crystallin (Novocastra) were applied on deparaffinized sections at recommended dilutions after appropriate pretreatments. Phosphate-buffered saline or normal rabbit serum was used instead of the primary antibody for control of specificity.

INDUCTION OF OSTEOCLASTIC CELLS (IV)

PBMC from PLOSL patients and two healthy individuals were isolated from buffy coat cells over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). PBS washed cells were resuspended in α -MEM (GibcoBRL), Fetal Calf Serum and antibiotics. 5×10^6 cells/well were allowed to adhere to 6-well plates for 1 h at + 37 °C. Nonadherent cells were washed away with PBS, and adherent cells were stimulated for 1, 3, 7, 14 and 21 days with 25 ng/ml M-CSF (R&D Systems) and 40 ng/ml RANKL (Alexis Biochemicals). The media with cytokines was replaced twice a week.

IN VITRO AND IN VIVO ANALYSIS OF BONE RESORPTION (IV)

In vitro bone resorption analysis was performed by first stimulating PBMC from three DAP12-deficient and four healthy individuals with M-CSF and RANKL for 7 or 21 days. 4×10^5 cells were then transferred to well 7.5mm in diameter containing a dentin slice 5mm in diameter (Immunodiagnostic Systems). Cells were incubated on dentin in the presence of media with cytokines (replaced twice a week) for 7 days, fixed and stained for TRAP. The number of multinucleated osteoclasts and nuclei per cell were calculated using a light microscope. The cells were then brushed away and the dentin slices were stained with toluidine blue to visualize the resorption pits. The surface area and depth of the resorption pits were determined using AnalySIS 3.2 software (Soft Imaging System) and a confocal microscope, respectively. Bone resorption in vivo was determined by serum CrossLaps ELISA in four DAP12- and one TREM2 -deficient patients and in four healthy individuals as previously described (Rosen et al. 2000).

HISTOCHEMISTRY AND IMMUNOFLUORESCENCE STAININGS (IV)

Staining for TRAP was performed using a TRAP staining kit (Sigma-Aldrich). The cytoskeletal actin was stained using Alexa Fluor 633 phalloidin reagent according to instructions provided by the manufacturer (Molecular Probes). The nuclei were visualized using DAPI reagent (Sigma-Aldrich). The staining for cathepsin K was performed using polyclonal anti-human cathepsin K antibody (Santa Cruz Biotechnology).

GENBANK ACCESSION NUMBERS (I, II, IV)

The genomic sequence encompassing the critical region was constructed from cosmids F19541 (current name U95090), R33502 (AC002133), R28051 (AD000864), F19399 (AD000833), R31158 (AF038458), R31874 (AD000823) and R28125 (AD000827). *DAP12* genomic sequence, AF019563; *DAP12* cDNA sequence, N41026, AF019562 and AA481924; *DAP10* genomic sequence, AF072845; *APLP1* genomic sequence, NM_005166; *NPHS1* cDNA sequence, AF035835; *DAP12* probe, N41026; *TREM2* cDNA, BF343916, *TRAP* cDNA, J04430; *CACLR* cDNA, NM_001742; β -actin (*ACTB*) cDNA, X00351; *PBGD* cDNA, M95623; *TA-NFKBH*, NM_032721; *RANK* cDNA .

DAP12 KNOCKOUT MOUSE

Dap12 loss-of-function mutant mouse strain (*Dap12* ^{Δ ex3-4}) was generated by Dr Lewin Lanier and his colleagues in DNAX, Palo Alto, California, USA (Bakker et al. 2000). In this mouse strain, exons 3 and 4 coding for the transmembrane domain as well as part of the cytoplasmic domain of Dap12 including the first tyrosine of the intracellular tyrosine-based activation motif (ITAM) have been deleted. The Dap12 knockout mouse strain is a hybrid of C57BL/6 and 129 Ola mouse strains. The mice for this study were obtained on a collaborative basis.

RESULTS AND DISCUSSION

IDENTIFICATION OF THE FIRST PLOSL GENE (I)

Analysis of the Critical PLOSL Region

The critical region for PLOSL in the Finnish patients had been previously assigned to chr. 19q13.1 using genome-wide linkage, linkage disequilibrium and haplotype analyses. The critical region encompassed 153 kb and was restricted by markers D19S1175 and D19S608 (Fig. 3) (Pekkarinen et al. 1998a; Pekkarinen et al. 1998b). The genomic sequence over the critical region was constructed as a contig of cosmids available at the NCBI database (Pekkarinen et al. 1998b). Since multipoint association analysis suggested that the mutated gene was located in the vicinity of marker D19S610 (Pekkarinen et al. 1998b), we concentrated on the telomeric 90 kb part of the critical region restricted by the marker D19S1175 and *StyI* restriction enzyme polymorphism. This region contained three known genes: *NPHS1* (Kestila et al. 1998), *APLP1* (Lenkkeri et al. 1998) and *DAP12* (Lanier et al. 1998b). In order to identify new putative genes in the critical region, we searched the EST and nr databases available at NCBI using the BLASTN algorithm. We also analyzed the genomic sequence using several different gene prediction programs (Genscan, Grail, MZEF, Fgenes, Genotator). With these methods we identified three previously unknown putative transcripts or EST contigs. Since then, one of these putative transcripts has been published as *DAP10* (*KAP10*) (Chang et al. 1999; Wu et al. 1999). Another predicted transcript has recently been cloned *in silico* as *FILTRIN* (Ihalmo et al. 2003). The last putative transcript has not been cloned but the EST contig has been designated as a putative T-cell activation NFKB-like protein (*TA-NFKBH*).

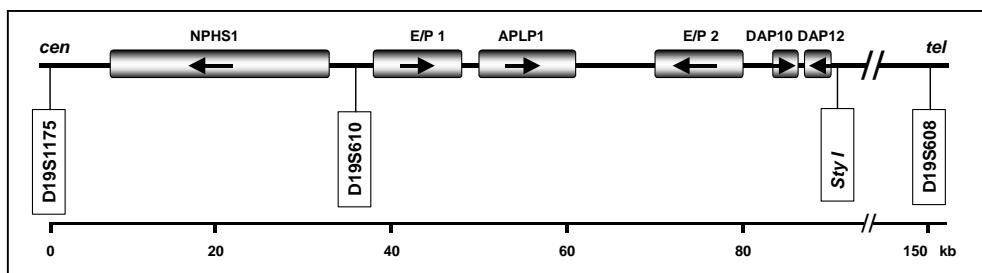


Figure 3. The known genes and predicted transcripts in the critical PLOSL region in chr. 19q13.1. E/P, EST contig or predicted transcript. The polymorphic markers restricting the region are indicated as their D-names. *StyI*, restriction enzyme polymorphism. The arrows indicate the orientation (*cen-tel* or *tel-cen*) of the genes. E/P 1: *FILTRIN*, E/P 2: *TA-NFKBH*. The scale indicates the distance from the centromeric border of the critical region restricted by marker D19S1175.

Identification of the First PLOSL Gene as DAP12

In order to identify the defective gene in the Finnish PLOSL patients, we exploited the positional cloning approach. The only promising regional candidate gene was the amyloid-precursor -like protein gene *APLP1* because of its homology to the amyloid precursor protein (*APP*) gene that is mutated in patients with familial Alzheimer's disease type I (Lendon et al. 1997). Sequence analysis of the genomic DNA (Pekkarinen et al. 1998b) and northern analysis of the steady state mRNA did not reveal mutations in *APLP1* in the Finnish patients. Because no other functional candidates existed in the critical region we started systematically to analyze the known genes as well as predicted transcripts in the critical region. Sequencing of the amplified coding regions of the candidate genes revealed no disease-associated DNA variants, except in the last regional candidate gene, *DAP12* (also known as *TYROBP* or *KARAP*).

We failed to obtain products by PCR-amplifying the coding region of *DAP12* of the genomic DNA of Finnish PLOSL patients with primers flanking exons 1–4 of the 5 exons of *DAP12*, whereas exon 5 was amplified normally. This indicated that the patients carry a homozygous deletion encompassing exons 1–4 of *DAP12*. In order to determine the 5' and 3' boundaries of the identified deletion, we PCR-amplified genomic DNA of Finnish patients across the deletion. We obtained a product of 699 bp, instead of the expected 6 kb, with a primer pair del_C/del_T (Fig. 11, p. 55). Sequencing of this PCR product revealed the 5' deletion breakpoint 2900 bp upstream from the initiation methionine, and the 3' breakpoint in the last intron of *DAP12*, 1300 bp upstream from the termination codon. The genomic deletion is 5265 bp and includes 343 bp (exons 1–4 and 5' UTR) of the 604-bp transcribed region of *DAP12*. All Finnish PLOSL patients, as well as one Swedish patient with known ancestors in Finland are homozygous for this deletion (I) (Nylander et al. 1996; Pekkarinen et al. 1998a). The same mutation was also found in a Norwegian family without known ancestors in Finland (Schrader et al. 2000; Tranebjaerg et al. 2000). The PLOSL_{Fin} deletion breakpoints are flanked by *Alu* repetitive elements that share an identical 23 bp sequence. As the deletion breakpoints occur in an identical nucleotide sequence, the precise deletion breakpoints cannot be defined. The PLOSL_{Fin} deletion probably arose from homologous recombination between two direct *Alu* repeats (5.3 kb apart) and resulted in the generation of a fusion *Alu* element in the disease allele.

Other patients we have found to carry mutations in *DAP12* are from Sweden (PLOSL_{Fin}, one family) (I), Norway (PLOSL_{Fin}, one family) (I) (Tranebjaerg et al. 2000), Japan (PLOSL_{Jpn}, 141delG, one family) (I), Portugal (145G>C) (Baeta et al. 2002), the UK (154ins42bp, unpublished), and Brazil (~8kb deletion encompassing exons 1–4, one family) (II) (Table 6, p. 39).

Expression of DAP12 and DAP10 in Finnish PLOSL patients

We did not detect steady-state mRNA corresponding to *DAP12* in the lymphoblasts of Finnish PLOSL patients using northern-blot analysis. Amplification by RT-PCR failed to produce any signal from lymphoblast RNA of Finnish patients. Western-blot analysis of the immunoprecipitates from peripheral blood mononuclear cells (PBMC) of the Finnish patients using a monoclonal antibody specific to DAP12 revealed no signal. Therefore, Finnish PLOSL mutation results in a complete loss of expression and function of DAP12.

DAP12 and DAP10 are both cell-surface adapter proteins. Their genes are located on the same chromosomal DNA strand in the opposite transcriptional orientation, and their translation termination codons are separated by only 408 bp. The PLOSL_{Fin} deletion does not include the known sequence of *DAP10*, but given the proximity of the genes, we explored the possibility that the deletion affects transcript levels of *DAP10*. Both patient and control lymphoblast lines yielded abundant *DAP10* transcripts; no differences were observed. Similarly, western-blot analysis of DAP10 polypeptides in PBMC revealed no difference between the patient and control. Moreover, a normal level of the DAP10-associated receptor NKG2D was found in the PLOSL patient.

Phenotypic and Functional Analysis of PBMC in PLOSL patients

Since DAP12 is an activating NK cell receptor, we investigated NK cell function and number in Finnish PLOSL patients with loss-of-function mutations in *DAP12*. Immunophenotyping of PBMCs from six Finnish PLOSL patients revealed that the frequency of NK cells (CD3-CD56+) and CD56+ T cells (CD3+CD56+) were within normal limits. Moreover, there were no abnormalities in the number or frequency of T lymphocyte subpopulations (CD3+CD4+, CD3+CD8+). Furthermore, we detected no differences between the patients and controls in either spontaneous or interleukin-2 activated cytotoxicity of the lymphocytes against the K562 leukemic cells. These studies imply the normal function of NK cells in PLOSL patients. Accordingly, PLOSL patients do not suffer from problems arising from defective NK cell function, such as herpes virus infections or an increased frequency of malignancies. Taken together, our finding of the normal NK cell function in PLOSL patients suggests functional redundancy or developmental compensation in target cell recognition by NK cells.

