

Loss-of-function mutations in *TYROBP* (*DAP12*) result in a presenile dementia with bone cysts

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Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS; MIM 221770), also known as Nasu-Hakola disease, is a recessively inherited disease characterized by a combination of psychotic symptoms rapidly progressing to presenile dementia and bone cysts restricted to wrists and ankles^{1–3}. PLOS has a global distribution, although most of the patients have been diagnosed in Finland⁴ and Japan, with an estimated population prevalence of 2×10^{-6} (ref. 2) in the Finns. We have previously identified a shared 153-kb ancestor haplotype in all Finnish disease alleles between markers *D19S1175* and *D19S608* on chromosome 19q13.1 (refs 5,6). Here we characterize the molecular defect in PLOS by identifying one large deletion in all Finnish PLOS alleles and another mutation in a Japanese patient, both representing loss-of-function mutations, in the gene encoding TYRO protein tyrosine kinase binding protein⁷ (*TYROBP*; formerly *DAP12*). *TYROBP* is a transmembrane protein that has been recognized as a key activating signal transduction element in natural killer (NK) cells⁸. On the plasma membrane of NK cells, *TYROBP* associates with activating receptors recognizing major histocompatibility complex (MHC) class I molecules^{7,9}. No abnormalities in NK cell function were detected in PLOS patients homozygous for a null allele of *TYROBP*.

The critical DNA region for PLOS (for clinical phenotype, see Table 1) on chromosome 19q13.1 contains four known genes, *APLP1* (ref. 10), *DAP10* (refs 11,12), *TYROBP* and *NPHS1* (ref. 13), as well as three predicted transcripts or EST contigs. Sequencing of the amplified coding regions of the regional candidate genes revealed no disease-associated DNA variants, except in *TYROBP*. We failed to obtain products by PCR-amplifying the coding region of *TYROBP* in the genomic DNA of Finnish PLOS patients with primers flanking exons 1–4, whereas exon 5 was amplified normally. This indicated that the patients carry a homozygous deletion encompassing exons 1–4 of *TYROBP* (Fig. 1a).

To determine the 5' and 3' boundaries of the identified deletion, we PCR-amplified genomic DNA of PLOS 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. 1a). Sequencing of this PCR product revealed the 5' deletion breakpoint 2,900 bp upstream from the initiation methionine, and the 3' breakpoint in the last intron of *TYROBP*, 1,300 bp upstream from the termination codon (Fig. 1a,b). The genomic deletion is 5,265 bp and includes 343 bp (exons 1–4 and 5' UTR) of the 604-bp transcribed region of *TYROBP*. All 26 Finnish PLOS patients, as well as 2 Swedish patients with known ancestors in Finland^{5,14}, are homozygous for the PLOS_{Fin} deletion. Southern-blot analysis of the parents of Finnish PLOS patients revealed the genomic deletion in the heterozygous form. We found no evidence of the mutation in our control sample pool of 120 unrelated Finns.

Using northern-blot analysis, we did not detect steady-state mRNA encoding *TYROBP* in the lymphoblasts of Finnish PLOS patients (Fig. 2a). 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 *TYROBP* revealed no signal (Fig. 2b).

Sequence analysis of the 342-bp coding region of *TYROBP* from the genomic DNA of a Japanese PLOS patient revealed a homozygous single-base deletion in exon 3 (Fig. 1a). This mutation creates a frameshift in the ORF, predicting premature termination of the polypeptide chain 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 *TYROBP* homodimer and the associated ligand-binding receptors on the NK cell surface⁷. Mutations of

Table 1 • Clinical and histopathological features of PLOS

Symptoms	Age of onset	Histopathological findings
Bones pains in ankles and wrists pathological fractures of ankles and wrists	20–30 y	bone cysts in ankles and wrists in X-ray loss of trabecular bone
CNS psychotic periods loss of social inhibitions euphoria progressive dementia	30–40 y	frontally accentuated loss of myelin astrocytic gliosis enlarged ventricles calcifications and atrophy of basal ganglia
myoclonic twitches convulsions gait disturbance primitive reflexes premature death	40–50 y	atrophy of corpus callosum thick-walled or obliterated small arterioles and capillaries

Based on data in refs 1–3,28–30.

