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Diagnosing XLP1 in patients with hemophagocytic lymphohistiocytosis

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Abstract

Background—Hemophagocytic Lymphohistiocytosis (HLH) is a life-threatening, heterogeneous, hyper-inflammmatory disorder. Prompt identification of inherited forms resulting from mutation in genes involved in cellular cytotoxicity can be crucial. X-linked lymphoproliferative disease 1 (XLP1), due to mutations in *SH2D1A* (Xq25) encoding SAP, may present with HLH. Defective SAP induces paradoxical inhibitory function of the 2B4 co-receptor and impaired NK (and T) cell response against EBV-infected cells.

Objective—To characterize a cohort of HLH patients with XLP1 for SAP expression and 2B4 function in lymphocytes, proposing a rapid diagnostic screening to direct mutation analysis.

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Methods—We set-up rapid assays for 2B4 function (degranulation or ^{51}Cr-release) to be combined with intracellular SAP expression in peripheral blood NK cells. We studied twelve patients with confirmed mutation in *SH2D1A* and some family members.

Results—The combined phenotypic/functional assays allowed efficient and complete diagnostic evaluation of all XLP1 patients, thus directing mutation analysis and treatment. Nine cases were SAP^{-}, two expressed SAP with MRFI values below the range of healthy controls (SAP^{dull}) and one, carrying the R55L mutation, was SAP^{+}. NK cells from all patients showed inhibitory 2B4 function and defective killing of B-EBV cells. Carriers with *SH2D1A* mutations abolishing SAP expression and low percentage of SAP+ cells, showed neutral 2B4 function at the polyclonal NK cell level. Three novel *SH2D1A* mutations have been identified.

Conclusion—Study of SAP expression is specific but may have insufficient sensitivity for screening XLP1 as a single tool. Combination with 2B4 functional assay allows identification of all cases.

Keywords

HLH; XLP1; SAP expression; 2B4 function; NK cells

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal hyperinflammatory syndrome, triggered by common pathogens and characterized by excessive activation of macrophages, T and NK cells.1,2 In a proportion of cases, defined as “primary” or “familial” HLH (FHL), this results from constitutional defects involving cellular cytotoxicity; although it is usually diagnosed in children, later onset is increasingly reported, including rare cases of adult patients.3 Among the inherited forms different genetic subtypes have been identified so far: FHL types 2-5, Griscelli syndrome type 2 (GS2), Chediak-Higashi syndrome (CHS), and Hermansky-Pudlak syndrome (HPS), caused by mutations in genes (*PRF1*, *UNC13D*, *STX11*, *STXBP2, RAB27A*, *LYST*, and *AP3B1*, respectively) encoding proteins required for lymphocyte cytotoxicity.2,4 Furthermore, patients with X-linked lymphoproliferative disease (XLP) may present with HLH. XLP2 results from mutations in the gene encoding the X-linked inhibitor of apoptosis (XIAP). XLP1 (Duncan disease, OMIM#308240) is a rare congenital immunodeficiency caused by mutations in *SH2D1A* (Xq25), the gene encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP).5,6 The estimated incidence of XLP1 is of 1-3 per million in males. In 1978 an XLP registry was established7 and as of the year 2000, 309 males with XLP phenotypes from 89 unrelated families had been registered.8

SAP is a cytoplasmic adaptor protein that is recruited by members of the SLAM family, among which is 2B4 (CD244), a co-receptor surface molecule expressed in NK and T lymphocytes.9 In healthy subjects 2B4, upon recognition of CD48, which is up-regulated in EBV-infected B cells, recruits SAP and delivers activating signals. In NK cells, these support the function of the activating receptors NKp46, NKp30 and NKp44 (collectively termed natural cytotoxicity receptors, NCR), involved in the recognition of B-EBV cells. In the absence of SAP binding, as in XLP1, 2B4 associates with protein tyrosine phosphatases and

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delivers inhibitory signals that result in impaired NK and $CD8^+$ T cell-mediated cytolytic responses against EBV infections.10,11 This leads to B cell accumulation and persistence of reactive inflammatory responses.12–14 Thus, in XLP1 patients EBV infection may have a fulminant course or alternatively induce B-cell lymphomas, lymphoproliferation, dysgammaglobulinemia, or an HLH clinical picture.15–18

Among patients with newly diagnosed HLH, rapid identification of cases with a genetic defect is crucial to direct treatment including indication to hematopoietic stem cell transplantation, the only curative treatment available so far.19,20 Screening methods have been developed over the last decade to select patients for mutation analysis, which is time-consuming and costly.21,22 Flow-cytometry evaluation of SAP expression has been proposed as a rapid diagnostic assay to screen XLP1 patients; a rat (KST-3)23,24 and, more recently, a murine (1C9)14 anti-SAP mAb have been made available. However, the reliability of flow cytometry alone could be hampered by the existence of *SH2D1A* mutations that, rather than disturb SAP transcription or translation, shorten the half-life of the protein or affect its function.6

We have developed a rapid phenotypic/functional diagnostic protocol effective to diagnose XLP1 and the carrier status (Figure E1). It combines the analysis of the intra-cytoplasmic SAP expression by flow cytometry with the analysis of 2B4 function in cytolytic assays. In order to verify the sensitivity and specificity of our diagnostic approach, we reviewed and updated our experience gained in patients with genetically documented XLP1. We provide evidence that flow-cytometry study of SAP expression is very specific but may have insufficient sensitivity as a single tool for screening XLP1; in contrast, the 2B4 dysfunction turned to be highly predictive of XLP1 in all cases.

Methods

Patients

Starting from 1984 we established a Registry for HLH19 (www.orpha.net) and a Biobank (located in Florence, Italy) that centralize patient information and biologic samples from all Italian centers of pediatric hematology-oncology and confirm the clinical diagnosis by immunological and genetic studies. HLH is defined by the diagnostic criteria established by the Histiocyte Society.20 Furthermore information and samples from additional patients are received from other countries, including England, India, Egypt, Belgium, Spain.

Out of 900 patients referred to the Registry between 1985 and 2013, encoded by unique patients number (UPN), 787 were diagnosed as HLH, and in 585 the genetic study could be performed. In 242 of them (41%), we identified a genetic marker, and among them, we selected 12 cases (5%) (from seven different centers) on the basis of an algorithm for diagnostic approach to XLP1 (Figure E1). Of these, 3 patients were exclusively studied for mutation analysis because we had received DNA only; EDTA-peripheral blood samples of 9 patients were shipped at room temperature (24 hour-express courier) to the Registry site (Florence) for mutation analysis and to the reference Immunology laboratory (Genoa, Italy) for phenotypic and functional studies. Furthermore, we also included 3 patients diagnosed as XLP1 at the reference laboratory for pediatric primary immune deficiencies

(PID) (University of Brescia, Italy) that had been previously described.10,25 They were selected due to the availability of frozen samples.

Participants gave written informed consent. Institutional review boards at the A.O.U. Meyer (Florence) and the Gaslini Institute (Genoa) approved the study.

Rapid combined cytofluorimetric and functional assay

To evaluate intracellular SAP expression, peripheral blood mononuclear cells (PBMC) were stained with anti-CD3-PerCP (SK7, IgG_1) and anti-CD56-APC (NCAM16.2, IgG_{2b}), fixed, permeabilized (cytofix/cytoperm, BD Bioscience, San Jose, CA, USA) and stained with anti-SAP (1C9, IgG_{2a}, Abnova, Taipei, TW) or isotype-matched control mAb, followed by anti-mouse IgG_{2a}-PE (SouthernBiotech, Birmingham, AL). Perforin, CD107a and XIAP intracellular stainings were performed as described.22 Resting NK ($CD3^-CD56^+$) and T ($CD3^+$) lymphocytes were evaluated. Mean relative fluorescence intensity (MRFI) indicates the ratio between MFI of cells stained with the relevant mAb and that of cells stained with the isotype-matched negative control. iNKT cells were identified using 6B11 mAb (Miltenyi Biotec, Bergish Gladbach, Germany). Data were acquired on a FACSCalibur cytometer (BD) or MACSQuant Analyzer (Miltenyi Biotec), and analyzed using FlowJo Version 8.8.6 (TreeStar).

To evaluate 2B4 function, PBMC cultured for 3-5 days in the presence of rIL-2 (600 IU/mL) (Proleukin, Chiron Corp., Emeryville, USA) were used as effectors in reverse antibody dependent cellular cytotoxicity (R-ADCC) assays against ^{51}Cr-labelled P815 ($Fc\gamma Rc^+$) murine targets, in the presence of functional grade purified anti-2B4 mAb (PP35, e-Bioscience, San Diego, CA), alone or in combination with the anti-NKp46 mAb (9E2, Miltenyi Biotec; or BAB281).26 All mAb were used at final concentration of 0.5 μg/mL. In addition, the same R-ADCC was also evaluated as degranulation assay using rIL-2 overnight activated PBMC and measuring by cytofluorimetric analysis surface CD107a (H4A3-PE, BD Bioscience) expression on NK cells ($CD3^-CD56^+$). Samples of patients and healthy donors were analyzed in the same daily session of phenotypic and functional assays.