IDENTIFICATION OF THE SECOND PLOSL GENE AS TREM2 (II)

We did not detect mutations in *DAP12* in a Norwegian and a Swedish family. In addition, linkage to chr. 19q13.1 could be excluded in these families (Pekkarinen et al. 1998a). These observations indicated genetic heterogeneity in PLOSL. Since *DAP12* codes for a cell-surface receptor element that interacts with many different receptors, we used a genetic strategy to identify genes mutated in PLOSL patients with no mutations in *DAP12*. We initially analyzed two informative families (Norwegian and Swedish) showing exclusion of linkage to the PLOSL locus on chromosome 19q13.1, for segregation of the marker haplotypes flanking genes that encode the polypeptides interacting with human *DAP12*. These genes included those for human *DAP12*-associated receptors *SIRPB1* (chromosome 20p13) (Dietrich et al. 2000), *TREM1* (chromosome 6p21.2), *TREM2* (chromosome 6p21.2) (Bouchon et al. 2000), *NKp44* (chromosome 6p22.1) (Vitale et al. 1998), *MDL1* (chromosome 7q33) (Bakker et al. 1999), *CD94* (chromosome 12p13.3), *NKG2C* (chromosome 12p13.1) (Lanier et al. 1998a) and *KIR2DS2* (chromosome 19q13.4) (Lanier et al. 1998b). Furthermore, we analyzed haplotypes of chromosomal regions containing genes for the intracellular protein tyrosine kinases (PTKs) *SYK* (chromosome 9q22.1) and *ZAP70* (chromosome 2q11.2) (Lanier et al. 1998b; McVicar et al. 1998) of the downstream *DAP12* signal-transduction pathway for cosegregation. For haplotype construction, we selected two or three polymorphic markers flanking each candidate gene. The only chromosomal region showing complete cosegregation with PLOSL was on chr. 6p21 encompassing approximately a 10 cM DNA region. This region contains genes coding for the Triggering receptor expressed on myeloid cells (*TREM*) 1 and 2 as well as *NKp44* (Table 5). Sequencing of these genes revealed homozygous mutations in *TREM2*. The

TABLE 5. Cosegregation of Marker Haplotypes in *DAP12*-Associated Loci in Patients Not Linked to Chr. 19q13.1

<u>Gene</u>	<u>Chr.</u>	<u>Haplotype Cosegregation</u>
ZAP70	2	-
<u>TREM1</u>	<u>6p21</u>	+
<u>TREM2</u>	<u>6p21</u>	+
<u>NKp44</u>	<u>6p21</u>	+
MDL1	7	-
SYK	9	-
NKG2C	12	-
CD94	12	-
KIR2DS2	19	-
SIRPB1	20	-

The loci of the genes have been determined using UCSC Human Genome Browser (August 6, 2001, draft assembly)

Swedish family had a homozygous nonsense mutation at position 233 (233G>A), changing tryptophan 78 to a translation termination codon (W78X). In the Norwegian family, a 558G>T mutation was found, leading to conversion of lysine 186 to asparagine (K186N). Neither of these mutations was found in a control panel of 100 Scandinavian DNA samples.

Since then, we have been able to identify mutations in *TREM2* in all patients with no *DAP12* mutations (Table 6). Families with mutations in *TREM2* are from Germany (40G>T) (IV), Belgium (C97>T) (unpublished), Bolivia (132G>A) (II), Sweden (233G>A) (II) (Nylander et al. 1996), France (267delG) (unpublished), Germany (313delG) (unpublished), Sri Lanka (377T>G) (unpublished), the United States (401A>G) (II) (Bird et al. 1983), Italy (482+2T>C) (II), Norway (558G>T) (II) (Edvardsen et al. 1983).

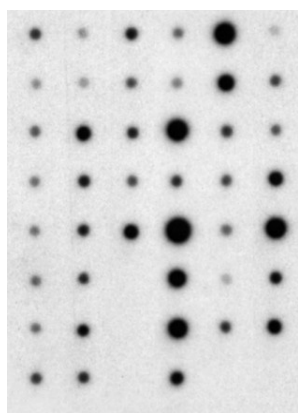
TABLE 6. Identified PLOSL mutations in *DAP12* and *TREM2*

<i>DAP12</i>			
<u>Country</u>	<u>Type of mutation</u>	<u>Exon(s)</u>	<u>N (patients)</u>
Finland, Sweden, Norway	<i>Del 5.3kb</i>	Ex 1-4	27
Brazil	<i>Del 8kb</i>	Ex 1-4	1
Japan	<i>2T>C/Met1Thr^a</i>	Ex 1	2
Japan	<i>141delG / TP^b</i>	Ex 3	4
Portugal	<i>145G>C / Gly49Arg</i>	Ex 3	2
Scotland	<i>154 ins 42bp</i>	Ex 3	1
<i>TREM2</i>			
Germany	<i>40G>T / Glu14Stp</i>	Ex 1	1
Belgium	<i>C97>T / Gln33Stp</i>	Ex 2	2
Bolivia	<i>132G>A / Trp44Stp</i>	Ex 2	1
Sweden	<i>233G>A / Trp78Stp</i>	Ex 2	2
France (Turkey)	<i>267delG / TP</i>	Ex 2	1
Germany	<i>313delG / TP</i>	Ex 2	1
Sri Lanka	<i>377T>G / Val126Gly</i>	Ex 2	1
USA (Slovakia)	<i>401A>G / Asp134Gly</i>	Ex 3	1
Italy	<i>482+2T>C / SP</i>	Int 3	2
Norway	<i>558G>T / Lys186Asn</i>	Ex 4	2

^aReported by (Kondo et al. 2002). ^bReported by us and (Kondo et al. 2002). TP, the mutation results in a truncated protein; SP, splicing mutation. The number of patients is likely to have been underestimated because mutations in some patients with clinical diagnosis could not be verified due to bad quality DNA.

Expression of DAP12 and TREM2

In order to determine the expression pattern of *DAP12* and *TREM2* in different human tissues, we performed northern-analyses of multiple human tissues and cell lines. *AP12* was found to be expressed in all human tissues studied. This suggests a fundamental role for *DAP12* in humans. However, the strongest steady-state *DAP12* mRNA signal was observed in hematological cells and tissues such as bone marrow, spleen, and peripheral blood leukocytes. A strong *DAP12* signal was also observed in tissues that form a barrier to the environment outside the body (lung, intestines etc.) (Fig. 4). Within the hematological compartment, the expression of *TREM2* was more specific, *TREM2* signal was detected only in lymph nodes. In the CNS, the intensities of the steady-state mRNA signals of *TREM2* closely followed those of *DAP12*, suggesting a regional co-expression of these two genes (Fig. 5). In the CNS, relatively strong northern-blot signals of *DAP12* and *TREM2* could be detected in the basal ganglia (putamen and caudate nucleus), medulla, spinal cord, and corpus callosum (Figs 4-5).



whole brain	Cerebellum left	Substantia nigra	Kidney	Lung	Fetal brain
cortex	Cerebellum right	Accumb. nucleus	Skeletal muscle	Placenta	Fetal heart
Frontal lobe	Corpus callosum	Thalamus	Spleen	Bladder	Fetal kidney
Parietal lobe	Amygdala	Spinal cord	Thymus	Uterus	Fetal liver
Occipital lobe	Caudate nucleus		Periph. Leukocyte	Prostate	Fetal spleen
Temporal lobe	Hippocamp.		Lymph node	Testis	Fetal thymus
Paracentr gyrus	Medulla oblongata		Bone marrow	ovary	Fetal lung
Pons	putamen		trachea		

Figure 4. Expression of *DAP12* in human tissues. The size and intensity of each dot indicate the steady state transcript level in the corresponding tissue.

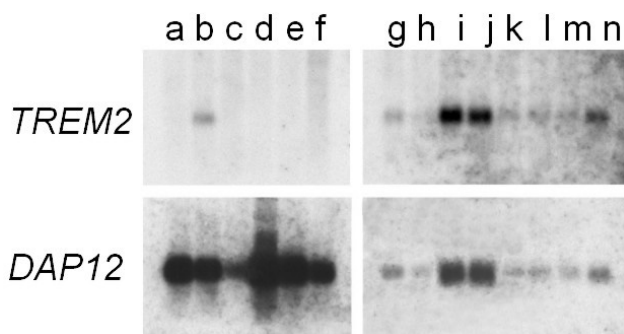


Figure 5. a. spleen, b. lymph node, c. thymus, d. leukocytes, e. bone marrow, f. fetal liver, g. cerebellum, h. cerebral cortex, i. medulla, j. spinal cord, k. occipital pole, l. frontal lobe, m. temporal lobe, n. putamen.

DAP12 and TREM2 Mutations Result in an Identical Phenotype

To see if there are differences in the clinical picture of PLOSL between patients with *DAP12* and *TREM2* mutations, we compared the disease manifestations with each other. Remarkably, we did not observe differences in the clinical symptoms or signs in these two patient groups (Table 7). Thus, PLOSL is an interesting example of how mutations in different components of a receptor signaling complex results in an identical disease phenotype. However, we studied only the clinical symptoms and we do not know if some minor differences are present at histological level. However, according to the published literature that necessarily consist of both patients with *DAP12* and *TREM2* mutations, the histopathological manifestations are also uniform.

TABLE 7. Comparison of PLOSL manifestations in patients with mutations in either *TREM2* or *DAP12*

Symptoms	Patients	
	<i>TREM2</i>	<i>DAP12</i>
<i>Bones</i> (3 rd decade)		
Skeletal pain	+	+
Bone lesions or fractures	+	+
<i>CNS</i> (4-5 th decades)		
Frontal lobe syndrome ^a	+	+
Memory disturbance	+	+
Other disturbances of higher cortical functions ^b	+	+
Convulsions	+	+
Primitive reflexes	+	+
Diffuse slowing in the EEG	+	+
Brain atrophy	+	+

+ = present; - = absent. Data on patients with *DAP12* mutations are based on reports by (Hakola 1972; Hakola and Partanen 1983; Hakola 1990b; Hakola and Puranen 1993; Paloneva et al. 2000; Paloneva et al. 2001). ^aEuphoria and loss of social inhibitions. ^bAgnostic-aphasic-apraxic symptoms.

NATURAL COURSE OF PLOSL (III)

In order to characterize the natural course of PLOSL in more detail we correlated the clinical course of the disease to neuroradiological, neurophysiological, and neuropathological progress in a series of Finnish patients.

Clinical Picture

The early development of all eight patients studied had been normal. All were employed, and except one who was engaged, all were married and had children.

The first symptoms of PLOSL appeared in early adulthood. In their early 20s, four patients had had pain and tenderness in their ankles and feet after strain or a minor accident. The first fractures occurred at the mean age of 27 years (range, 18–33 years), most commonly in the bones of the extremities. Radiographs showed trabecular loss in the distal ends of the long tubular bones and symmetric cyst-like alterations in the phalanges of fingers and toes, in the heads of metacarpals and metatarsals, as well as in the carpals (Fig. 6), and tarsals, where a fall-in fracture of the talus was typical. The fractures healed well. Remarkably, two of eight patients had not had skeletal problems although cystic bone lesions were radiologically diagnosed at the time of neurological evaluation.