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this residue have been shown to disrupt both the complex formation with the associated receptors^{15,16} and the cell-surface expression of TYROBP (refs 17,18). It is therefore probable that, similar to the Finnish patients, no functional TYROBP polypeptide exists in the Japanese PLOSL patient (no cell lines or RNA are available).

One Norwegian and one Swedish patient with the typical clinical phenotype of PLOSL (refs 14,19) have shown exclusion of linkage to chr 19q13.1 (ref. 5); we found no sign of mutation of TYROBP in them or their families. Genes encoding TYROBP-associated molecules such as the KIR2DS2 (refs 7,9), KLRD1 (CD94; ref. 17), KLRC2 (NKG2C; ref. 17), SIRPB1 (ref. 20) and CLECSF5 (MDL-1; ref. 16) polypeptides are potential candidates for the second PLOSL gene.

TYROBP 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 termination codons are separated by only 408 bp (Fig. 1a). The PLOSL_{Fin} deletion does not include 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 (Fig. 2a). Similarly, western-blot analysis of DAP10 polypeptides in PBMC revealed no difference between the patient and control (Fig. 2b). Moreover, a normal level of the DAP10-associated receptor KLRC4 (NKG2D; ref. 11) was found in the PLOSL patient (data not shown). We cannot exclude the possibility that the deletion changes the expression pattern of other genes further upstream or downstream of the deletion site.

TYROBP is expressed mostly in haematological cells and tissues such as peripheral blood leukocytes and spleen⁷. We observed a distinct, steady-state mRNA signal of lower intensity in the brain samples, especially in those parts of the brain consisting purely or mostly of white matter, such as the corpus callosum and spinal cord (Fig. 3a,b). Our semi-quantitative RT-PCR analysis also produced signals from mRNA of primary cultures of microglial cells and astrocytes prepared from four-day-old mice, as well as from the mRNA of primary neurons isolated from mouse and rat embryos²¹ (data not shown).

Previous studies of TYROBP have emphasized its function in NK cells and myeloid cells¹⁶. NK cells are CD3-CD56+ lymphocytes, representing 10–15% of circulating blood mononuclear cells, which spontaneously lyse malignant and virus-infected cells. Activated NK cells also produce cytokines, making them regulators of acquired immune response²². TYROBP has been suggested to have a crucial role in the activation cascade of NK cells^{7,9}. TYROBP is a 12-kD protein predicted to consist of a leader peptide, a short extracellular domain followed by a transmembrane and a cytoplasmic domain⁷. The functional molecule is a disulphide-bonded homodimer linked by two cysteines in the extracellular domain. On the cell membrane of NK cells, the TYROBP homodimer forms a complex with several activating receptors recognizing MHC class I molecules: the KLRD1/KLRC2 (C-type lectin receptor) for HLA-E and the killer-cell Ig-like receptors (KIR) for HLA-C (refs 7,17). The cytoplasmic domain of TYROBP contains an immunoreceptor tyrosine-based activation motif (ITAM), which, upon receptor engagement, becomes tyrosine phosphorylated and binds the cytoplasmic protein tyrosine kinases SYK and ZAP70 (refs 7,15). This interaction results in intracellular calcium mobilization and subsequent cellular activation¹⁵.

We investigated NK cell function and number in Finnish PLOSL patients. Immunophenotyping of PBMCs from six Finnish PLOSL patients revealed