Statistical analysis

Statistical analyses were performed using Graphpad software Version 6.0. The utilized tests are indicated in the figure legends. Not significant (n.s.); $****P < .0001$; $***P < .001$; $**P < .01$; and $*P < .05$.

Mutation analysis, MLPA analysis, assays on expanded activated NK and T cells, analysis of SH2D1A transcript, HEK-293T transient transfections and Immunoblotting can be found in the Online Repository Materials.

Results

SH2D1A mutation analysis and characterization of the mutations

Twelve XLP1 were identified among 242 HLH patients in whom a genetic marker could be assigned (Table I). Ten different *SH2D1A* mutations were identified in patients analyzed, and seven were previously described.5,11,27,28 The c.137+3_+6delGACT

mutation (UPN722) disrupts the donor splice site of exon 1, causing the absence of a stable transcript, and was recently described.29 Three mutations were novel. The c.89T>C mutation (UPN218) results in amino acid substitution (p.L30S) in the SH2 domain, predicted to be pathogenic by in silico analysis. The c.162C>G mutation (UPN562) produces a premature stop codon. The other two mutations result in loss of ORF transcript. In particular, UPN489 lacked the active transcript at the cDNA level, with the only presence of the Δ55 isoform; this result was confirmed by a Real-Time Taqman experiment using a specific probe spanning exon 3-4 junction.

The c.138(-2)A>G mutation found in UPN627 was absent in his mother. Extensive analysis suggested that the mutation might have occurred *de novo* in the patient, although we could not definitely rule out minimal maternal mosaicism.

Cytofluorimetric analysis of peripheral blood lymphocytes

We evaluated intracellular SAP expression in peripheral blood lymphocytes of XLP1 patients and their relatives. NK cells of UPN360 showed no staining (Fig 1, *A*); his mother's NK cells revealed a bimodal distribution with SAP^+ and SAP^- cells, in keeping with her carrier status; his father's and sister's NK cells showed a unimodal, positive staining, with MRFI values falling within the range of healthy controls (5÷21).

In UPN674, NK cells showed a homogeneously weak SAP expression (SAP^{dull}) (Fig 1, *B*); his mother and sister 2 showed a bimodal expression profile with SAP^+ and SAP^{dull} cells, compatible with their carrier status; SAP expression was normal in his father and sister 1.

NK cells of patients and female carriers expressed normal levels of intracellular CD107a, perforin (defective in FHL2)21,22 and XIAP (defective in XLP2)30 (Fig E2, *A*). SAP expression in resting T cells of the patients and their family members paralleled that detected in NK cells (Fig E2, *B* and data not shown).

In patient UPN627, NK cells did not express SAP; unexpectedly however, maternal NK and T cells showed a normal unimodal, positive profile conflicting with her carrier status (Fig E3) but in keeping with the finding, at mutation analysis, of no evidence of the familial mutation (see above).

Rapid functional analysis of 2B4 in peripheral blood lymphocytes

We evaluated the 2B4 function in a standard ^{51}Cr-release assay. PBMC from XLP1 or healthy donors, short term cultured with rIL-2, were used as effectors in R-ADCC assays against the ($Fc\gamma R^+$) P815 target cell line, either in the absence or in the presence of the anti-2B4 mAb, alone or in combination with anti-NKp46 (activating NK receptor) mAb (Fig 1 and Fig E4, *A*). In UPN360 NK cells, anti-2B4 mAb inhibited both spontaneous and NKp46-induced target cell lysis (Fig 1, *C*). 2B4 engagement strongly enhanced paternal NK cytotoxicity, while in the mother expressing SAP in a minority of NK cells, 2B4 engagement resulted in a neutral (neither activating nor inhibitory) effect. 2B4 had no functional impact in SAP^{dull} UPN674 (Fig 1, *D*), while in his mother it enhanced spontaneous and NKp46-mediated cytotoxicity. Interestingly, this family was characterized by a missense mutation (p.S28R) (Table I), previously reported to shorten the half-life of the protein.31

Similar results were obtained when performing R-ADCC assays followed by cytofluorimetric detection of CD107a exposure on the NK cell surface during degranulation. In healthy controls, 2B4 engagement enhanced NKp46-mediated degranulation ($P<0.0001$, Fig 2 and Fig E4, *B*), while in UPN360 it decreased CD107a expression, and in his mother it had a neutral effect. PIDs other than XLP1 are shown for comparison (Fig 2). XLP2 and FHL2 samples showed normal activating 2B4 function; in FHL3, consistent with the degranulation defect, 2B4 as well as NKp46 engagement could not induce CD107a expression. Notably, UPN925 (XLP2) was studied during active EBV infection and NK cells appeared activated with high degranulation capability.

SAP expression and 2B4 function in polyclonal activated NK cells from XLP1 patients and relatives

NK cells from UPN360, UPN674 and their family members were expanded in culture to obtain polyclonal activated NK cell populations. Cytofluorimetric analysis showed a SAP expression pattern similar to that detected in peripheral blood NK cells (Fig E5). However, in R-ADCC, 2B4 inhibited the NK-mediated lysis not only in UPN360 but also in UPN674, in whom the 2B4 function resulted neutral using as effectors short term activated PBMC (Fig 1, *D*). On the other hand, in the mother of UPN360 (SAP^+/SAP^-) neither 2B4-mediated activation nor inhibition could be observed even using polyclonal activated NK cells (Fig E5).

2B4 always exerted an inhibitory function in all UPN patients included in the study, regardless of whether they carried mutations that caused absent or reduced expression of SAP (Table I). This 2B4-mediated inhibitory function was never been observed in other PIDs as well as in patients with chronic active EBV infection (CAEBV) or lymphoma (Fig E6).

Identification of a *SH2D1A* mutation leading to normal SAP expression but defective 2B4 function

HEK-293T cells transfected with plasmid coding for the different SAP mutated proteins identified in UPN patients were characterized by a negative staining (not shown). This was expected to occur also in the previously described10,25 XLP1 patients (identified as BS), not referred to the HLH registry. Notably however, cells transfected with plasmid carrying the c.164G>T *SH2D1A* mutation,32 identified in BS-2, showed a positive SAP staining (Fig 3, *A*). Accordingly, both resting and activated BS-2 NK cells expressed SAP with MRFI values falling within the range of healthy controls (Fig 3, *B*). Normal SAP expression was confirmed in western blot analysis (Fig 3, *C*). Importantly, in BS-2 NK cells 2B4 clearly demonstrated an inhibitory role (Fig 3, *D*).

SAP was not detected in NK cells of BS-1 (Fig 3, *B*) and BS-3 patients (Table I). Interestingly, patient BS-1 had a minority of T cells (12%) expressing normal levels of SAP (Table I; Fig 3, *B*). This finding is consistent with previous report of somatic reversion in $CD8^+$ T cells under selective pressure by EBV infection in patients with a missense or nonsense *SH2D1A* mutations.33

Reduced repertoire of immune-competent NK cells in SAP^{+}/SAP^{-} female carriers

Polyclonal NK cell populations derived from SAP^{-}, SAP^{dull} and loss-of-function mutated SAP^{+} (i.e. BS-2) XLP1 patients, displayed poor cytolytic activity against 221 B-EBV cells (LU_{30}=31±4.9; mean±SEM) as compared with healthy controls (LU_{30}=492±68.6) (Fig 4, *A*). To analyze the cytotoxic capabilities of carriers, we obtained SAP^{+} or SAP^{-} NK cell clones from UPN360's mother. In R-ADCC assays 2B4 crosslinking increased the cytotoxicity of SAP^{+} while decreasing that of SAP^{-} clones (Fig 4, *B*). Moreover, SAP^{-} NK cell clones displayed reduced or absent cytotoxicity against CD48^{+} 221 cells (Fig 4, *C*), while their ability to kill CD48^{-} K562 target cells was comparable to that of SAP^{+} NK cell clones (Fig 4, *D*).

Data at clonal level show that carriers of XLP1 mutations abolishing SAP expression have a reduced repertoire of immunocompetent NK cells. This likely explains the neutral 2B4 function at the polyclonal cell level (Fig 1, Fig 2 and Fig E5).