A change of personality began to develop insidiously in the fourth decade at the mean age of 33 years. The patients showed progressive loss of judgment, leading to serious social problems: divorces, unemployment, financial troubles, and suicide attempts. Subsequently, the full-blown picture of the frontal lobe syndrome appeared in all patients: loss of judgment, euphoria, disinhibition, disturbance of concentration, as well as lack of insight and libido. Progressive signs of upper motor neuron involvement (spasticity, Babinski's sign) were noticed at the same age as the change of personality in all patients. With advancing disease, a lack of initiative and activity concealed the aforementioned symptoms.

Memory disturbances set in approximately at the same age as the personality change, and were best detectable by psychometric tests (Benton's Visual Retention Test, ten-word test, and WMSc). The memory disturbance was initially less severe than the personality change, and the patients were able to maintain their most important personal data (name etc.) until the last stage of the disease.

Other disturbances of higher cortical functions, such as motor aphasia, agraphia, acalculia, and apraxia, appeared only at the last stage of the disease. A very conspicuous feature was postural dyspraxia. The patients walked or sat in peculiar skewed postures, for example, with the upper part of the body tilted laterally up to 20 degrees. Finally, the patients lost their ability to walk at the mean age of 42.5 years (range, 34–47 years). At the final stage, the patients lay without any active movement in an apallic state. Primitive reflexes, such as the visual and tactile grasp and mouth opening reflexes, as well as the sucking reflex, became noticeable.

In some patients, impotence or lack of libido and urinary incontinence were among the first symptoms. Involuntary slow choreiform movements or myoclonic twitches were

common. Almost all the patients who had reached their mid-30s had epileptic seizures. Five patients had died at the mean age of 43 years (range, 37 to 48 years).

Neuroimaging

Cerebral atrophy (dilated ventricles and sulci) was found in all examinations. It was mild in three patients aged 28 to 33 years and moderate to severe in five patients aged 32 to 47 years. A slow progression was seen during follow-up examinations. Cerebellar atrophy was observed in some patients. Calcifications were found in the basal ganglia in all patients examined with CT. Most often they were situated in the putamina. The basal ganglia, the putamina in particular, showed very low signal intensities on T2-weighted MR images. This finding was probably caused by calcifications. The heads of the caudate nuclei were small on MRI, and the bicaudate ratios were abnormally high for age (Aylward et al. 1991) and increased progressively. The signal intensity of the cerebral white matter was increased on T2-weighted images, being higher than that of the cortex in some regions. The signal intensity of deep gray matter was lower than that of the surrounding white matter. The white matter changes were diffuse and, apart from the frontal lobes, had no predilection regions. They usually were centrally located, sparing most of the arcuate fibers, but in some instances also extended up to the cortex. In some patients, the white matter changes were more limited in extent and asymmetric. All post mortem MRIs showed white matter abnormalities similar to those in the living patients. One patient had no white matter changes at the examination performed before the manifestation of CNS symptoms but developed them later. However, the bicaudate ratio was abnormal even at the first examination. Only in one patient with CNS symptoms did the white matter look normal. The basal ganglia calcifications could be demonstrated by neuroimaging before the clinical manifestation of CNS symptoms suggesting early basal ganglia involvement in PLOSL.

Neurophysiology

At the early stage of the disease, the EEGs were normal. Patients with moderate to severe mental symptoms showed accentuation of the theta and delta activities. Initially, the theta was often rhythmic, 6 to 7 Hz, dominating in the centroparietal areas, but later on diffuse slowing was evident. At the late stage of the disease, there usually was irritative activity in the EEG. Paroxysmal EEG changes were associated with epilepsy.

Macroscopical Autopsy Findings

The neuropathological findings were similar in all patients who underwent autopsies. The brain weights (1170, 1170, and 910 g) were markedly below normal, and there was generalized cerebral gyral atrophy with frontal accentuation. On coronal sections, particularly the frontal deep white matter was atrophic and tough with an uneven grayish discoloration, whereas the cerebral cortex was generally of normal color and thickness. The corpus callosum was abnormally thin and grayish. The basal ganglia, especially the caudate nuclei, were variably reduced in size. All three patients showed marked hydrocephalus *e vacuo* (Fig. 6). The cut surfaces of the cerebellum, brain stem, and spinal cord were unremarkable.

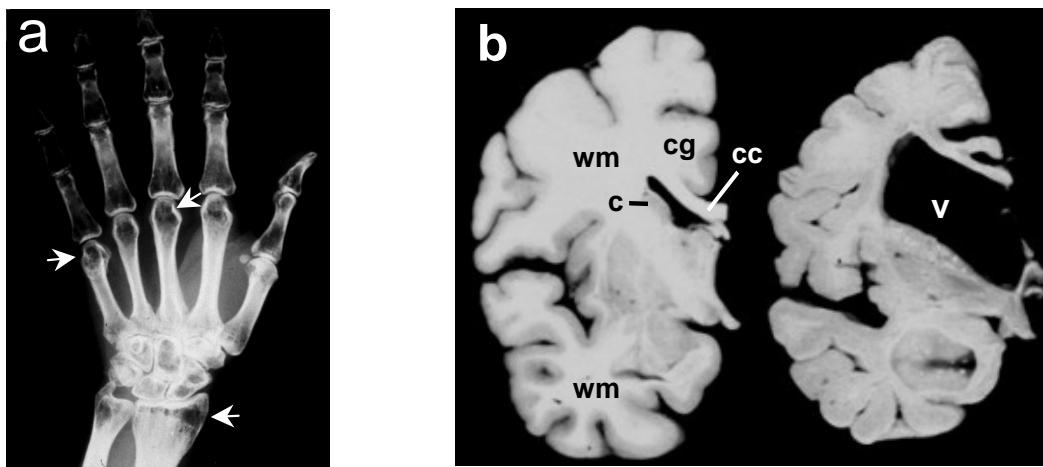


Figure 6. a) Radiograph shows typical cystic lesions (arrows) in addition to trabecular loss in the hand of a PLOSL patient. b) Coronal section of a control (left) and PLOSL (right) hemispheres. There is a profound reduction of the deep frontal and temporal white matter (wm) in the PLOSL brain. Note the severe affection of the cingulate gyrus (cg) and corpus callosum (cc). The caudate nucleus (c) is shrunken and the lateral ventricle (v) much enlarged. (Fig. 6b modified from a figure kindly provided by Dr. Haltia)

Histological and Immunohistochemical Findings

The cerebral neocortical cytoarchitecture as well as the hippocampus were generally well preserved without obvious neuronal loss. In all patients, scattered large neurons in the deeper cortical laminae showed rounded contours, marginalization of the Nissl granules, and eccentric nuclei, i.e., features of chromatolysis. Apart from a few neurofibrillary tangles and neuropil threads in the entorhinal cortex, no other intraneuronal or glial pathologic inclusions, immunoreactive for phosphorylated tau, α -synuclein, or ubiquitin, were present. There were no cortical or nigral Lewy bodies,

neuritic or diffuse plaques, or congophilic angiopathy. The chromatolytic neuronal cell bodies did not stain for phosphorylated neurofilament protein or α -B-crystallin. There was slight to moderate astrocytic proliferation and hypertrophy and neuronal loss in the caudate nuclei as well as scattered calcospherites, particularly in the putamina and globi pallidi.

There was variable neuronal loss in the thalamic nuclei and slight to moderate loss of the Purkinje cells in the cerebellar cortex. At the brain stem and spinal cord levels features of chromatolysis were observed in neurons, particularly in the pontine and cranial nerve nuclei as well as in the spinal anterior horns.

All patients showed an advanced loss of axons and myelin, accompanied by scattered axonal spheroids, some lipid-laden macrophages, and pronounced astrocytic reaction in the centrum semiovale of the frontal and temporal lobes with moderate involvement of the gyral white matter. In addition, there was widespread activation of microglia in the cerebral white matter. The parietal and occipital white matter was slightly to moderately affected. Myelin loss was found in the corpus callosum and in the cerebellar white matter. All patients showed degeneration of the lateral corticospinal tracts and medial parts of the posterior columns.

Vascular alterations were observed in all patients in the deep frontal and temporal white matter. The alterations affected scattered small arterioles and capillaries and consisted of concentric thickening of the vascular wall with narrowing or obliteration of the lumen. Immunostaining for collagen type IV, a blood vessel wall component, showed thickened and multiple basement membranes. No congophilic deposits or basophilic, PAS-positive material between vascular smooth muscle cells were detected.

OSTEOCLAST DIFFERENTIATION AND FUNCTION IN PLOSL PATIENTS (IV)

The cystic bone lesions and loss of trabecular bone in PLOSL are potentially caused by dysfunction of osteoclasts, the cells responsible for resorption and remodeling of bone (Paloneva et al. 2000). To test this hypothesis, we studied the differentiation and bone resorption capability of osteoclasts derived from peripheral blood mononuclear cells (PBMC) from four Finnish and one German PLOSL patient with loss-of-function mutation in *DAP12* or *TREM2*.

DAP12- and TREM2-Deficient Monocytes Inefficiently Differentiate into Osteoclasts

Osteoclasts are multinucleated giant cells that are formed by the fusion of mononuclear precursor cells that circulate in the monocyte fraction (Fujikawa et al. 1996; Heymann et al. 1998; Suda et al. 1999). In order to generate osteoclasts, we stimulated the PBMC of patients with homozygous mutations in *DAP12* (PLOS_{Fin}) and *TREM2* (40G>T) and healthy individuals using a cytokine combination consisting of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) known to induce functional osteoclasts from PBMC or bone marrow cells (Quinn et al. 1998; Suda et al. 1999). Stimulation with M-CSF and RANKL generated multinucleated osteoclasts with >10 nuclei in 3-7 days from control PBMC. Notably, the differentiation of *DAP12*- and *TREM2*-deficient PBMC into multinucleated osteoclasts was seriously impaired (Fig. 7). After 7 days stimulation the number of multinucleated (≥ 3 nuclei) cells generated by *DAP12*- and *TREM2*-deficient PBMC was only 10% of that of control cells ($p < 0.01$). A majority of these genetically deficient multinucleated cells had 3-4 nuclei. The percentage of osteoclasts with ≥ 5 nuclei was only 6% of the controls ($p = 0.01$). After stimulation for 14 days the number of genetically deficient multinucleated cells increased to 18% ($p < 0.01$), and the proportion of cells with ≥ 5 nuclei to 20% of that of controls

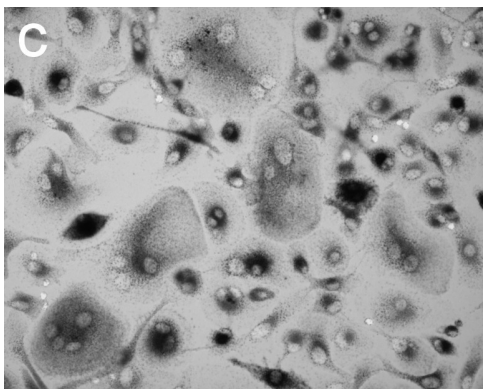
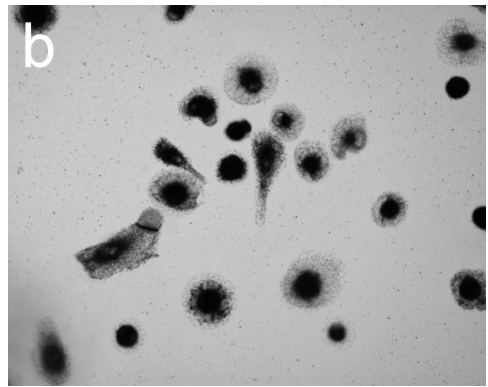
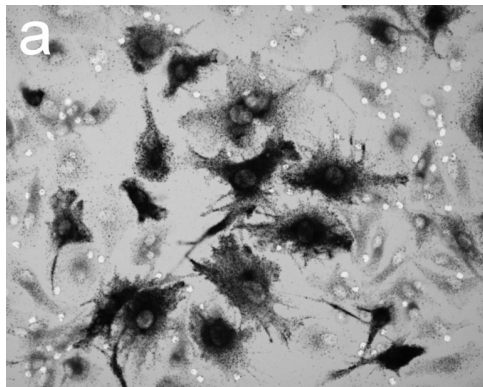


Figure 7. *DAP12*- (a) and *TREM2*- (b) deficient monocytes inefficiently differentiate into multinucleated osteoclasts in 7 days, whereas control cells (c) efficiently form multinucleated giant cells in 7 days (TRAP staining). Note the numerous processes in *DAP12*-deficient cells.