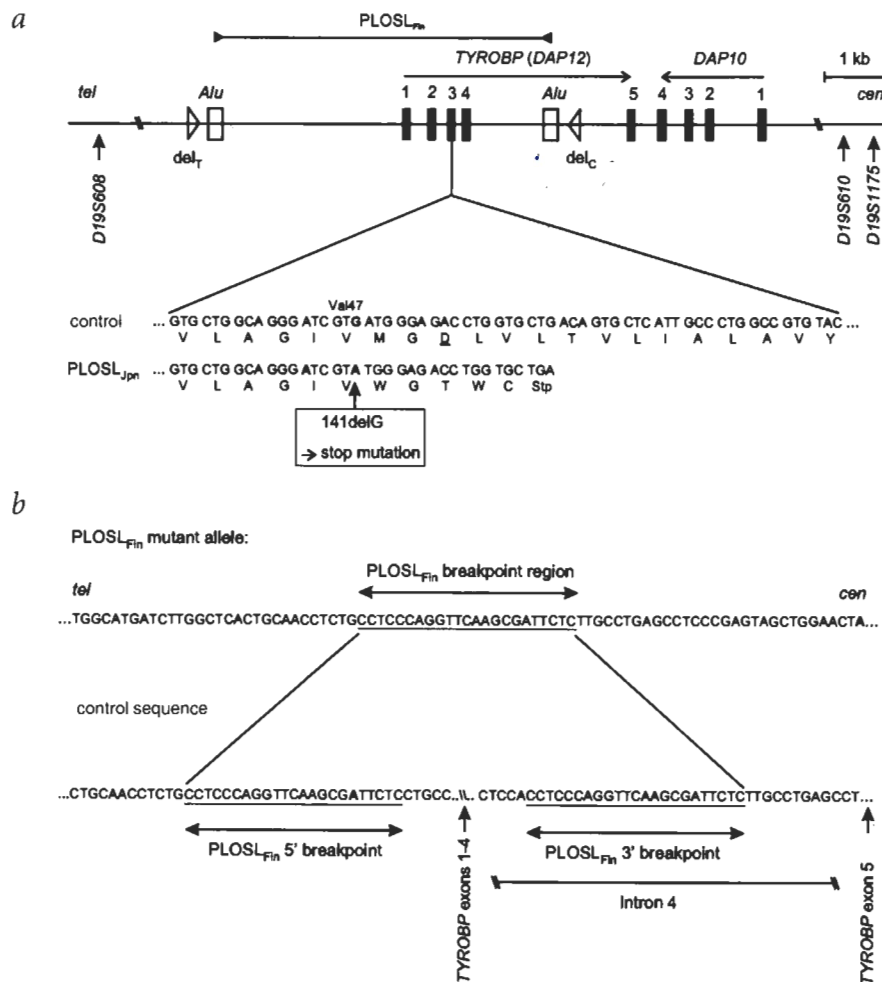


Fig. 1 TYROBP and DAP10, located in opposite transcriptional orientation on chromosome 19q13.1, and identified PLOSL mutations in TYROBP. **a**, We performed the PCR across the PLOSL_{Fin} deletion from genomic DNA. Primer pair *del_T*/*del_C* revealed a 5.3-kb deletion in all Finnish PLOSL alleles and *Alu* sequences flanking the deletion. The PLOSL_{Jpn} mutation was found in the sequence analysis of the PCR product of exon 3 of TYROBP. The mutation disrupts the reading frame, resulting in a premature stop codon yielding in a 52-aa polypeptide. The functionally critical aspartic acid (D, underlined) at position 50 is changed to threonine (T). The critical markers are indicated. **b**, The PLOSL_{Fin} deletion includes exons 1–4 of TYROBP. The deletion breakpoints are flanked by *Alu* repetitive elements sharing an identical 23-bp sequence (underlined). Because 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.

that the frequency of NK cells (CD3-CD56+) and CD56+ T cells (CD3+CD56+) were within normal limits (Table 2). Moreover, there were no abnormalities in the number or frequency of T lymphocyte subpopulations (CD3+CD4+, CD3+CD8+; Table 2). Furthermore, we detected no differences between the patients and the controls in either spontaneous or interleukin-2 activated cytotoxicity of the lymphocytes against the K562 leukaemic cells (Table 2). 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 herpesvirus infections or an increased frequency of malignancies. Further, normal levels of granulocytes, monocytes and lymphocytes were observed in the routine leukocyte differential counts of the patients. Our finding of normal NK cell function in PLOSL patients suggests functional redundancy or developmental compensation in target cell recognition by NK cells.

Consistent with the deletion phenotype in PLOSL patients, the development of NK cells and their frequency in peripheral lymphoid tissues are not affected in recently produced *Tyrobp*-deficient mice. (A.B.H.B. *et al.*, unpublished data). Preliminary analyses of young mice homozygous for targeted disruption of *Tyrobp* on a mixed genetic background have not indicated pathology in the bones or brain. Because the symptoms of PLOSL are of late onset in humans, further studies with older mice are warranted.