Discussion

Reference clinical centers dealing with HLH and congenital immunodeficiencies may be asked to provide a rapid and differential diagnosis. Yet, in some cases HLH may represent an XLP1 phenotype.27 Mutation analysis remains the gold standard. However, it is costly and not universally, rapidly available. Specific expertise is required, with more than 70 reported mutations, including exon(s) deletions, nonsense, small insertion/deletion, missense, and splice site mutations.6,16 Furthermore, novel mutations are increasingly reported, as in the present series, and the recent report of intronic mutation extends the standard of mutation analysis.34

In this study, we describe our experience in the investigation of patients who presented with HLH syndrome and needed differential diagnosis between "secondary" and "primary/genetic" HLH and, in the latter case, discrimination among the various genetic subtypes. Seven HLH patients were characterized by the lack of SAP expression and were clearly XLP1. In two patients however, NK cells showed SAP expression but with MRFI values below the range of healthy controls (<5÷21, SAP^{dull}). In UPN674, the c.84C>G missense mutation caused a single amino acid change (S28R) that resulted in reduced protein half-life;31 in UPN562 the c.162C>G p.Y54X mutation produced a premature stop codon. Notably, XLP1 patient BS-2 had MRFI values overlapping those of normal controls. Intriguingly, the mutation R55L detected in this patient, as previously described by Pasquier et al (R55P),35 does not affect SAP expression and detection; yet, it strongly affects the SAP function (Fig 3). Thus, caution should be used before ruling out the diagnosis of XLP1 based on the gross cytofluorimetric analysis of SAP expression in peripheral blood NK (or T) cells. In addition, some patients were tested for presence of iNKT cells which were nearly completely absent (Table I), in keeping with previous data.35,36

Importantly, all our patients were clearly classified as XLP1 by the detection of 2B4 dysfunction, regardless of the result of flow-cytometry SAP detection, ranging from negative to markedly positive (BS-2). Investigation of the 2B4 receptor function in peripheral blood lymphocytes by the CD107a assay is a reliable, rapid, easy and inexpensive method to

document the molecular dysfunction characterizing XLP1 (Fig 2). It provided normal results in all controls, including patients referred for acute, persistent of clinically aggressive EBV infection, with the only exception of XLP1 patients.

Thus, our approach based on both phenotypic and functional assays can be used by reference immunology laboratories to precisely identify all XLP1 patients and differentiate between various inherited immunodeficiencies, implementing the already described diagnostic algorithm.22 The use of transfectant cells may allow full investigation also of some patients for whom only genetic material, but no more viable cells, are available.

The genetic study of our patients confirms the lack of recurrent mutations in XLP1 and provides identification of three novel SAP mutations. Furthermore, we had two unexpected findings. First, in UPN489 we described at cDNA level the absence of the active transcript with the only presence of the Δ55 isoform.11 Second, although mothers of XLP1 patients are expected to be obligate carriers, the UPN627 mother lacked the mutation documented in her son. We have not been able to discriminate between a *de novo* mutation and the alternative hypothesis of germinal mosaicism; this event had been previously reported in a single case, in whom haplotype analysis showed the paternal origin of the mutation.37 Analysis of SAP expression and 2B4 function might be a screening tool for XLP1 female carriers. It is of note however that at polyclonal cell level the 2B4 dysfunction is manifest only in carriers characterized by the lack of SAP expression in the majority of NK cells (as in mother of UPN360).

In conclusion, we considered that the diagnostic approach to HLH has been for a long time an unmet need. Screening tools are now available for selection of patients to be addressed to genetic analysis. In this frame, the testing for XLP1 has previously been less than optimal and there was therefore a need for improvement of the diagnostic algorithm. Thus, the aim of our work is to improve the efficiency of the diagnostic screening of patients with suspected HLH, which will comprise a subpopulation of patients with XLP1 (Figure E1). This method will also be suitable for those settings in which patients with later and/or non HLH-like onset will be assessed. This study has limitations: in particular, since XLP1 is a rare disease, the number of patients tested is relatively low; thus generalization of the testing strategy proposed to all XLP1 remains to be confirmed in a larger population which might be collected by a cooperative group or consortium. Nevertheless, our data confirm that the cytofluorimetric analysis of SAP expression in peripheral blood lymphocytes has a good specificity for prediction of genetic diagnosis of XLP1, but in rare cases its sensitivity may be insufficient. Thus, patients with a compatible clinical picture, expressing SAP at flow-cytometry screening, should also be investigated by a second-level method, consisting of rapid evaluation of the 2B4 receptor inhibitory effect followed by mutation analysis, before safely excluding the diagnosis of XLP1 (Fig E1). Complete characterization of the genotype of patients with XLP1 will also be useful in the development of a future gene transfer therapeutic approach.38

Online Repository Materials

Mutation analysis

Genomic DNA was isolated from peripheral blood samples using Qiacube Workstation (Qiagen, Jesi, Italy). The four coding exons and exon-intron boundaries of the SH2D1A gene were amplified and directly sequenced, in both directions, with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). Amplification reactions were performed with 60 ng of DNA, 10 ng of each primer, 200 mM dNTPs, 1X PCR reaction buffer and 2.5U Taq polymerase in a final volume of 25 ml; primers are available upon request. Sequences obtained using an ABI Prism 3130XL Sequence Detection System (Applied Biosystems) were analyzed and compared with the reported gene structure (NG_007464.1, NCBI) using the dedicated software SeqScape (Applied Biosystems).

In silico analysis was performed using the web query tools (Pmut, Polyphen, SIFT).

To confirm the disruptive effect of the mutation c.137+3_+6delGACT, Real-Time PCR experiment were performed with primer/probes spanning exon 3-4 junction to investigate the presence or absence of SH2D1A mRNA expression; GAPDH was used as endogenous control and a healthy donor as reference sample.

MLPA analysis

MLPA (Multiplex ligation-dependent probe amplification) was performed on genomic DNA according to the manufacturer's instructions (http://www.mrc-holland.com), by P205 SALSA MLPA probe; it includes two probes for each of the four exons of SH2D1A, 7 reference probes on the X chromosome, two autosomal reference probes, and a Y chromosome specific probe. Reaction products were run on ABI Prism 3130XL Sequence Detection System. Normalization was obtained by dividing the peak area of each probe's amplification product by the combined peak areas of all probes (intra-normalization). Inter-sample normalization was obtained by dividing normalized probe ratios of a sample by the average ratio of all reference samples (DNA samples from healthy individuals). Threshold values of loss and gain of genetic material were set at 0.7 and 1.30, respectively. All samples showing evidence for deletions or duplications were replicated at least twice.

Assays on expanded activated NK and T cells

Purified NK cells (RosetteSep method, StemCell Technologies, Vancouver, BC, Canada) were cultured on irradiated feeder cells in the presence of phytohemagglutinin (PHA) (2 μg/mL) (Sigma-Aldrich, Irvine, UK) and rIL-2 (600 IU/mL) to obtain polyclonal NK cell populations or, after limiting dilution, clonal NK cells. To obtain expansion of activated T lymphocytes, PBMC were cultured with rIL-2 (100 IU/ml) and PHA (1.25 μg/ml). Intracellular SAP expression was evaluated as described above. In ^{51}Cr-release assays, activated NK cells were analyzed for 2B4 function in R-ADCC, and cytotoxicity against 721.221 ($CD48^+$, hereafter termed 221) B-EBV and K562 ($CD48^-$) cell lines. Lytic units (LU) at 30% lysis were calculated.

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Analysis of SH2D1A transcript

Total RNA was extracted from polyclonal NK populations using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The cDNA coding for the different SAP proteins were amplified,8 cloned into pcDNA 3.1/V5-His5-TOPO expression vector using the Eukaryotic TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and sequenced.

HEK-293T transient transfections

HEK-293T cells were transiently transfected with the plasmids coding for different SAP proteins (either w.t or mutated) using the linear polyethylenimine derivative jetPEI transfection reagent (Polyplus, New York, NY). Forty-eight hours after the transfection, the cells were intracellularly stained with the anti-SAP 1C9 mAb followed by anti-IgG_{2a}-PE. Staining with anti-vimentin mAb (V9, IgG1, Santa Cruz Biotechnology, Santa Cruz, CA) was used to assess permeabilization of the samples.

Immunoblotting

Cell lysates from activated lymphocytes were separated on 15% acrylamide gels and transferred to nitrocellulose. Membranes were blocked with PBS + 5% skimmed milk powder + 0.2% Tween 20, incubated overnight with rabbit anti-SH2D1A (Cell Signaling Technology, Hitchin, UK), washed with PBS + 0.2% Tween 20, incubated for 45-60 min. with anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), washed and then developed using Uptilight (Cheshire Sciences, Chester, UK). Membranes were re-probed with mouse anti-β actin (Sigma-Aldrich, St. Louis, MO) and anti-mouse HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories).