($p < 0.05$). The fusion process of DAP12- and TREM2-deficient cells appeared to be comparable to each other. The genetically deficient osteoclastic cells were positive for osteoclastic markers tartrate-resistant acid phosphatase (TRAP) and cathepsin K. The calculated cell density of cultured DAP12- and TREM2-deficient PBMC was similar to the controls at all time points, indicating that the genetically deficient cells were able to proliferate in a similar manner as the controls. Thus, the DAP12/TREM2 complex mediates the differentiation of osteoclasts. Further, in the absence of DAP12/TREM2 signaling, the fusion is very inefficient, but proceeds in the course of time.

Aberrant Morphology of DAP12- and TREM2-Deficient Osteoclasts

In order to characterize the morphology of the induced osteoclastic cells in more detail we stained the cells for cytoskeletal actin. An actin ring is a functional characteristic of resorbing osteoclasts and delineates the bone resorption area between an osteoclast and bone. The actin ring consists primarily of F-actin filaments (Vaananen and Horton 1995; Salo et al. 1997; Teitelbaum 2000). After 7 days the typical single large actin ring following the contours of the cell margin was seen in control osteoclasts, whereas even after stimulation for 14 days the multinucleated DAP12-deficient osteoclastic cells contained several small unorganized granular actin clusters. The TREM2-deficient osteoclastic cells generated only one granular ring-like actin cluster after 14 days. The transmembrane adaptor molecule DAP12 forms a complex with several different activating receptors depending on the cell type (Lanier and Bakker 2000). In DAP12-deficient cells, all DAP12-associated cell surface receptors are likely to be inactive. The potential costimulatory function of other DAP12-associated cell surface receptors in TREM2-deficient cells could explain the slightly different morphology of actin rings in DAP12- and TREM2-deficient osteoclastic cells.

Quantitative RT-PCR Analysis

In order to study the molecular basis for resorption capability and osteoclastic character, we analyzed the expression of cathepsin K, receptor activator of nuclear factor- κ B (*RANK*) (Nakagawa et al. 1998), *TRAP*, and calcitonin receptor (*CALCR*) (Zaidi et al. 2002) in cells stimulated for 1, 3, 7, and 21 days using quantitative RT-PCR. *Cathepsin K* transcripts became strongly upregulated after stimulation for 3 days ($p < 0.001$). No difference between DAP12/TREM2-deficient and control cells was observed. The transcript level of the gene encoding RANK, the receptor for RANKL, became upregulated in both genetically deficient and control cells after stimulation for 1 day. There was no significant difference between the patient and control cells, although the *RANK* transcript level in genetically deficient cells appeared higher compared to the

controls. *TRAP* transcript level was initially low but became upregulated after stimulation for 3-7 days. No differences between the patients and controls were observed. Both DAP12- and TREM2-deficient cells expressed *CALCR*, the receptor regulating the resorption process of osteoclasts. Reliable quantitation could not be performed due to a low transcript level but no obvious differences were observed between the genetically deficient and control cells. Taken together, the delayed differentiation and impaired resorption function of genetically deficient osteoclasts is not caused by a loss of *RANK*, *CALCR*, or *TRAP* expression. There were no significant differences between DAP12/TREM2-deficient and control cells, suggesting that the steady state expression levels are not dependent on DAP12 and TREM2.

The transcript level of *DAP12* and *TREM2* in normal cells followed each other and increased progressively during the stimulation for up to 21 days. A 5-fold increase in *DAP12* ($p < 0.01$) and a 39-fold increase in *TREM2* transcript level ($p < 0.001$) was observed between stimulations for 1 and 21 days. TREM2 protein has previously been shown to be undetectable in macrophages derived by stimulation of monocytes with M-CSF alone (Bouchon et al. 2001b). Therefore, osteoclasts are likely to express *DAP12* and *TREM2*, since the expression of *DAP12* and *TREM2* in PBMC differentiating along the osteoclastic lineage became progressively upregulated parallel with the increasing number of multinucleated osteoclasts. The transcript level of these genes did not show signs of downregulation as would have been expected if DAP12 and TREM2 were involved only in the fusion process of prefusion osteoclasts.

DAP12-Deficient Osteoclasts Resorb Bone

In order to assess the bone resorption capability of DAP12-deficient multinucleated cells, we performed an *in vitro* bone resorption assay. We first induced DAP12-deficient and control PBMC with M-CSF and RANKL for 7 days and then transferred the cells on dentin slices for 7 days. Multinucleated DAP12-deficient cells stimulated for 7 days before transferring on dentine slices were capable of bone resorption. Although the same number of DAP12-deficient and control cells was transferred per dentin slice, the total surface area resorbed by DAP12-deficient osteoclasts was dramatically reduced, being only 0.03% (a 3500-fold difference) of that of control osteoclasts ($p < 0.01$). Similarly, a striking 1000-fold difference in the average resorbed surface area per multinucleated osteoclast between DAP12-deficient and control osteoclasts was observed ($36 \mu\text{m}^2$ and $35700 \mu\text{m}^2$, respectively, $p < 0.05$). Remarkably, the surface area per resorption pit generated by DAP12-deficient and control osteoclasts was the same. However, the resorption pits generated by DAP12-deficient osteoclasts were 25% deeper compared to the controls ($25 \mu\text{m}$ and $20 \mu\text{m}$, respectively, $p < 0.0001$) (Fig. 8).

In order to address the bone degradation rate *in vivo* we measured the concentration of type I collagen carboxy-terminal crosslinks (CTX), in serum of DAP12- and TREM2-deficient patients. CTX assay is considered as a specific method to determine the bone degradation rate *in vivo* (Rosen et al. 2000). The average CTX concentration in the serum of the patients (mean age 36 years) was 40% higher than that of healthy individuals (mean age 36 years) ($p < 0.05$). Taken together, the capability of bone resorption definitively confirms that DAP12-deficient

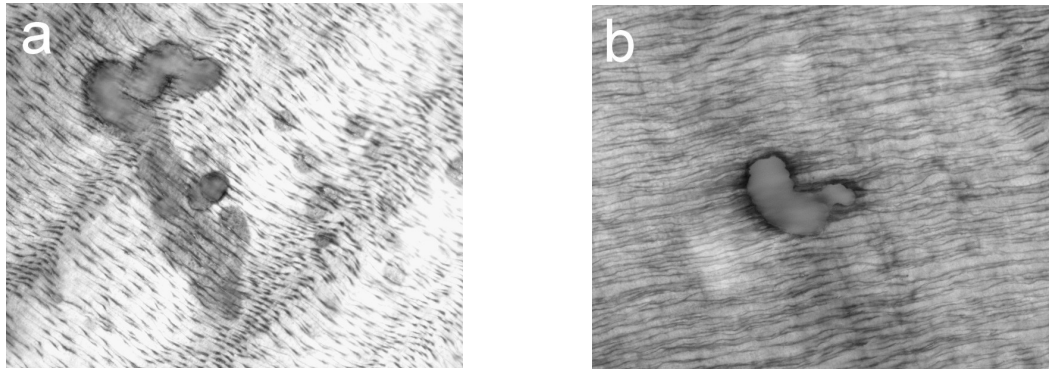


Figure 8. DAP12-deficient osteoclasts are capable of bone resorption. (a) Control osteoclasts generated long continuous resorption pits. (b) The average surface area per resorption pit generated by DAP12-deficient osteoclasts is similar to the controls. Note the sharp-looking edges of the resorption pit generated by genetically deficient osteoclast indicating that the pit is deeper compared to controls (toluidine blue staining).

multinucleated cells are osteoclasts. Furthermore, the resorption capability of DAP12-deficient osteoclasts is seriously impaired. This is potentially explained by the impaired actin ring in the DAP12-deficient osteoclasts since the actin ring is known to be a prerequisite for efficient bone resorption (Salo et al. 1997). On the other hand, increased CTX concentration suggests that the cathepsin K mediated bone resorption rate, and thereby the osteoclastic activity, is abnormally high in the patients. This discrepancy between *in vitro* and *in vivo* studies could be explained by the possibility that in the course of time the genetically deficient osteoclasts finally differentiate into efficiently resorbing osteoclasts. However, the striking difference in the resorbed surface area per cell between DAP12-deficient and control osteoclasts may be artefactual to some extent since the efficient resorption by control osteoclasts causes a significant increase in extracellular calcium concentration that has been shown to induce apoptosis in osteoclasts (Lorget et al. 2000). Therefore, the resorbed area per cell cannot be accurately determined.

DAP12- and TREM2-Deficient PBMC Migrate Efficiently

In order to determine if the differentiation of DAP12- and TREM2-deficient osteoclast precursor cells is delayed due to reduced motility we performed an *in vitro* motility assay. We cultured DAP12-, TREM2-deficient, or control PBMC on coverslips with a cylindrical silicone object firmly attached to the center of each coverslip before plating. The object was removed after 24h stimulation. After 7 days, the genetically deficient cells effectively migrated to the center of the cover slip, and multinucleated osteoclasts were found at the center. A notable difference was observed in the control cell density between the center and surrounding areas ($p < 0.01$), whereas there was no significant difference in the density of genetically deficient cells between these areas ($p > 0.05$). This indicates that DAP12- and TREM2-deficient PBMC migrate efficiently and that the delayed fusion of prefusion osteoclasts is not caused by impaired motility.

Osteoclasts in PLOSL Patients Are Multinucleated and Resorptive

In order to see whether the bone cells of PLOSL patients show features similar to osteoclastic cells *in vitro*, we analyzed bone samples from two patients with homozygous deletion of exons 1-4 of *DAP12* for osteoclast morphology and bone resorption capability. As expected, the osteoclasts were multinucleated, and their size appeared to be normal. The osteoclasts in the patients generated extensive Howship's lacunae (resorption pits) with normal ruffled border membranes at light microscope level (Fig. 9). Unfortunately, bone samples from patients with *TREM2* mutations were not available. However, it is likely that the osteoclasts in patients with *TREM2* mutations show features similar to *DAP12*-deficient patients because the clinical phenotype of patients with *DAP12* and *TREM2* mutations is identical (III).

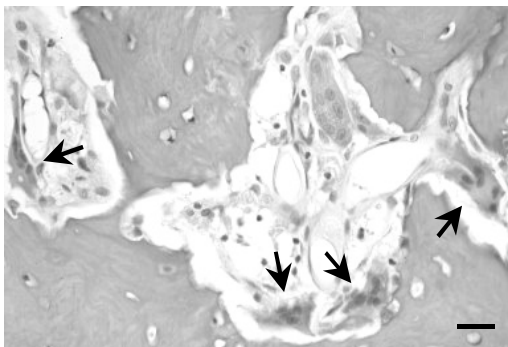


Figure 9. Multinucleated bone-resorbing osteoclasts (arrows) in talus of a PLOSL patient. The scale bar corresponds to 25 μm (H&E).