Despite of the characterization of the primary cause of PLOSL, the molecular and cellular pathogenesis remains unknown. The expression of TYROBP in monocyte/macrophage lineage may provide a link between lesions in the brain and bone. Microglial cells in the CNS are phagocytic cells of haematopoietic origin differentiating from the monocyte/macrophage lineage²³. Osteo-

clasts (cells responsible for resorption of bone tissue) also share a common differentiation pathway with macrophages²⁴. The CNS pathogenesis of PLOSL may arise from the inability of microglial cells to remove apoptotic tissue in the brain. Bone cysts may result from chronic dysfunction of osteoclasts, resulting in a defective remodelling process of bones. It thus seems that the CNS and skeletal system are more rigidly dependent on the normal function of TYROBP than the activation of NK cells. Identification of the *TYROBP* mutations as the molecular defect in PLOSL not only provides new clues to the pathogenic mechanisms of dementias, but should also stimulate a search for new biological functions of the TYROBP molecule.

Methods

Sequence and mutation analysis. To identify the genes in the critical DNA region, we used several different gene prediction computer programs: Fgenes (<http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html>), Genotator²⁵ and Genscan²⁶. We also searched EST databases (GenBank, EMBL and DDBJ) using the BLASTN algorithm at NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/BLAST/>). We designed primers flanking the coding regions based on the predictions and EST-database searches. The PCR-amplified coding regions were sequenced by automated sequencing (ABI377; Perkin-Elmer -Applied Biosystems) using the PCR primers as sequencing primers.

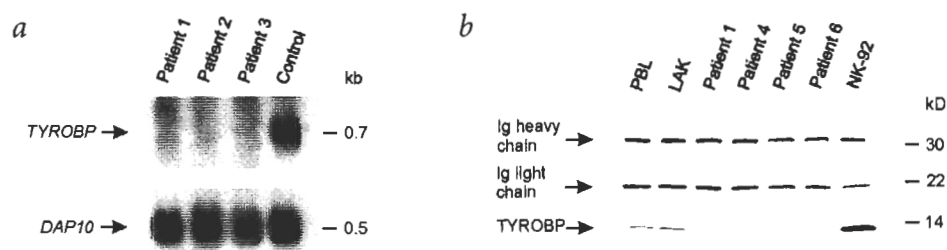


Fig. 2 Northern (a) and western (b) analysis of *TYROBP* and *DAP10* transcripts and corresponding polypeptides of patients carrying PLOSL_{Fin} mutations. **a**, We analysed lymphoblast poly(A)⁺ blot using radiolabelled human *TYROBP* (encompassing exons 1–5) and *DAP10* (encompassing exons 1–4) cDNA as probes. **b**, *TYROBP* and *DAP10* polypeptides were immunoprecipitated with anti-*TYROBP* monoclonal antibody or an affinity-purified polyclonal antiserum against *DAP10*, or with a control mouse IgG1 monoclonal antibody or control rabbit IgG (cIg), respectively. Polypeptides from the Finnish PLOSL patients and control individuals were immunoprecipitated from the lysates of PBMC (*TYROBP*) and lymphoblasts (*DAP10*). Immunoprecipitates were then analysed by western blot using anti-*TYROBP* and anti-*DAP10* antibodies. PBL, control peripheral blood leukocytes; LAK, control interleukin-2 activated killer cells; NK-92, control NK cell line.

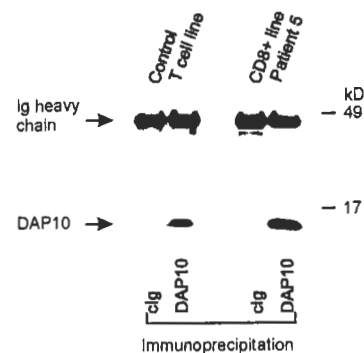


Fig. 3 Northern-blot analysis of *TYROBP* in multiple human tissues. **a**, Selected dots from a human multiple tissue mRNA expression array hybridized using radiolabelled human *TYROBP* cDNA as a probe. 1, peripheral blood leukocytes; 2, spleen; 3, fetal spleen; 4, thymus; 5, bone marrow; 6, placenta; 7, lung; 8, fetal lung; 9, liver; 10, jejunum; 11, erythroleukaemia cell line K562; 12, whole brain; 13, fetal brain; 14, frontal lobe; 15, parietal lobe; 16, cerebellum; 17, cortex; 18, corpus callosum; 19, nucleus caudatus; 20, putamen; 21, thalamus; 22, hippocampus; 23, spinal cord; 24, genomic DNA (500 ng). **b**, Northern-blot analysis of different human tissues using the same probe as in (a) on multiple-tissue northern blots. Each lane contains 2 μ g poly(A)⁺ RNA from human tissues. Abundant steady-state mRNA was observed in haematological cells and tissues such as peripheral blood leukocytes and spleen. *TYROBP* transcripts were also found in almost equal quantity in bone marrow, lymph nodes, placenta, lung and liver. A distinct, but lower intensity, steady-state mRNA signal was detected in different parts of the brain, especially in the basal ganglia (nucleus caudatus and putamen) which are affected at an early stage of PLOSL, and in the corpus callosum. Relatively strong northern-blot signals were also detected in the medulla and spinal cord. PBL, peripheral blood leukocytes.