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Extended Data

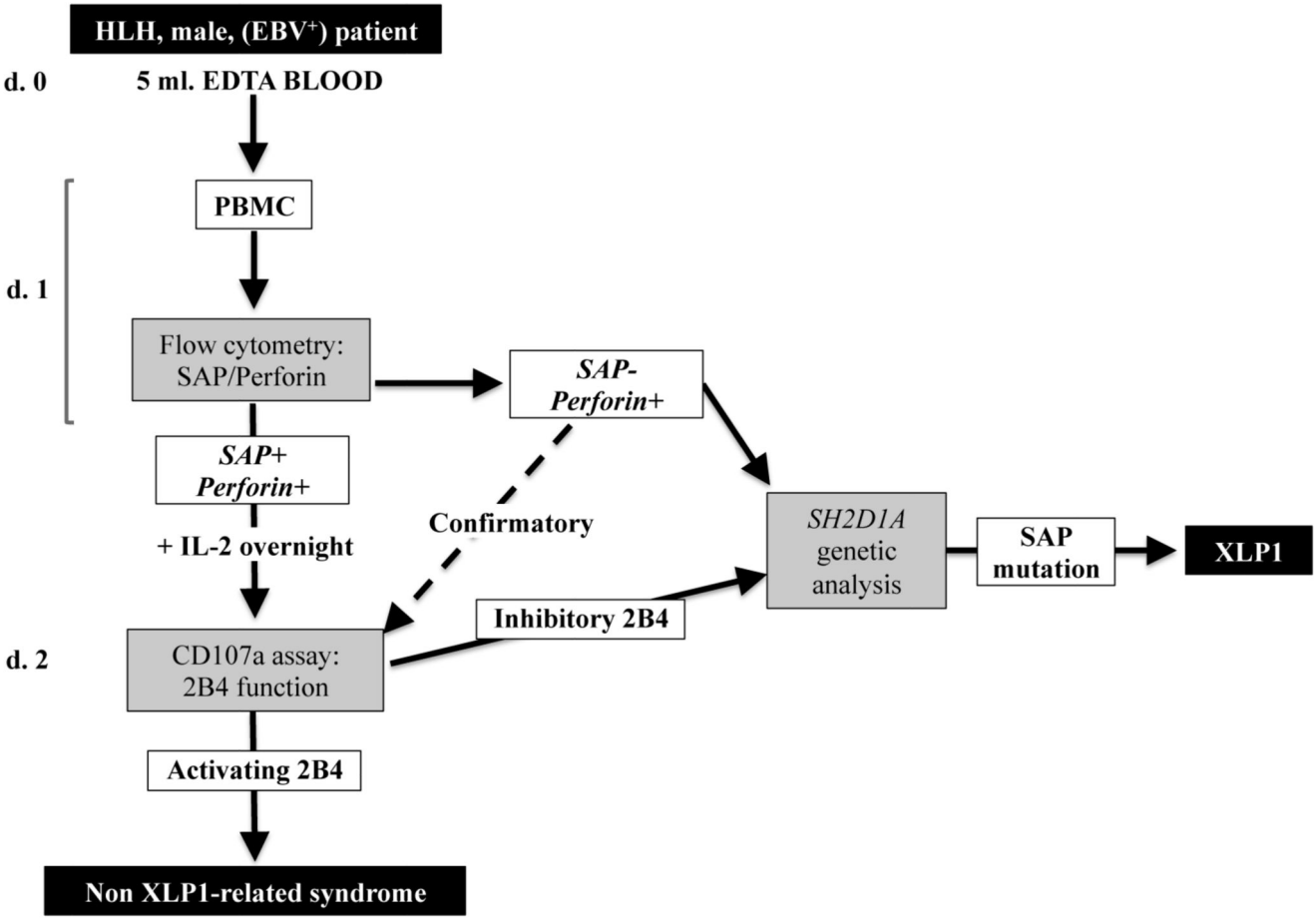

Fig E1.

Proposed rapid XLP1 diagnostic algorithm based on the combined cytofluorimetric analysis of SAP and CD107a expression for patients presenting with HLH. Specialized immunology centers receiving blood samples can suspect XLP1 in (perforin+) PBMC characterized by SAP^{-} or SAP^{dull} phenotype. Analysis of 2B4 function (CD107a) is also recommended. Patients with defective SAP expression and/or inhibitory 2B4 are directed to *SH2D1A* mutation analysis.

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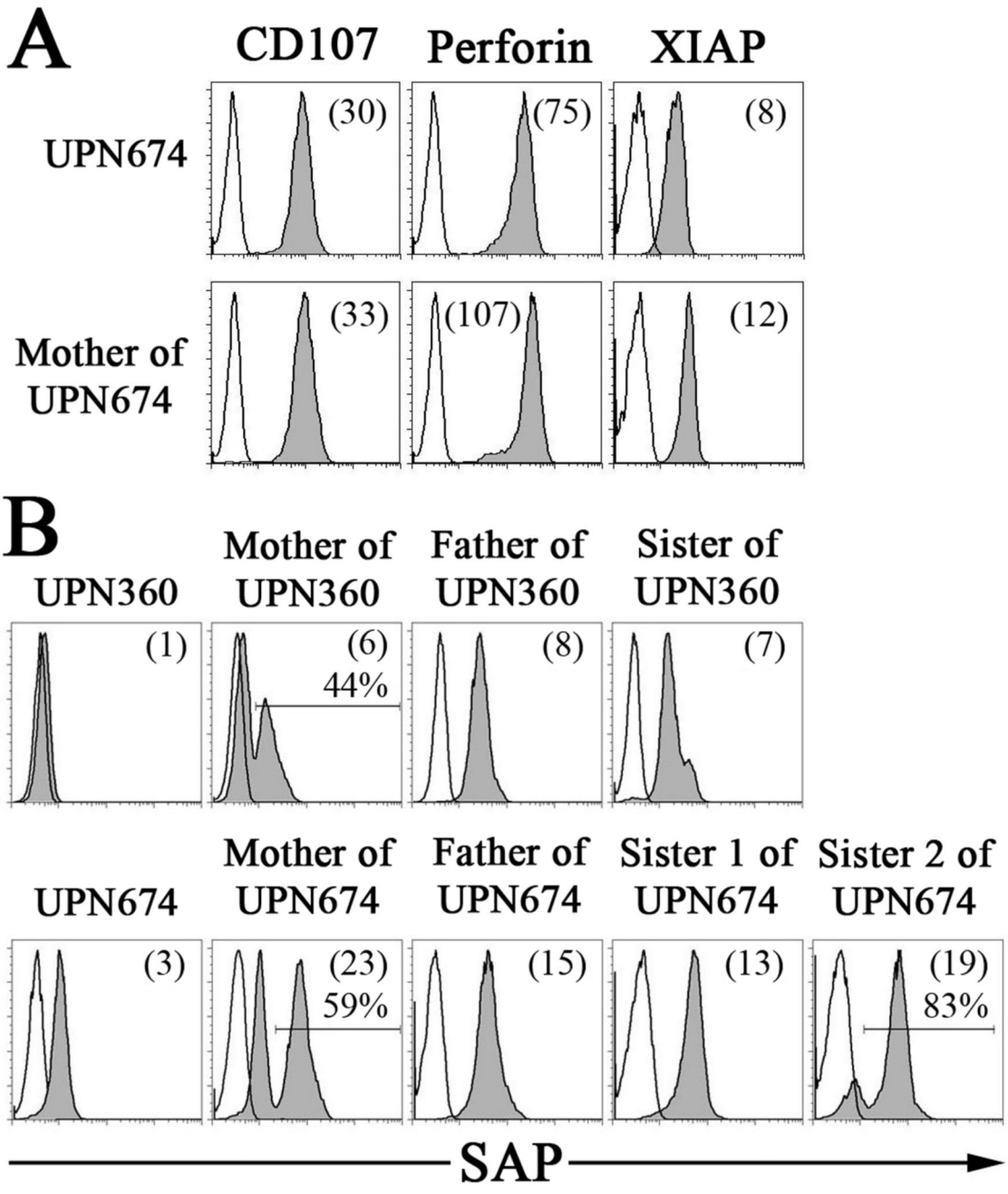

Fig E2.

A, CD107, perforin and XIAP expression in peripheral blood NK cells of UPN674 and his mother (flow cytometry). Empty profiles represent the isotype matched negative controls. MRFI (numbers in brackets) are indicated. **B**, SAP expression in peripheral blood T cells of UPN360, UPN674 and their family members (flow cytometry). The percentage of SAP^{+} (mother of UPN360) or SAP^{bright} cells (mother and sister 2 of UPN674) are indicated.