ANALYSIS OF DAP12 KNOCKOUT MOUSE (UNPUBLISHED)

In order to address the significance of Dap12 in mouse, we performed a detailed histological and functional long-term follow-up analysis of the central nervous and skeletal systems of mice carrying targeted loss-of-function mutation in *Dap12* in both alleles. In this *Dap12* knockout mouse strain (*Dap12*^{Δex3-4}), produced by Lanier and his colleagues, exons 3 and 4 of the 5 exons of *Dap12* have been deleted (Bakker et al. 2000). Exons 3 and 4 code for the transmembrane and part of the cytoplasmic domain as well as the first tyrosine in the intracellular tyrosine-based activation motif (ITAM). Therefore, *Dap12*^{Δex3-4} mice correspond to the loss-of-function genotype of *Dap12* we have observed in PLOSL patients (I). We analyzed brain and bone samples taken at several ages ranging from 6 to 19 months. The brains were analyzed using histological and immunohistochemical methods in order to study the tissue pathology, and magnetic resonance imaging (MRI) techniques to further evaluate the CNS manifestations of adult *Dap12* knockout mice. Behavioral tests were performed to assess the CNS affection of mice aged 1-11 months. In order to evaluate the bones, histological and radiological analyses were performed. Surprisingly, we did not observe any signs of brain atrophy, loss of myelin, astrocytic gliosis, neuronal loss or activation of microglia in the brain of *Dap12* knockout mice. Similarly, the bones did not show signs of cyst-like bone lesions or obvious changes in bone mass. Further, there were no significant differences between homozygous and heterozygous control mice in prepulse inhibition test that measures sensorimotor gating mechanisms. However, *Dap12* knockout mice show impaired immune responses, as previously reported (Bakker et al. 2000) (see pp. 62-63).

GENERAL DISCUSSION

THE POWER OF DIFFERENT APPROACHES FOR FINDING DISEASE GENES

In this thesis, two approaches for finding disease genes were utilized. The first PLOSL gene, *DAP12*, was identified by using the positional cloning approach. This was the only useful gene identification method since only little was known about the defective biochemical pathways in PLOSL. The order in which the candidate genes were analyzed was determined according to the known or hypothesized function in addition to the expression pattern of the genes in the candidate region. This proved to be quite inefficient since *DAP12* was the last gene in the critical region with the lowest priority of all candidates.

The candidate gene approach was successfully utilized in the identification of the second PLOSL gene, *TREM2*. Systematical sequencing of all genes coding for *DAP12*-associated proteins would have been very time-consuming and laborious. Furthermore, an advantage of this approach was that only a small amount of DNA was needed because very little DNA was left from some individuals that were essential for the analyses. The segregation analysis of marker haplotypes of the candidate genes was performed in two informative unrelated families with a total of 4 and 7 individuals and by analyzing two or three markers per candidate locus. A complete co-segregation of marker haplotypes in the *TREM2* locus spanned a large, approximately 10 cM, genomic region. Identification of the second PLOSL gene is a good example of how the candidate gene approach can powerfully be exploited to find disease genes when enough is known about the pathogenic mechanisms of the disease.

STRUCTURE AND FUNCTION OF *DAP12/TREM2* SIGNALING COMPLEX

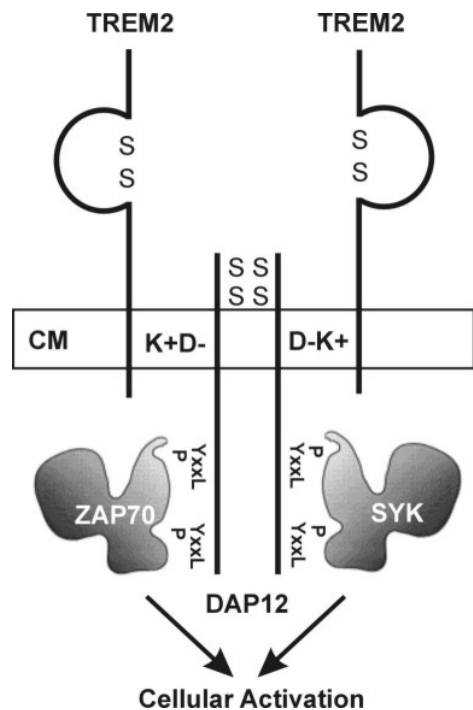
The 230-amino-acid (aa) (~40 kD) *TREM2* polypeptide belongs to the immunoglobulin superfamily (Ig-SF). It is predicted to consist of a 13-aa signal peptide followed by a 154-aa extracellular domain encoded by exons 2 and 3, with two cysteines potentially involved in generating an intrachain disulfide bridge of the Ig-SF V-type fold. The 33-aa transmembrane domain is followed by a short, 30-aa cytoplasmic domain (Bouchon et al. 2000).

DAP12 is a 113 aa (12 kD) protein that consists of a 27 aa leader peptide, a short 14 aa extracellular domain followed by a 24 aa transmembrane segment and a 48 aa cytoplasmic domain. The leader peptide and extracellular domains are encoded by exons 1-2. Exons 3 and 4 code for the transmembrane as well as part of the cytoplasmic domains. On the cell surface *DAP12* is expressed as a disulphide-linked

homodimer mediated by two cysteine residues in the extracellular domain. The transmembrane domain of DAP12 contains a negatively charged residue that mediates the interaction with a wide variety of associated receptors, TREM2 being one of these. This interaction is mediated by oppositely charged amino-acids in the transmembrane domains of these proteins; one of these amino-acids is a positively charged lysine in TREM2, and the other is a negatively charged aspartic acid in DAP12 (Campbell and Colonna 1999; Bouchon et al. 2001b).

The interaction between TREM2 and the so far unidentified ligand results in the phosphorylation of tyrosine residues in the intracellular tyrosine-based activation motif (ITAM) of DAP12. Phosphorylated DAP12 transmits activating signals by providing a docking site for the SH2 domains of the cytoplasmic protein tyrosine kinases (PTKs), preferentially to SYK but also to some extent to ZAP70. This interaction leads to an increase in intracellular Ca^{2+} concentration, and subsequent cellular activation (McVicar et al. 1998; Lanier and Bakker 2000) (Fig. 10).

Figure 10. The DAP12/TREM2 signaling complex. On the cell membrane (CM) DAP12 is expressed as a homodimer linked by cysteines in the extracellular domain of DAP12. DAP12 dimer is linked to a dimer of TREM2 polypeptides. This interaction is mediated by oppositely charged amino acids in the transmembrane domains of DAP12 (D) and TREM2 (K). The interaction of TREM2 and an unidentified ligand results in the phosphorylation of the intracellular tyrosine-based activation domain (ITAM, consensus sequence YxxL/Ix₆₋₈YxxL/I, where x denotes any amino acid) of DAP12. Phosphorylated ITAM recruits the cytoplasmic protein tyrosine kinases ZAP70 and SYK. This interaction leads to an increase in the intracellular Ca^{2+} concentration and subsequent cellular activation. The stoichiometry between DAP12 and TREM2 polypeptides has not been established and is only hypothetical. Modified from (Campbell and Colonna 1999)



MUTATIONS IN GENES OF THE DAP12 SIGNALING PATHWAY

We have identified mutations in all 42 patients with PLOSL who were available to us; 35 (75%) carry a mutation in *DAP12*, and 12 (25%) in *TREM2*. To date, six different mutations have been reported in *DAP12* (I, II, unpublished, Kondo et al. 2002), whereas 10 different mutations have been found in *TREM2* (II, IV). The molecular genetic background of PLOSL appears to be explained by these two genes.

The relatively high number of PLOSL patients in Finland explains the higher proportion of patients carrying mutations in *DAP12* than in *TREM2* because all of our 25 Finnish patients are homozygous for the same 5.3 kb deletion in *DAP12* (PLOSL_{Fin}) (I). Mutations in *DAP12* are typically found in Scandinavia and Japan, whereas *TREM2* is mutated in other countries more frequently than *DAP12*. It thus seems that *TREM2* is more susceptible to mutations because the number of different *TREM2* mutations is higher.

There appears to be two major mutations in PLOSL: PLOSL_{Fin} is found in all Finnish patients and 141delG (PLOSL_{Jpn}) in most Japanese patients (I) (Kondo et al. 2002). All other mutations in *DAP12* and *TREM2* are sporadic and found in single families only, except for the Swedish Trp78Stp mutation which has been found in two families (II). All patients carry homozygous mutations, compound heterozygotes have not been found.

The 5.3kb (PLOSL_{Fin}) and ~8kb (PLOSL_{Bra}) gross deletions in *DAP12* encompass exons 1-4 of the five exons of *DAP12* and result in a total loss of *DAP12* expression and function. A Met1Thr missense mutation in a Japanese patient (Kondo et al. 2002) disrupts the translation initiation methionine and therefore the whole polypeptide since no other putative translation initiator codons are present in the vicinity of this amino-acid. Three of the identified *DAP12* mutations disrupt the transmembrane domain (Gly49Arg, 141delG, 154ins42nt). The single-nucleotide deletion (141delG) found in a number of Japanese patients creates a frameshift predicting premature termination of translation after 52 amino-acids. The mutation also changes the aspartic acid residue (D) in the transmembrane domain, which is essential in mediating the interaction between the *DAP12* and the associated ligand-binding receptors. The missense mutation (145G>C) in the Portuguese patient results in conversion of Glycine 49 to Arginine one amino-acid before the functionally critical aspartic acid (Asp50), and insertion of 42 bp after nucleotide 145 in the immediate vicinity of Asp50 potentially disrupt the conformation of the transmembrane domain, and subsequently the association of *DAP12* with receptors (Fig. 11).

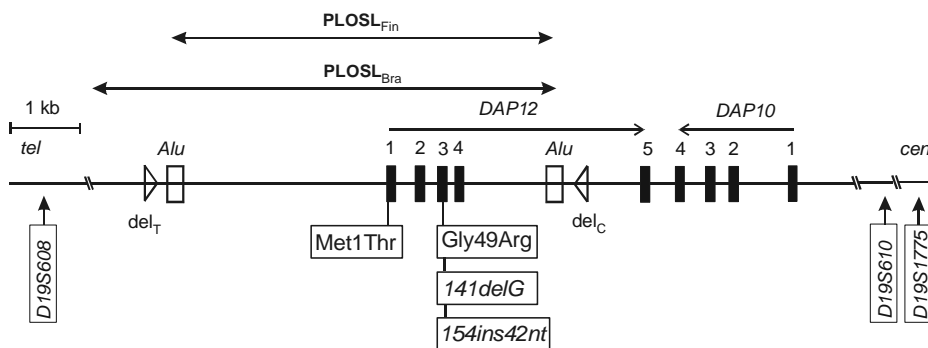


Figure 11. Identified PLOSL mutations in *DAP12*

Nine of ten of the identified mutations in *TREM2* have occurred in the extracellular ligand-binding domain (Fig. 12). The four nonsense mutations (*40G>T* /Glu14Stp, *C97>T* /Gln33Stp, *132G>A* /Trp44Stp, and *233G>A* /Trp78Stp) result in a truncated protein with no transmembrane domain. Two frameshift mutations due to single-nucleotide deletion in the extracellular domain of *TREM2* (*267delG* and *313delG*) both result in a premature translation termination codon at position 188 in the amino-acid chain. The exact mechanism by which the two missense mutations *377T>G* /Val126Gly and *401A>G* /Asp134Gly result in an impaired function of *TREM2* cannot be predicted. The conversion of nucleotide T to C at the second position of splice-donor consensus site in intron 3 (*482+2T>C*) is likely to result in skipping of exon 3 from the mature mRNA. The only mutation in the transmembrane domain of *TREM2* (*558G>A* /Lys186Asn) disrupts the functionally critical positively charged amino-adic Lysine that is essential for the association of *TREM2* with *DAP12*.