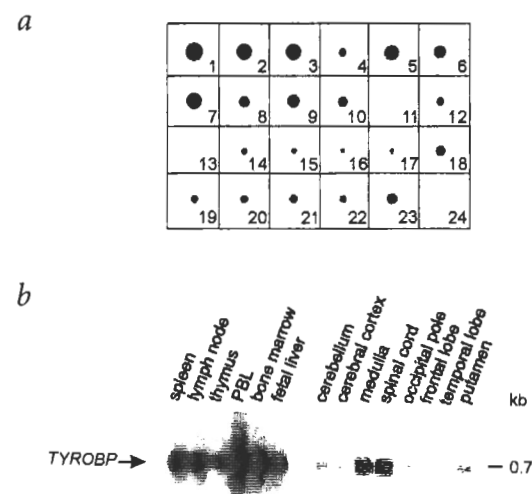


Table 2 • Number and function of NK cells

Immunophenotyping of PBMC of PLOSL patients							
Patient	Cell surface antigen						
	CD3+	CD4+	CD8+	CD16+	CD56+	CD3+CD56+	CD3-CD56+
1	88	67	23	3.5	18	15	3.5
2	73	42	25	12	21	3.9	17
3	68	24	46	37	37	18	20
4	75	48	31	18	23	9.1	14
5	61	32	14	13	17	7.4	9.6
6	77	42	18	19	24	11	13
mean	73.5	42.3	26.3	17.1	23.4	10.6	12.8
σ	9.2	18.8	11.5	11.1	7.4	5.0	5.7
Controls, $n = 16$							
mean	74.8	52.3	24.2	14.7	20.0	7.2	12.8
σ	8.0	8.8	5.8	5.0	6.5	3.4	4.9
Cytotoxicity assays of PBMC of PLOSL patients							
Effector:target cells Patient	Non-activated			IL-2 activated			
	(50:1)	(25:1)	(12.5:1)	(50:1)	(25:1)	(12.5:1)	
1	9.1	6.2	3.0	27	18	9.7	
2	45	30	22	67	48	37	
3	58	44	34	78	65	48	
4	29	13	11	57	50	29	
5	17	13	5.6	30	19	14	
6	26	19	10	62	51	32	
mean	30.8	20.7	14.4	52.2	41.7	28.3	
σ	18.1	14.0	11.8	22.7	19.1	14.4	
Controls, $n = 4$							
mean	28.4	19.9	12.2	44.8	36.2	23.5	
σ	3.4	1.2	3.1	17.4	16.0	8.6	

PBMC were immunophenotyped and sorted by flow cytometry. The numbers represent percentage values of total PBMC. n , number of control subjects; σ , standard deviation. Cytotoxicity assays of PBMC of patients and controls were performed against K562 leukaemic cells at different effector to target cell ratios. Percentage cytotoxicity was determined using ^{51}Cr release assay. IL-2 treatment was used to increase the cytolytic activity of PBMC by generating IL-2-activated killer cells.

We amplified and sequenced *TYROBP* using the following primers: exon 1, sense primer, 5'-TGGGGACGGAGGTGAAGTTT-3', antisense primer, 5'-CCCATCCCAACACCCACTTT-3'; exon 2, sense, 5'-GCCTGTGGGT TTCTCCCAGA-3', antisense, 5'-GGCAGGGAGGTTTGAAAGG-3'; exon 3, sense, 5'-CCGTCTCTCCACACCCTT-3', antisense, 5'-CCTC CATTACCATCCCTTTGGA-3'; exon 4, sense, 5'-GGGCTGGGTAACTC CCAGA-3', antisense, 5'-CCCAGCCCCTTTCACACAT-3'; exon 5, sense, 5'-GCAGAGGAGAAGGGGGAACA-3', antisense, 5'-AGTATT GGGGAGCGGTCTGG-3'. The amplification and sequencing across the PLOSL_{Fin} deletion was carried out with primers del_C (5'-GGAACATTGA CAGAGAGGGGG-3') and del_T (5'-TTGGGACCCCTTGGACTTTC-3').