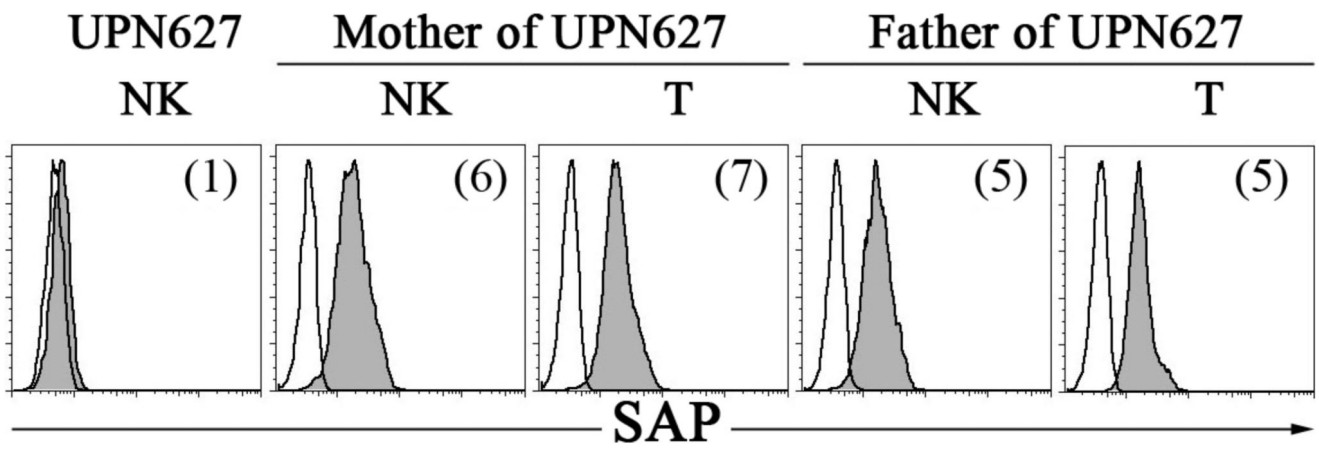

Fig E3.

SAP expression in polyclonal activated NK cells from patient UPN627 as compared with peripheral blood NK and T cells from his mother and father (flow cytometry). MRFI (numbers in brackets) are indicated.

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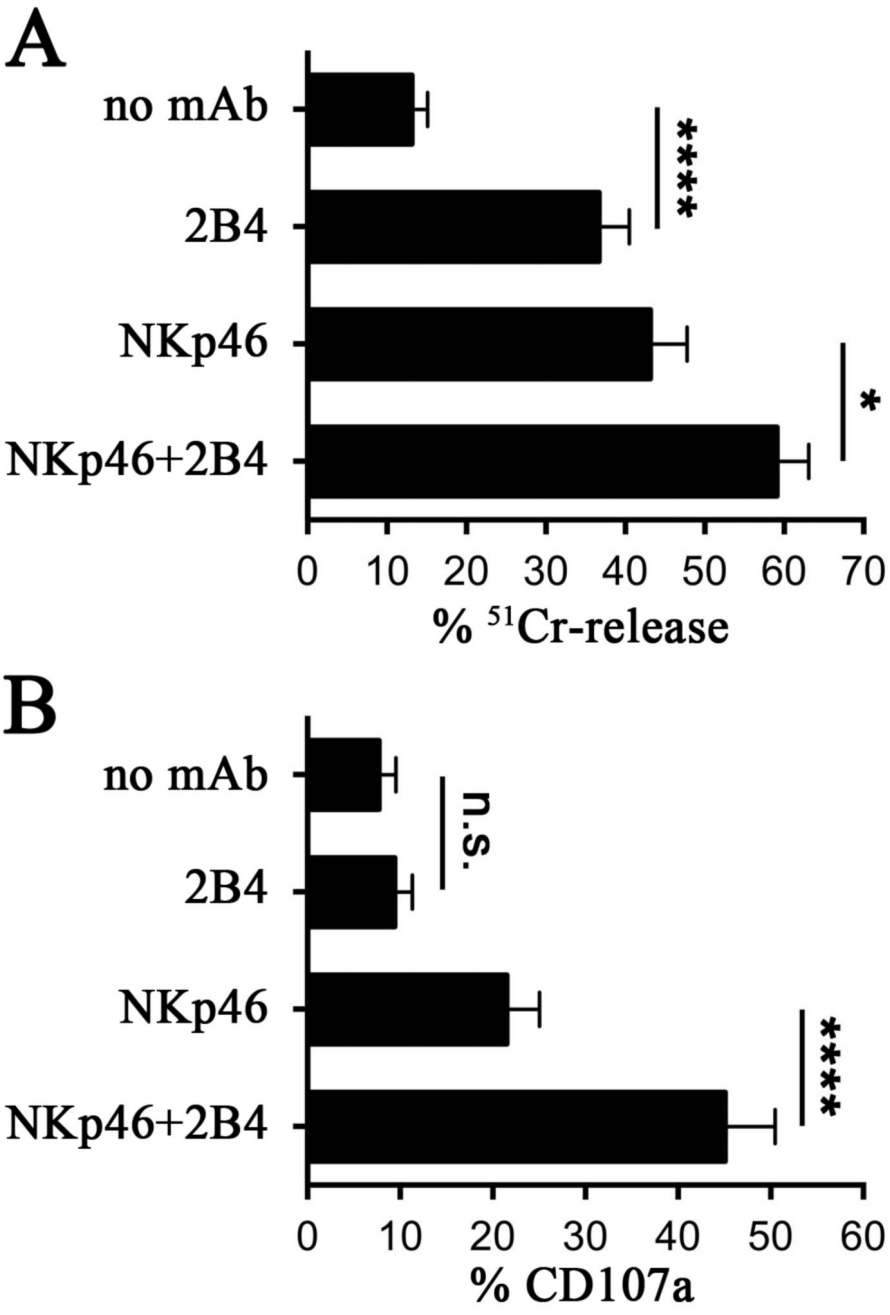

Fig E4.

Rapid 2B4 function evaluation in healthy controls. R-ADCC assays against P815 target cells with or without mAbs to the indicated molecules were evaluated in healthy controls, using either **(A)** rIL-2 short term cultured PBMC from 22 donors (^{51}Cr-release assay, E:T ratio 10:1), or **(B)** rIL-2 overnight activated PBMC from 11 donors (CD107a assay). Mean with SEM and significance are shown (ANOVA 1-way and Bonferroni's post-test).