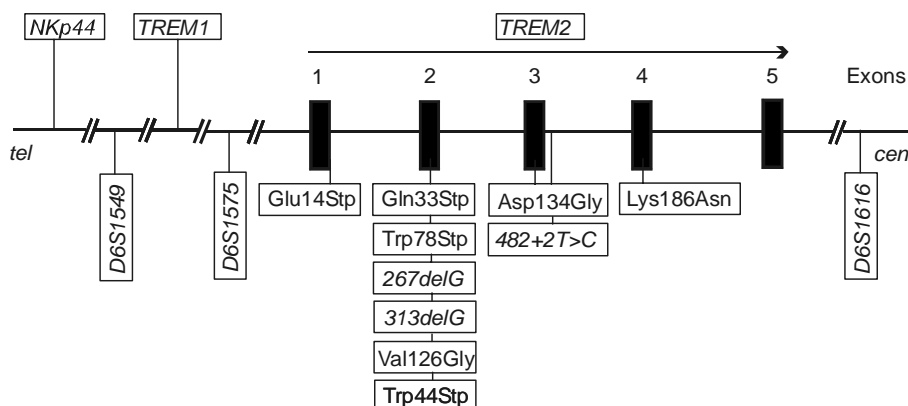


Figure 12. Identified PLOSL mutations in *TREM2*.

Mutations in genes coding for DAP12 –associated cell surface receptors other than TREM2 have been reported only in NKG2C (Hikami et al. 2003). A large deletion encompassing the whole NKG2C gene is found in ~5% of the Japanese population. The deletion does not appear to cause any symptoms. Mutations in *ZAP70* encoding the protein tyrosine kinase (PTK) of the downstream signal transduction pathway result in severe combined immunodeficiency (SCID) syndrome in humans. The function of T cells in SCID patients is severely impaired; the patients lack peripheral CD8+ T cells and their CD4+ T cells are dysfunctional due to defective T cell receptor (TCR) signaling (Arpaia et al. 1994; Chan et al. 1994; Elder et al. 1994). Germline mutations in *SYK*, the second DAP12-associated PTK, have not been described, but somatic *SYK* deficiency has been associated with childhood pro-B cell acute lymphoblastic leukemia (Goodman et al. 2001).

ITAM DOMAIN IN HEMATOPOIETIC CELL SIGNALING

DAP12 belongs to a family of intracellular tyrosine-based activation motif (ITAM) - bearing polypeptides. *SYK* and *ZAP70* play a crucial role in the signal transduction of hematopoietic cells (Chu et al. 1998). The cytoplasmic domain of the cell surface receptors utilizing *SYK* and *ZAP70* do not usually contain the ITAM required for binding these PTKs. Therefore, these receptors use transmembrane adaptor molecules as intermediates (Lanier and Bakker 2000). All these adaptor molecules have small extracellular regions that are not capable of interacting with ligands. The adaptors typically have cysteine residues in the extracellular domains and are expressed on the cell surface as disulfide-bonded dimers (Campbell and Colonna 1999). The ITAM-bearing transmembrane adaptor family is divided into four categories: 1) DAP12; 2) the CD3 γ , δ , ϵ and ζ subunits of the T-cell receptor (TCR) complex; 3) the α and β subunits (CD79) associated with surface immunoglobulin (Ig) in the B-cell receptor complex; and 4) the γ subunit associated with the high affinity IgE receptor (Fc ϵ R1 γ), the low affinity IgG receptor (CD16) and the TCR complexes (Lanier and Bakker 2000). Most of the ITAM-bearing molecules contain multiple copies of ITAM (Love and Shores 2000) whereas DAP12 contain a single ITAM domain. The prototypic ITAM sequence is YxxL/Ix₆₋₈YxxL/I, where x denotes any amino-acid (Reth 1989).

DAP12 -MEDIATED SIGNAL TRANSDUCTION COMPLEX

DAP12 transmits activating signals from a wide variety of receptors depending on the cell type. It is apparent that only very little is known about the biological role of DAP12 - mediated signaling. New receptors that utilize DAP12 are being continuously reported.

These receptors which signal via DAP12 show some common characteristics. First, all DAP12-associated receptors transmit activating signals. Second, the cytoplasmic domain of all these receptors lack structures required for downstream signaling. Third, all receptors contain a positively charged amino acid residue in the transmembrane domain, usually lysine (K+), or, in some cases, arginine (R+) that is required for the association with DAP12. Several of these receptors belong to a larger family including both activating and inhibitory receptors. The inhibitory forms typically carry an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the transmembrane domain. Despite the characterization of the expression pattern of the receptors, the biological significance of most of these molecules has not been determined.

The receptors can be roughly categorized into two groups according to their expression pattern: TREM1, TREM2, TREM3, MDL1 and SIRPB1 are mainly expressed in myeloid cells, and KIR2DS2, NKp44, CD94, NKG2C and members of the Ly49 receptor family in NK cells. However, transcripts corresponding to some myeloid receptors have also been detected in neutrophils or some B- and T- cell lineages (Bouchon et al. 2001a; Chung et al. 2002). Most of these DAP12-associated receptors are closely homologous to each other in human and mouse. In the next section, the known structure and function of these genes and proteins are discussed. Most of the commonly used names of the genes and proteins of the DAP12 signaling pathway are aliases and not the official names. The approved names (in parenthesis) and loci of the genes have been determined by using the Ensembl, GeneCards, Genome Database and Mouse Genome Informatics web pages.

TREM1

Triggering receptors expressed on myeloid cells (TREM) are located on chr. 6p21.1-21.2. Like the other DAP12-associated receptors in this locus (TREM2 and NKp44), TREM1 carries a single V-type Ig-like extracellular domain. The gene coding for *TREM1* consists of five exons that code for a 234 aa polypeptide. Gingras and colleagues have reported a splice variant of *TREM1* that lacks the transmembrane domain. This potentially represents a soluble variant of *TREM1* (Gingras et al. 2002). TREM1 is expressed on neutrophils and monocytes and mediates the activation of these cells. TREM1 becomes strongly upregulated in neutrophils as a response to infections by bacteria such as *Pseudomonas aeruginosa* and *Stafylococcus aureus*. TREM1 amplifies inflammation and has been reported to mediate septic shock in patients with bacterial sepsis. Interestingly, blockade of TREM1 has been shown to protect mice against septic shock. Therefore, TREM1 is a potential therapeutic target for septic shock (Bouchon et al. 2000; Bouchon et al. 2001a).

TREM2

The *TREM2* gene, located on chr. 6p21.1, consists of five exons that encode 690 bp cDNA. *TREM2* displays a single V-type Ig-like extracellular domain and belongs to the immunoglobulin superfamily (Bouchon et al. 2000). The structure of *TREM2* has been discussed elsewhere in this study (pp. 52-53). Despite their common names, *TREM1* and *TREM2* are only weakly homologous to each other. Two different *Trem2* (*Trem2a* and *Trem2b*) genes have been reported in mouse. These isoforms differ from each other only by three amino acids (six base pairs) scattered throughout the polypeptides, and are therefore not alternatively spliced variants (Daws et al. 2001; Chung et al. 2002). Chung and colleagues have suggested the presence of two *Trem2* genes in C57BL/6 and BALB/c mice and one in 129, AJ and DBA mice (Chung et al. 2002). However, aligning of mouse *Trem2b* cDNA against the available NCBI mouse genome sequence database covering over 96% of the genomic sequence of the C57BL/6 strain, in addition to sequences of the 129S1/SvImJ, 129X1/SvL, DBA2/J and A/J2 mouse strains (Celera web pages) using BLASTN algorithm revealed only a single locus in mouse chr. 17c, syntenic to human *TREM2* in chr 6p21, and no evidence of the presence of two *Trem2* loci in C57BL/6 or other mouse strains. Therefore, different variants most likely represent differences between mouse strains or mutations in the cDNA libraries.

Schmid and colleagues have reported a splice variant of mouse *Trem2b* in C57BL/6 mouse strain. The alternative splice site in intron 3 is located 55 nucleotides upstream of the 5' boundary of exon 4 and causes a frameshift. The alternatively spliced *Trem2* is 22 aa longer than the main product. The *Trem2* variant lacks the transmembrane domain required for the association with *Dap12*, and is therefore predicted to represent a soluble form of the protein (Schmid et al. 2002). Splice variants of human *TREM2* have not been reported.

Trem3

Triggering receptor expressed on myeloid cells 3 has been reported only in mouse (mouse chr. 17). *Trem3* is expressed mainly in the monocyte/macrophage lineage, but has also been detected in T cell lines at a low level (Chung et al. 2002).

MDL1

MDL1 (CLECSF5, C-type [calcium dependent, carbohydrate-recognition domain] lectin, superfamily member 5; human chr. 7q33, mouse chr. 6) is found in both the human and the mouse. *MDL1* mediates the activation of monocytes and macrophages and is associated with myeloid differentiation (Bakker et al. 1999).

SIRPB1

SIRPB1 (chr. 20p13) has been reported to be expressed in human monocytes and dendritic cells (Dietrich et al. 2000; Tomasello et al. 2000a). SIRPB1 belongs to a family of SIRP molecules, and shows a high homology to an ITIM-containing inhibitory receptor SIRP α (PTPNS1, protein tyrosine phosphatase, non-receptor type substrate 1) (Cant and Ullrich 2001).

KIR2DS2

KIR2DS2 (killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2; chr. 19q13.4) belongs to a multi-member family of killer cell immunoglobulin-like receptors. This family contains both activating as well as ITIM-bearing inhibitory receptors. KIR2DS2 is the only member in this family that associates with DAP12. KIR2DS2 triggers natural cytotoxicity in NK cells (Lanier et al. 1998b).

NKp44

NKp44 (NCR2, LY95, Natural cytotoxicity triggering receptor 2; chr 6p21.1) is specifically expressed on all resting and activated NK cells. NKp44 is located in the same locus as the TREM molecules and carries a single V-type Ig-like extracellular domain similarly to TREM1 and TREM2. NKp44 triggers cytotoxicity by NK cells (Vitale et al. 1998; Bouchon et al. 2000).

CD94 and NKG2C

CD94 (KLRD1, killer cell lectin-like receptor subfamily D, member 1) and NKG2C (KLRC2, killer cell lectin-like receptor subfamily C, member 2), both located in human chr. 12p13.2-p12.3 and mouse chr. 6, are expressed as a disulfide-bonded heterodimer. This receptor complex participates in the activation of NK cells (Lanier et al. 1998a). A total of 4.3% of the Japanese population has been reported to carry a homozygous deletion encompassing the whole NKG2C gene without any obvious clinical manifestations (Hikami et al. 2003).

Ly49 Receptor Family

This major receptor family, found only in the mouse, includes several highly homologous members, Ly49A through V. Most members in this family contain an ITIM domain and are thus inhibitory receptors. Activating members of this family that are known to associate with Dap12 are Ly49D (Klra4, killer cell lectin-like receptor, subfamily A, member 4), Ly49H (Klra8, killer cell lectin-like receptor, subfamily A, member 8), and Ly49P located on mouse chr. 6. The interaction between Ly49 and Dap12 is mediated by the positively charged arginine (R) residue in the transmembrane domain (Smith et al. 1998; Makrigiannis et al. 1999; Silver et al. 2000).

SIGNIFICANCE OF DAP12 AND TREM2

An interesting finding is that the clinical phenotype of patients with either *DAP12* or *TREM2* mutations is identical. *DAP12* transmits activating signals from several cell surface receptors, and loss-of-function mutations in *DAP12* potentially disrupt the signaling of all these receptors. Therefore, one could hypothesize that the disease phenotype in PLOSL patients carrying *DAP12* mutations would be different and more severe than that in patients carrying mutations in only one of these receptors, namely *TREM2*. Furthermore, PLOSL patients homozygous for mutations in *DAP12* or *TREM2* do not show any primarily immunological symptoms. These observations suggest either significant functional redundancy or the presence of additional cell surface molecules capable of replacing the inactive *DAP12*-*TREM2* complex in cells of innate immunity.