Northern-blot analysis. Poly(A)⁺ RNA from the PLOSL and control lymphoblast cell lines was isolated using a Fast Track 2.0 kit (Invitrogen). We separated poly(A)⁺ RNA (9 μg) by electrophoresis and transferred it to a nylon membrane as described²⁷. We hybridized membranes with ^{32}P -labelled *TYROBP* and *DAP10* cDNA probes. The *TYROBP* probe was generated from a clone obtained from the IMAGE Consortium. The *DAP10* probe was generated from lymphoblast RNA by RT-PCR using an Enhanced Avian RT-PCR kit (Sigma). The RT-PCR primers used were 5'-CTCTGGAC CACAGTCTCTG-3' (sense) and 5'-CAATCCAAAAGTTGGGGCGG-3' (antisense). We performed hybridization using ExpressHyb hybridization solution (Clontech) according to the manufacturer's protocol. The probes were labelled using Prime-It II random primer labelling kit (Stratagene) and purified using NucTrap probe purification columns (Stratagene). We hybridized the human multiple-tissue mRNA expression array (Clontech) and multiple-tissue northern blots (Clontech) using a random-prime-labelled *TYROBP* cDNA probe according to the manufacturer's instructions.

Phenotypic analysis of PBMC. We labelled 1×10^5 PBMC for 20 min on ice with FITC- or phycoerythrin-conjugated monoclonal antibodies against CD3, CD4, CD8, CD16 and CD56 (Becton Dickinson) and immediately analysed them by flow cytometry using a FACScan (Becton Dickinson).

Cytotoxicity assay. We tested PBMC 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). We labelled the target cells for 3 h with sodium⁵¹chromate (100 μCi ; Pharmacia Biotech) at 37 °C, washed them 3 times, and exposed them to PBMC at effector to target cell ratios of 50:1, 25:1 and 12.5:1 in 96-well plates. We collected supernatant (100 μl) after 4 h incubation and measured the release of ^{51}Cr with a γ -counter (Wallac). We prepared triplicate wells for each effector to target cell combination. Per cent cytotoxicity (Cx) was calculated using the formula % Cx=(test release-spontaneous release (without effector cells)/maximal release (with 10% Triton X)-spontaneous release) $\times 100$.

Immunoprecipitation and western-blot analysis. PBMC were incubated overnight at 37 °C in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies), antibiotics and L-glutamine. We washed $2-3 \times 10^7$ cells once with PBS and lysed them with NP40 lysis buffer (1% NP40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA and protease inhibitors (5 $\mu\text{g}/\text{ml}$ each of chymostatin, pepstatin A, antipain HCl, and 10 mg/ml leupeptin hemisulphate)) for 15 min on ice. The protein concentration of lysates was adjusted to 2 mg/ml and the proteins were immunoprecipitated from lysates (500 μl) with the anti-human *TYROBP* monoclonal antibody DX37 (a mouse IgG1 generated by immunizing Balb/c mouse with a polypeptide corresponding to the cytoplasmic domain of human *TYROBP*). Immunoprecipitates were collected with protein A/G-Sepharose (50 μl ; Pharmacia Biotech) and washed three times with NP40 lysis buffer. The beads were boiled in sample buffer (250 μl ; Laemmli), and 40 μl aliquots were separated on 4-20% gradient SDS-PAGE gels (Readygel, BioRad) for western-blot analysis using the same monoclonal antibody. We visualized *TYROBP* 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. We analysed immunoprecipitates by western blot using the same rabbit anti-DAP10 antiserum¹¹.

GenBank accession numbers. We determined the genomic sequence encompassing the critical region from cosmids F19541 (U95090), R33502 (AC002133), R28051 (AD000864), F19399 (AD000833), R31158 (AF038458), R31874 (AD000823) and R28125 (AD000827). *TYROBP* genomic sequence, AF019563; *TYROBP* cDNA sequence, AF019562; *DAP10* genomic sequence, AF072845; *APLP1* genomic sequence,

NM_005166; *NPHS1* cDNA sequence, AF035835; *TYROBP* probe, N41026.

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