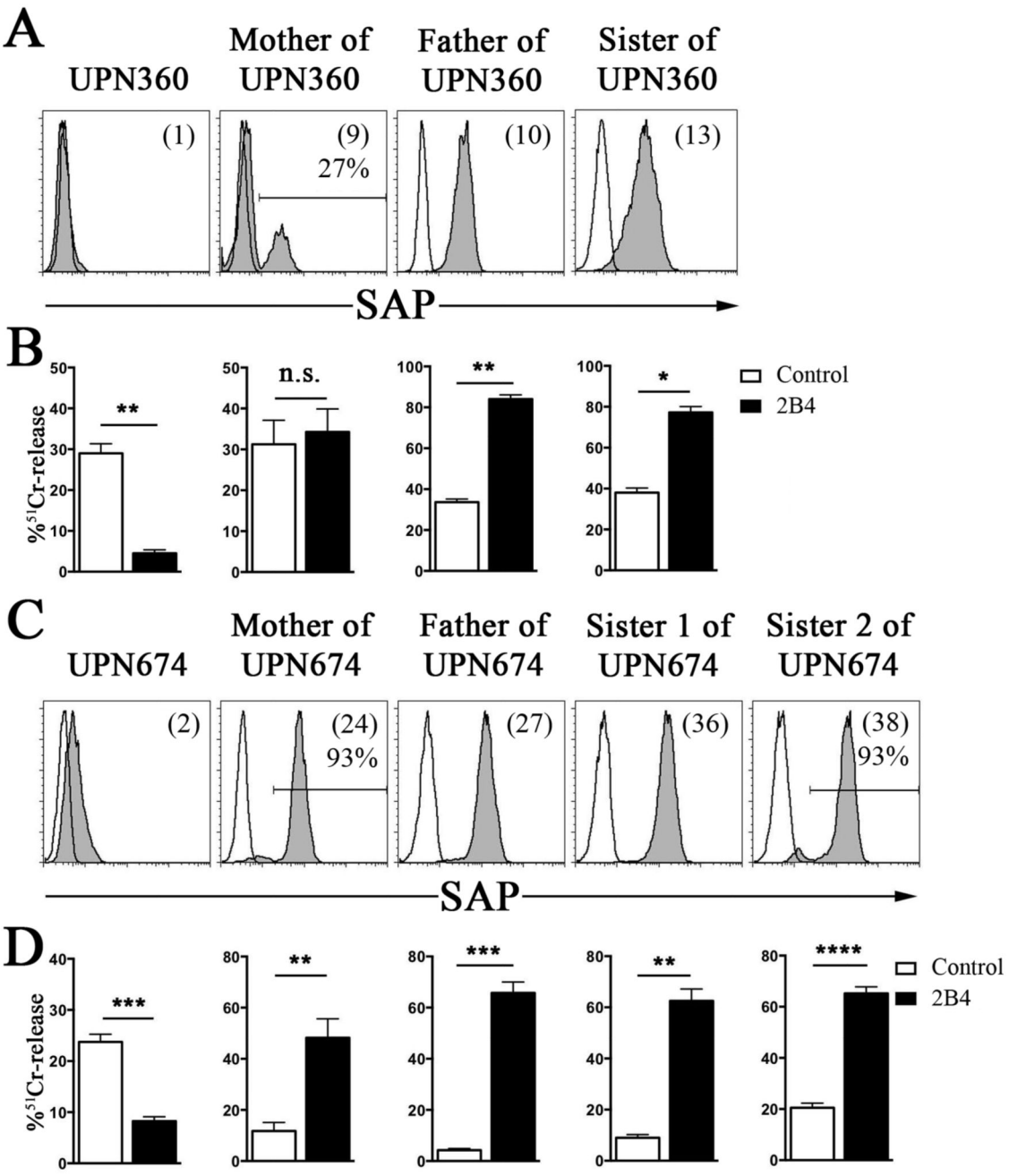

Fig E5.

A,C, SAP expression (flow cytometry) and **(B,D)** 2B4 function (^{51}Cr-release R-ADCC assays against P815 target cells, E:T ratio 4:1) in polyclonal activated NK cells from XLP1 patients and family members. (A,C) MRFI (Numbers in brackets) and the percentage of SAP^{+} or SAP^{bright} cells are indicated. (B,D) Mean with SEM and significance are shown (paired t test).

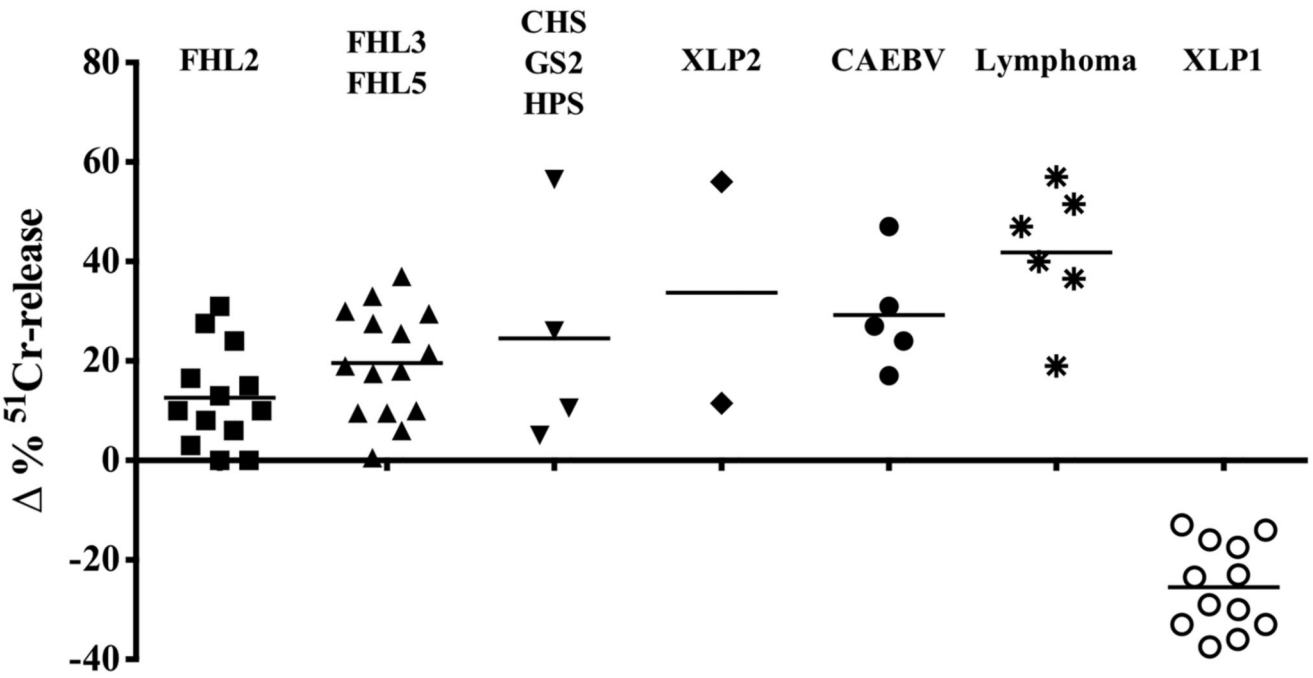

Fig E6.

2B4 inhibitory function selectively identifies XLP1. D % ^{51}Cr-release of lysis in the presence – in the absence of anti-2B4 mAb (R-ADCC assays against P815 target cells, E:T ratio 4:1) in polyclonal activated NK cells from different groups of patients and XLP1, as indicated. CAEBV represents patients with chronic active EBV infection. Each symbol represents a different case, lines indicate mean values.

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Abbreviations

HLH	Hemophagocytic lymphohistiocytosis
FHL	Familial Hemophagocytic lymphohistiocytosis
XLP	X-linked lymphoproliferative disease
SLAM	signaling lymphocyte activation molecule
SAP	SLAM-associated protein
EBV	Epstein-Barr virus

PID	primary immune deficiency
CAEBV	chronic active EBV infection

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Clinical Implications

The use of 2B4 receptor function assay, in combination with SAP expression in peripheral blood lymphocytes, may help to refine the selection of patients with XLP1 who deserve mutation analysis.

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Capsule summary

To rapidly and safely diagnose XLP1 and thus define indication to mutation analysis, transplantation and counseling, SAP expression screening should be integrated, in patients with compatible clinical picture, by analysis of 2B4 function.

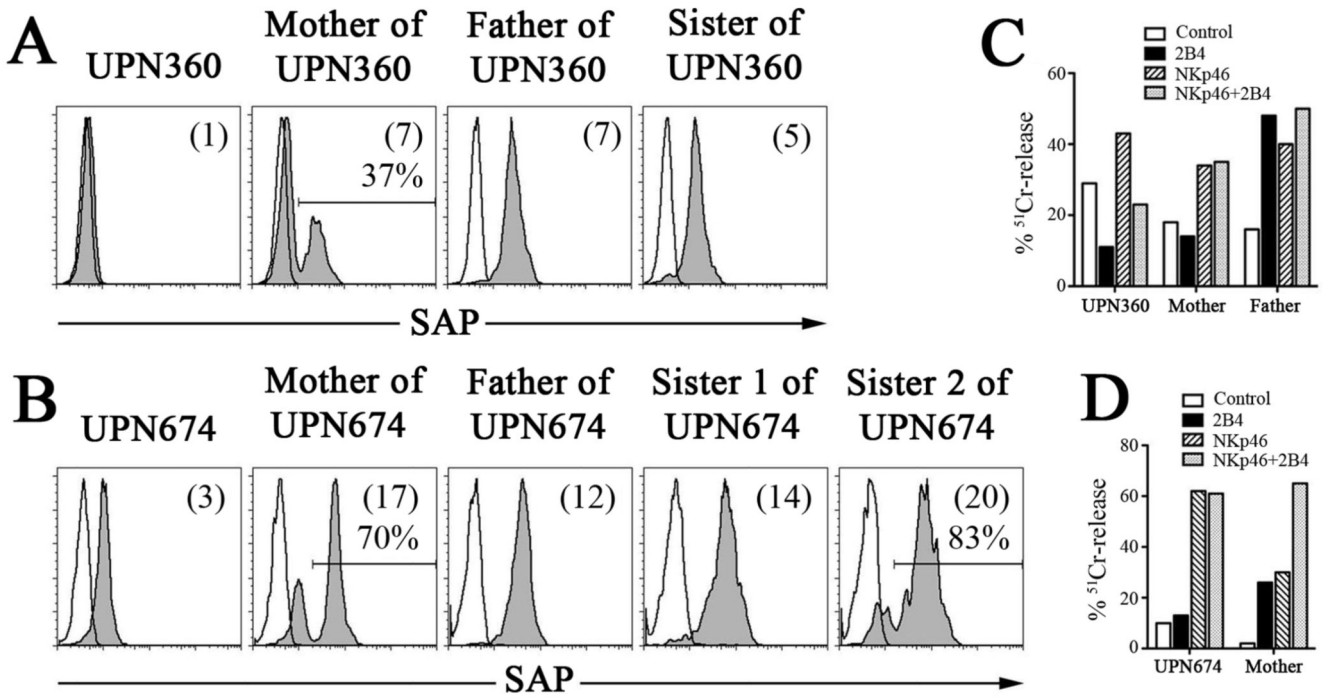

Figure 1. SAP expression and 2B4 function in peripheral blood mononuclear cells

(A,B) PBMC of XLP1 patients and relatives were analyzed for intra-cytoplasmic SAP expression in NK cells. MRFI (Numbers in brackets) and the percentage of SAP^{+} or SAP^{bright} cells are indicated. (C,D) rIL-2 short-term cultured PBMC were tested against ^{51}Cr-labeled P815 target cells (E:T ratio 10:1) either in the absence (Control) or in the presence of mAbs to the indicated molecules.