Our findings also provide direct evidence that *DAP12* and *TREM2* play a key role in the normal functions of cells not directly involved in immune responses. It seems that the CNS and skeletal system are more rigidly dependent on the normal function of *DAP12* and *TREM2* than the function of cells of the innate immune system. Determining the role of *DAP12*/*TREM2* signaling in other diseases of the CNS and bone, such as more common forms of dementia or osteoporosis, requires further studies

Mutations in *DAP12* and *TREM2* result in clinically identical disease phenotype. We are aware of one earlier example of an inherited human disease resulting from defects in different components of the same signaling pathway. Autosomally dominantly inherited holoprosencephaly (HPE), in which the developing forebrain fails to divide into two separate hemispheres and ventricles, is the most commonly occurring congenital structural forebrain anomaly in humans. This nonsyndromic holoprosencephaly is caused by mutations in genes encoding the signaling molecule, SHH, and its receptor, PTCH, in the sonic hedgehog signaling pathway. In contrast to PLOSL, the phenotype of holoprosencephaly is extremely variable, ranging from alobar HPE (a single ventricle and no separation of the cerebral hemispheres) to clinically normal with a minor anatomic defect even within the same family (Ming et al. 2002; Genereviews web site).

DAP12 KNOCKOUT MICE SHOW CONTRADICTIONARY MANIFESTATIONS

The *Dap12* knockout mouse strain we analyzed carries a deletion of exons 3-4 in both alleles (*Dap12*^{Δex3-4}). This mouse strain has been produced by Lewis Lanier and his colleagues (Bakker et al. 2000). Although the *Dap12*^{Δex3-4} mice correspond to the loss-of-function genotype of PLOSL patients homozygous for *DAP12* mutations, the mice do

not develop symptoms or signs similar to the disease phenotype observed in the CNS and bone of PLOSL patients. This is a commonly observed situation since several different mouse strains with targeted mutations in germ line develop no symptoms at all, or show totally different manifestations when compared to human patients.

Kaifu and his colleagues have recently reported the CNS and bone manifestations in their *Dap12* knockout mouse strain carrying deletion of the putative promoter and exons 1-3 of *Dap12* in both alleles (*Dap12*^{Δex1-3}) (Kaifu et al. 2003). This mouse model presents with a thalamus-accentuated reduction of myelin as early as 1.5 months of age, whereas the *Dap12*^{Δex3-4} mice we analyzed do not show any signs of myelin loss even when aged 19 months. Furthermore, their *Dap12*^{Δex1-3} mutant mice show behavioral changes (abnormal sensorimotor gating) not seen in *Dap12*^{Δex3-4} mice. Interestingly, the *Dap12*^{Δex1-3} mice produced by Kaifu and his colleagues present with a mild osteopetrosis (pathological increase of bone mass), whereas the human PLOSL patients show osteoporotic features, the opposite of osteopetrosis.

There are several potential explanations for the contradictory CNS manifestations between *Dap12*^{Δex3-4} and *Dap12*^{Δex1-3}. One could be the different targeted mutations; deletion of the transmembrane domain and part of the cytoplasmic domain including the first tyrosine of the ITAM domain in *Dap12*^{Δex3-4} mice, and deletion of the putative promoter as well as the extracellular and transmembrane domains in *Dap12*^{Δex1-3} mouse model. However, this seems unlikely because several studies have shown that the transmembrane domain as well as the complete ITAM domain are required for the complex formation of Dap12 with the associated receptors as well as for cell-surface transportation of the Dap12/receptor complex (Lanier et al. 1998a; McVicar et al. 1998; Smith et al. 1998; Bakker et al. 1999; Love and Shores 2000). Thus, the targeted mutations in both mouse strains are predicted to result in a total loss of Dap12 signaling. Along this line, no Dap12 polypeptide has been detected in tissues of either of these mouse strains (Bakker et al. 2000; Kaifu et al. 2003). Further, the targeted deletions in these two mouse strains are not known to affect the function of genes other than *Dap12*.

An alternative explanation for differences in the phenotype could be that the *Dap12*^{Δex3-4} and *Dap12*^{Δex1-3} mouse strains represent outcomes of different knockout strategies: Cre/lox strategy in *Dap12*^{Δex3-4} mice (Bakker et al. 2000) and the conventional *neo* cassette disruption of the *Dap12* genes in *Dap12*^{Δex1-3} mice (Kaifu et al. 2003).

The third explanation for the different manifestations in these two mouse strains could be the potential divergence in Dap12 signaling in different mouse strains. The genetic background of both mouse strains is heterogeneous. The *Dap12*^{Δex3-4} mice are hybrids of 129 Ola and C57BL/6 strains, whereas the *Dap12*^{Δex1-3} mouse strain is a hybrid of

129/SvJ and C57BL/6 strains. Furthermore, McVicar and his colleagues have demonstrated a signaling deficit within the NK cells in 129/J and 129Sv mouse strains downstream to Dap12 (McVicar et al. 2002). It is unknown whether this reflects a wider Dap12 signaling deficit in mice derived from 129 strain. It seems evident that detailed neuropathological and bone studies of congenic *Dap12* knockout mice are required to clarify the issue of the phenotype difference.

IMMUNOLOGICAL PHENOTYPE OF MICE WITH MANIPULATED DAP12 GENES

Previous reports demonstrate that mice with disrupted Dap12 molecules present normal myeloid and lymphoid development but show impaired immune responses (Bakker et al. 2000; Tomasello et al. 2000b). The immunological phenotype of the *Dap12*^{Δex3-4} knockout mouse strain we used has been reported by Bakker and his colleagues (Bakker et al. 2000). Interestingly, these *Dap12*^{Δex3-4} knockout mice are resistant to experimental autoimmune encephalomyelitis (EAE), a mouse model for the human disease multiple sclerosis (MS), induced by subcutaneous immunization with myelin oligodendrocyte glycoprotein (MOG) peptide. Further, while the NK cells of knockout mice recognize several mouse tumor cell lines normally, they show an impaired function by inefficiently lysing xenogeneic Chinese hamster ovary (CHO) cells, as reported by Bakker and his colleagues (Bakker et al. 2000).

In a mouse strain created by Tomasello and her colleagues the mutated Dap12 protein lacks the one tyrosine of the ITAM at position 75 (Y75) as well as the wild-type C terminus amino acids (Y75-R86) (*Dap12*^{KΔY75}) (Tomasello et al. 2000b). Tomasello and her colleagues have reported an accumulation of dendritic cells in muco-cutaneous epithelia, associated with an impaired hapten-specific contact sensitivity initiated by skin dendritic cells. The NK cells of *Dap12*^{KΔY75} mice show functional deficiency as determined by an impaired recognition and killing of some mouse tumor cell lines and CHO cells. Their mouse model also shows an impaired resistance against murine cytomegalovirus by NK cells (Sjolin et al. 2002).

The *Dap12* transgenic mouse model, created by Lucas and his colleagues, carry 11, 17 or 30 copies of human *DAP12* genes both in myeloid and lymphoid compartments. Overexpression of DAP12 has been shown to result in T and B cell lymphopenia and reduction in the number of NK cells and monocytes in a transgene dose-dependent manner. These mice also show an inflammatory syndrome associated with neutrophilia and lung infiltration by multinucleated macrophages (Lucas et al. 2002).

In conclusion, Dap12 signaling is required for the normal NK and dendritic cell function in the mouse. However, the immunological defects caused by Dap12 deficiency are rather mild.

PATHOGENIC MECHANISMS IN PLOSL

The pathogenesis of PLOSL is unknown. The hypotheses on the potential pathogenic mechanisms in PLOSL have evolved along with an increasing knowledge of the disorder. The first hypothesis considered an error in the systemic lipid metabolism to be responsible for PLOSL (Nasu et al. 1973; Wood 1978). The basic metabolic defect would then cause the breakdown of the myelin sheaths, but no unequivocal evidence has been presented in support of this theory (Akai et al. 1977; Kitajima et al. 1989). In addition, the asymmetry of the white matter changes, revealed by MRI, is not typical of systemic metabolic disorders. The second hypothesis suggested vascular damage with resultant blood–brain barrier breakdown and consequent chronic brain edema as the main pathogenic mechanism (Kalimo et al. 1994). In addition to the brain, abnormal blood vessels have also been reported in the bones, and immunohistochemical studies have shown extravasation of plasma constituents in the brain. The microvascular alterations observed in the cerebral white matter of our patients could conceivably lead to edema or ischemia and result in oligodendroglial and axonal damage, including spheroid formation, and widespread loss of axons and myelin sheaths. However, it is unknown whether the vascular changes are primary or secondary.

Our finding of defective DAP12/TREM2 signaling does not directly support either of these previous hypotheses. We have hypothesized that the connecting factor between lesions in the CNS and bones could be the common myeloid origin of cells potentially expressing DAP12 and TREM2. Microglia, the phagocytic cells in the CNS, differentiate from cells of monocyte/macrophage lineage (Cuadros and Navascues 1998) known to express DAP12 and TREM2. Similarly, osteoclasts, cells responsible for remodeling of bone tissue, are derived from the hematopoietic stem cell population in the bone marrow, which share a common differentiation pathway with macrophages (Vaananen and Horton 1995; Fujikawa et al. 1996; Heymann et al. 1998) (Fig. 13). Accordingly, analyses of the expression of *Dap12* and *Trem2* in the mouse brain have shown that these genes are expressed in microglial cells (Schmid et al. 2002; Kaifu et al. 2003; unpublished). Furthermore, our quantitative RT-PCR analyses show that *DAP12* and *TREM2* are expressed in PBMC differentiating to osteoclasts (IV). It should be noted that although DAP12 and TREM2 are expressed by microglial cells, the role of microglia in the CNS pathogenesis is unknown. Similarly, despite the characterization of a defect in osteoclast maturation and function, determining the true role of osteoclasts in the bone pathogenesis of PLOSL requires further studies.

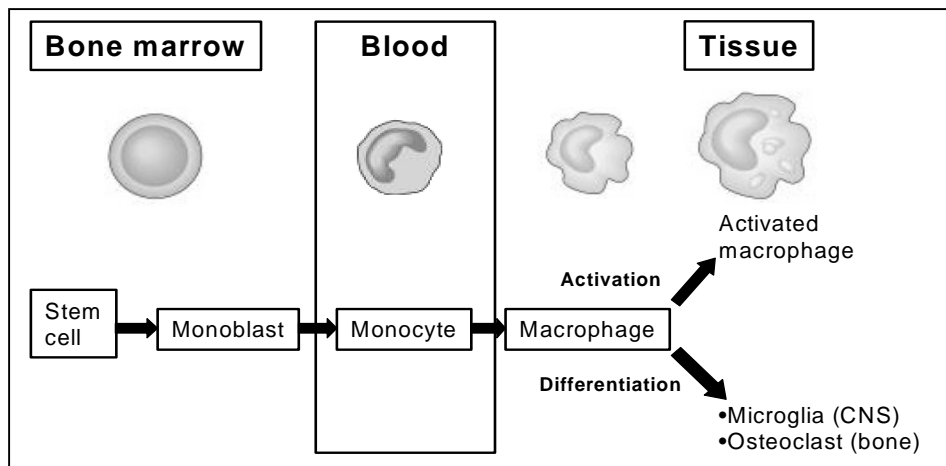


Figure 13. Microglial cells in the CNS and osteoclasts in bone originate from bone marrow and are differentiated from the same CD34+ progenitor cell type circulating in the monocyte fraction. Modified from (Abbas et al. 2000)

Kaifu and his colleagues have recently detected Dap12 polypeptides and transcripts in primary cultures of mouse oligodendrocytes and oligodendrocyte progenitors (Kaifu et al. 2003). This is a rather surprising finding since Dap12 has previously been reliably detected only in hematopoietic lineage, whereas oligodendrocytes are of neuroepithelial origin (Miller 2002). The role of oligodendrocytes in the CNS pathogenesis of PLOSL has to be studied carefully.