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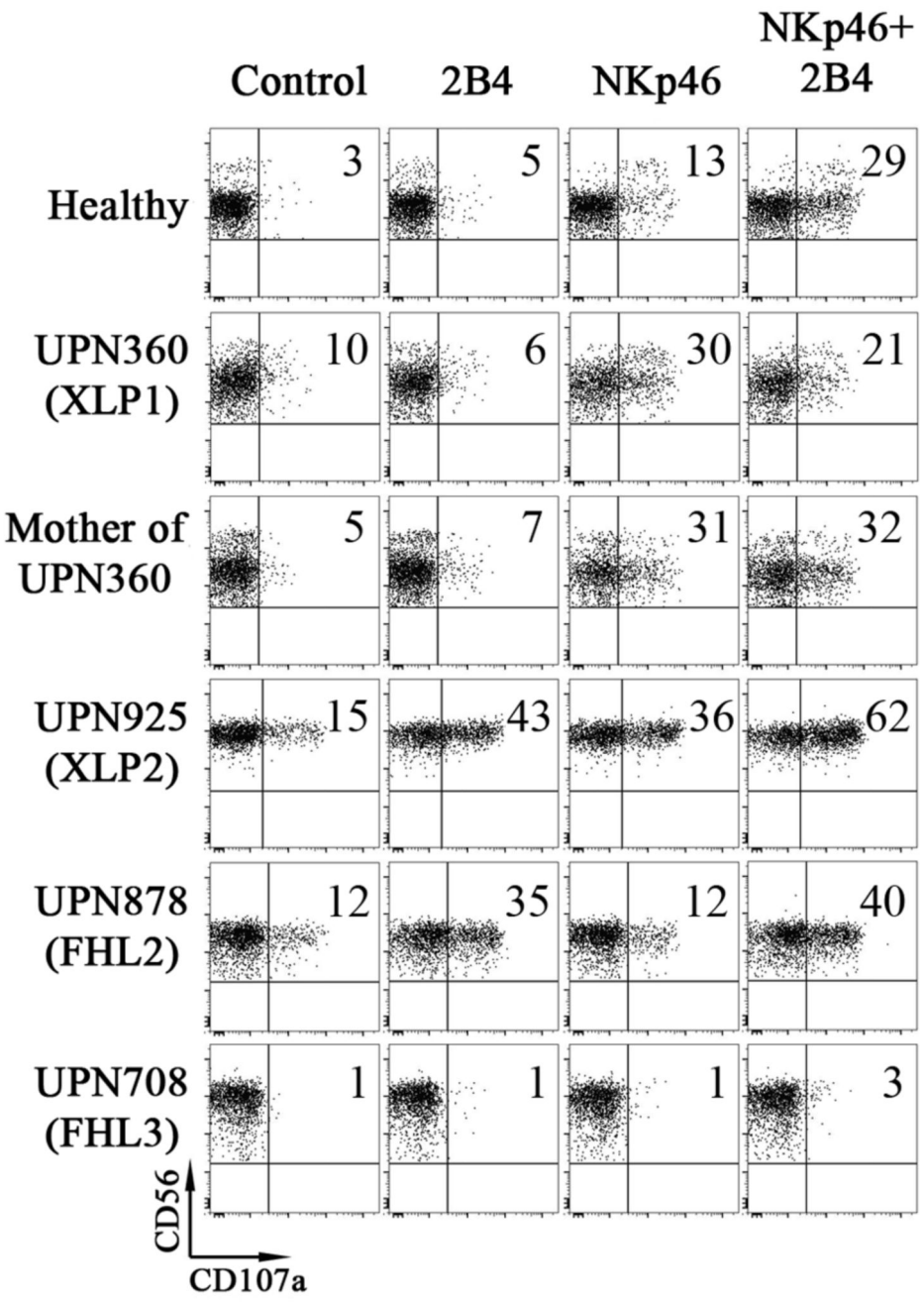

Figure 2. Rapid 2B4 function evaluation by CD107a assay

rIL-2 overnight activated PBMC of UPN360 and his mother were tested in R-ADCC against P815 target cells with or without mAbs to the indicated molecules. Percentage of $CD107a^{+}$ NK cells are indicated. An healthy control and other PID are shown for comparison.

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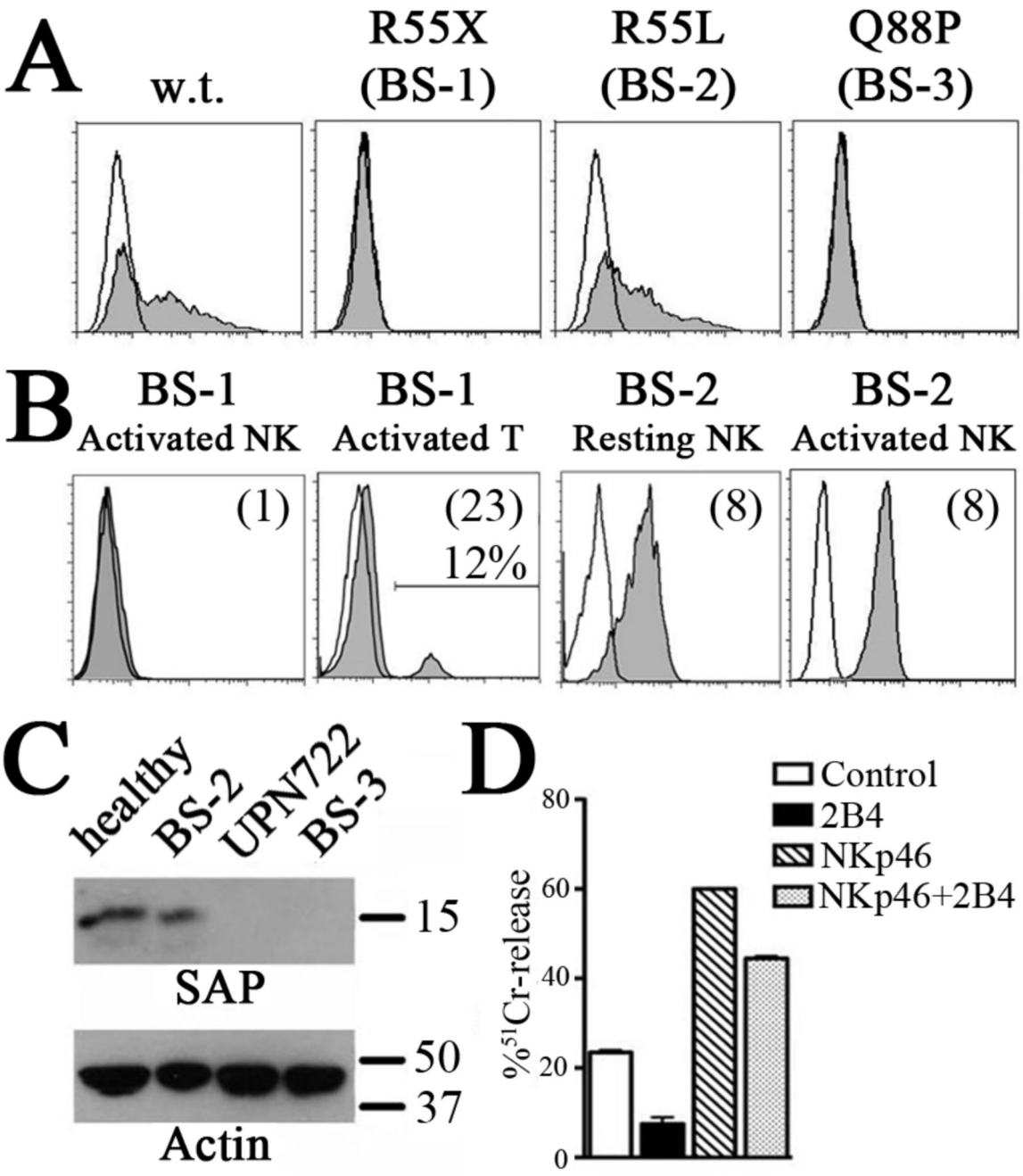

Figure 3. Unexpected discrepancy between normal SAP expression and 2B4 inhibitory function in NK cells from a patient carrying R55L mutation

Intracytoplasmic SAP expression (flow-cytometry) in (A) HEK-293T cells transfected with w.t or SAP mutants from BS patients, (B) BS-1 and BS-2 lymphocytes. (C) Western blot analysis of SAP expression in polyclonal NK cell lysates of the indicated patients and healthy control. (D) 2B4 function (R-ADCC assays against P815 target cells, E:T ratio 4:1) in BS-2 activated NK cells.