Development of the Bone Lesions

The first symptoms of PLOSL appear approximately at age 20 as pain and swelling in the ankles and feet following strain. Fractures in the bones of the hands and feet after minimal trauma occur a few years later due to symmetric bone cavities filled with lipid material that microscopically consists of convoluted 1-2 μm thick lipid membranes, amorphous lipid material and fat cells (Fig. 14) (Nasu et al. 1973; Paloneva et al. 2001). The lesions are found in all bones of the extremities, especially in the carpal and tarsal bones, metacarpals, metatarsals and phalanges. In the long tubular bones, the cavities are typically located at the distal end of the bones. In addition to bone cavities, the affected bones show pronounced focal osteoporotic features, i.e. marked loss of trabecular bone. The development of the bone lesions is slowly progressive, and the age of onset of the lesions is unknown (Makela et al. 1982). The normal average height and the macroscopic structure of the bones in PLOSL patients, apart from the cyst-like lesions and trabecular loss, indicates that bone remodeling by osteoclasts during growth

is not severely affected. In contrast to the phenotype with severe osteoclast failure, the bones are not osteopetrotic (de Vernejoul and Benichou 2001).

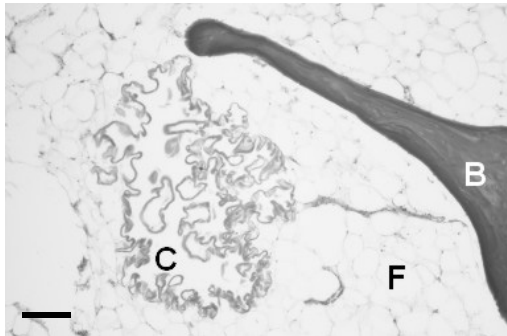


Figure 14. Contents of the bone lesion. The lesions consist of cavities that radiologically resemble cysts. Microscopically, the lesion cavities contain convoluted lipid membrane structures (C) filled with amorphous lipid substance, and fat cells (F). The bone trabeculae (B) are partially preserved. The scale bar corresponds to 125 μm (van Gieson).

The reason why the bone lesions are found only in the limb bones and especially in the distal end of the bones remains unclear. Bones develop either by endochondral or intramembranous ossification. In endochondral ossification a cartilage model serves as the precursor of bone, whereas intramembranous ossification occurs without an intervention of a cartilage precursor (Ross et al. 1995). Interestingly, all bones affected by PLOSL are formed by endochondral ossification. No cystic lesions or pathologic fractures have been found in the bones developed by intramembranous ossification (the skull, face, clavicle and mandible). However, this does not explain why the axial skeleton (vertebrae), developed by endochondral ossification, is unaffected. The pathogenic mechanism resulting in the accumulation of lipid material within the lesion cavities remains unestablished. The accumulated lipid membranes may represent incompletely processed breakdown products of cell membranes. It is unlikely that the lipid substance is transcytosed from the bone resorption area and secreted by osteoclasts because the resorbed bone matrix consists mostly of calcium, osteocalcin fragments and type I collagen fragments, but not lipids (Nesbitt and Horton 1997; Salo et al. 1997).

CLASSIFICATION OF THE BONE LESIONS

The current literature on PLOSL describes the bone lesions as bone cysts or cystic lesions. This is rather misleading since the lesions are not real cysts, that by definition have to be well-demarcated cavities lined by epithelium (Dorland 2000). Histologically, the bone lesions in PLOSL are poorly demarcated cavities with inhomogeneous contents. The bone cavities resemble cysts only in radiographs of bones (Fig. 6a, p. 44). It is actually possible that the cavities represent predilection regions of trabecular osteoporosis-type bone loss that is observed around the cavities.

NEUROPATHOLOGICAL MECHANISMS IN DIFFERENT FORMS OF DEMENTIA

Certain forms of both inherited and sporadic dementia and other neurodegenerative disorders are found to have several common neuropathological manifestations. For example, the two key pathological lesions in Alzheimer's disease (AD) are the plaques (aggregated β -amyloid) and neurofibrillary tangles (intraneuronal aggregations of tau protein). However, similar neurofibrillary tangles are also the neuropathological hallmark in frontotemporal dementia with parkinsonism linked to chr. 17 (FTDP-17). Furthermore, amyloid plaques may also occur in dementia with Lewy bodies as well as in transmissible spongiform encephalopathies. Further, Lewy bodies (intracellular aggregates of α -synuclein) are found in Lewy body dementia, Parkinson's disease, and some forms of AD (Hardy and Gwinn-Hardy 1998; Haltia 2000; Mirra and Hyman 2002; Ritchie and Lovestone 2002; Taylor et al. 2002; Tsuang and Bird 2002). Most dementias discussed above primarily affect the neurons. The neuropathology of PLOSL clearly differs from the brain lesions found in the above-mentioned neurodegenerative disorders. PLOSL does not seem to be a primary neurodegenerative dementia since the neuropathological hallmark of PLOSL is the severe loss of myelin, and no signs of intraneuronal or intragial inclusions have been identified, despite the ballooned contours found in some neurons. The presence of ballooned neurons might represent central chromatolysis as a sign of regenerative attempts of neurons in response to axonal damage due to myelin loss.

PSYCHIATRIC SYMPTOMS IN DEMENTING DISORDERS

PLOSL patients show a prominent frontal lobe syndrome with loss of judgment, euphoria, lack of social inhibitions (including moria, i.e. a tendency to joke), and disturbance of concentration. Such symptoms in dementing disorders are common. The frontal lobe syndrome is a typical manifestation in several neurodegenerative disorders, such as FTDP-17 and classical Pick's disease (Foster et al. 1997; Dickson 1998). Psychiatric symptoms including mood disturbances (euphoria, depression), personality change, behavioral difficulties (agitation, apathy, disinhibition, aberrant motor behavior), hallucinations, delusions, and eating disorders occur in up to 90% of dementia patients (Foster et al. 1997; Ritchie and Lovestone 2002).

DIFFERENTIAL DIAGNOSIS OF PLOSL

The combination of frontal-type dementia, debuting in the fourth decade, and radiologically demonstrable cyst-like osseous lesions is rather unique and makes it easy to clinically distinguish PLOSL from the established forms of sporadic and familial frontotemporal dementia such as Pick's disease, nonspecific frontal lobe degeneration,

and the various entities of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Foster et al. 1997), in several of which mutations of the *tau* gene have been reported (Hutton et al. 1998; Spillantini et al. 1998; Goedert et al. 1999). At the tissue level, there is no beta-amyloid accumulation, and the various synucleinopathies and tauopathies (Hardy and Gwinn-Hardy 1998) can be excluded by the absence of intraneuronal or glial deposits, immunoreactive for α -synuclein, phosphorylated tau, or ubiquitin (Dickson 1999). A lack of immunoreactivity for phosphorylated neurofilament epitopes or α B-crystallin distinguish the chromatolytic neurons of PLOSL patients from the ballooned cells seen in Pick's disease and several other dementing conditions (Dickson 1998). The brain pathology of PLOSL patients is dominated by sclerosing leukoencephalopathy but, in contrast to PLOSL, cyst-like bone cavities are not found in the known forms of adult-onset leukodystrophy. The white matter lesions in PLOSL are associated with vascular alterations, distinct from the various cerebral amyloid angiopathies and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Kalimo et al. 1999) by the lack of congophilia or granular basophilic and PAS-positive material in the thickened vessel walls. A negative history for elevated blood pressure precludes hypertensive small-artery disease.

It is important to note that, according to our data, up to 25% of the PLOSL patients do not have any clinical skeletal symptoms before the onset of the neurological symptoms, and some patients do not suffer from fractures at all, despite the presence of the pathognomonic bone lesions (III and unpublished). From the differential diagnostic point of view, it should also be noted that occasional patients the cerebral white matter looked normal when investigated by MRI at the time of the first clinical neurologic symptoms (III).

TREATMENT AND CLINICAL COUNSELLING

Currently, no therapy to delay or halt the progression of PLOSL is known. Therefore, only symptomatic treatment is available. In individual cases, orthopedic ankle surgery as well as supportive orthopedic devices may be of value. Epileptic seizures may worsen the patient's condition. Consequently, adequate antiepileptic medication is important. To prevent and minimize the severe social problems (divorces, unemployment, financial troubles, suicide etc.) caused by the frontal lobe syndrome, the family members should be well informed about the nature of the disorder (Hakola 1990a).

CONCLUDING REMARKS

This thesis presented molecular genetic, clinical and pathogenic aspects of polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL, Nasu-Hakola disease), a monogenic model for dementia and bone degeneration. In the first original publication the causative gene for PLOSL was identified as *DAP12*, and the mutations responsible for the disease in majority of the patients were established. In the second article the molecular genetic background of PLOSL was completed by the identification of mutations in the second PLOSL gene coding for the *DAP12*-associated receptor *TREM2*. The third article characterized the natural course of PLOSL in respect to the clinical, radiological, histopathological, and electrophysiological manifestations. The fourth original article characterized the role of *DAP12/TREM2* signaling in osteoclast differentiation and function, and provided an insight into the potential pathogenic mechanism of bone lesions in PLOSL.

With the identification of the *DAP12/TREM2* complex to be responsible for PLOSL, the basic tools for the elucidation of the pathogenesis of PLOSL have been established. Functional studies to follow will answer the essential questions concerning the molecular pathogenic mechanisms of PLOSL.

The biological significance of the *DAP12*-mediated signaling in innate immunity has just began to be understood. Based on this thesis, the significance of *DAP12* and *TREM2* in the normal function of the CNS and bone have been characterized. This study also links the immune system to CNS and bone disorders in an interesting way.

In the past decade, tremendous advances have been made in our understanding of the molecular mechanisms of disorders of the brain. I hope that this study will provide perspectives for the studies of other degenerative disorders of the central nervous system and bone such as more common forms of dementia and osteoporosis. Finally, I wish that some day our findings will benefit the PLOSL patients by promoting the development of a treatment for this dramatic disease.

ELECTRONIC DATABASE INFORMATION

Internet addresses for the databases, programs and organizations mentioned in the text are provided below.

BLAST, service at NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>

Celera, <http://www.celera.com>

Ensembl, <http://www.ensembl.org>

Fgenes, <http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html>

GenBank, NCBI, <http://www.ncbi.nlm.nih.gov/Genbank/>

GeneCards, <http://bioinfo.weizmann.ac.il/cards/index.html>

GeneReviews, <http://www.genereviews.org/>

Genome Data Base (GDB), <http://gdbwww.gdb.org/>

HUGO Gene Nomenclature Committee, <http://www.gene.ucl.ac.uk/nomenclature/>

Human Gene Mutation Database, <http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>

Human Genome Organization (HUGO), <http://www.gene.ucl.ac.uk/hugo/>

I.M.A.G.E. Consortium, <http://image.llnl.gov/>

Mouse Genome Informatics, <http://www.informatics.jax.org/>

National Center for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/>

National Human Genome Research Institute (NHGRI), <http://www.genome.gov/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

University of California Santa Cruz (UCSC) Genome bioinformatics,
<http://genome.ucsc.edu/>

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I thank the PLOSL patients and their families which with their positive attitude towards this study enabled the project. I wish that some day our findings will benefit them.

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Helsinki, May 16th, 2003

A handwritten signature in black ink, appearing to be 'M. M.', written in a cursive style.

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