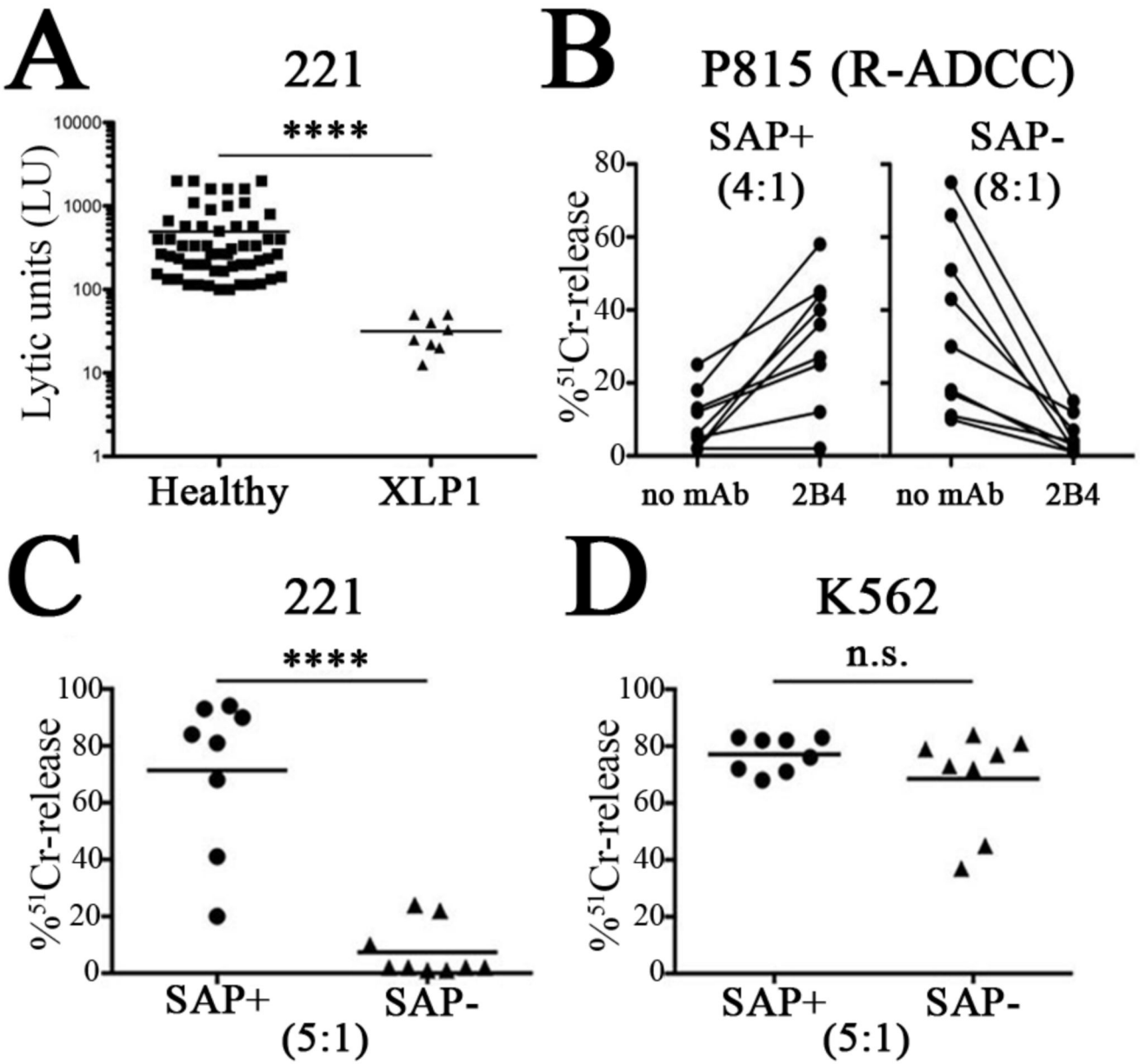

Figure 4. Decreased capability of SAP-defective NK cells from patients and carriers to lyse 221 B-EBV cell line

Cytolytic activity against the indicated targets of (A) polyclonal activated NK cells of patients (UPN360, UPN489, UPN562, UPN590, UPN627, UPN674, UPN722, BS-2) and 57 healthy donors, (B-D) SAP^+ and SAP^- NK cell clones from UPN360 mother. E:T ratios are indicated in brackets. Mean of the values and significance are shown (Mann-Whitney test).

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Table I

Results of mutation analysis, SAP expression and 2B4 function in XLP1 patients

Patient	Age at diagnosis (years)	Clinical picture and current status	Evidence of EBV infection (previous or recent)	*SH2D1A* mutations	Reference mutation/ Novel	SAP expression NK or T cells[§]	2B4 function NK cells R-ADCC[#]	iNKT[^]
UPN98 [*]	2.5	HLH (2 yr) SCT at 3.1 years; rejected. Dead of progressive disease at 8 years.	Yes	c.163C>T p.R55X	Ref. 5	n.d.	n.d.	n.d.
UPN199 [*]	18	HLH (18 yr). Dead of progressive disease	Yes	(entire exon 2 deletion)	Ref. 11	n.d.	n.d.	n.d.
UPN218	2	HLH (2 yr). Dead of progressive disease	No	c.89T>C p.L30S	Novel	1 (T cells)	Inhibitory	n.d.
UPN221	1	HLH (1 yr); hypogamma (3 yr). Alive, asymptomatic on replacement therapy (33 yr)	Yes	c.228T>A p.Y76X	Ref. 27	1 (T cells)	Inhibitory	n.d.
UPN222 [*]	2.2	HLH (2.2 yr). Dead of progressive disease	Yes	(entire exon 2 deletion)	Ref. 11	n.d.	n.d.	n.d.
UPN360	5.8	HLH (5.8 yr) with severe encephalopathy and secondary autism; entero-colitis (13 yr) requiring resection. Alive, no SCT due to severe neurologic sequelae (13.5 yr)	Yes	c.138_201del (entire exon 2 deletion)	Ref. 11	1 (NK cells)	Inhibitory	0.08
UPN489	4.4	HLH (4.4 yr) with severe encephalopathy. Dead of neurologic sequelae (5.4 yr)	Yes	loss of active transcript (only Δ55)	Novel	1 (NK cells)	Inhibitory	<0.05
UPN562	1.6	HLH (1.6 yr). Dead of progressive disease	Yes	c.162C>G p.Y54X	Novel	2 (NK cells)	Inhibitory	n.d.
UPN590	2.8	HLH (2.8 yr); SCT (3 yr). Alive, cured after SCT (6 yr)	Yes	Del exons 2-4	Ref. 28	1 (NK cells)	Inhibitory	<0.05
UPN627	13.1	IM (9 yr); Hypogamma (11 yr); SCT (15 yr). Alive, cured after SCT (16 yr)	Yes	c.138(-2)A>G (skipping of exon 2)	Ref. 28	1 (NK cells)	Inhibitory	0.08
UPN674	2.0	Asymptomatic, diagnosis in preclinical phase[¶]; SCT (2 yr). Alive, cured after SCT (4 yr)	No	c.84C>G p.S28R	Ref. 28	2 (NK cells)	Inhibitory	0.06
UPN722	12	Burkitt lymphoma (4 yr); encephalopathy (11 yr); HLH (12 yr); SCT (12 yr). Dead of SCT-related complications (13 yr)	Yes	c.137+3_+6delGACT (loss of transcript)	Ref. 29	1 (NK cells)	Inhibitory	<0.05
BS-1	36	Histiocytic lymphoma (14 yr); hepatitis (21 yr); recurrent pneumonia and sinusitis	Yes	c.163C>T p.R55X	Ref. 5	1 (NK cells) 23 (12% T cells)	Inhibitory	n.d.
BS-2	6	IM (4 yr); hypogamma; recurrent respiratory infections	Yes	c.164G>T p.R55L	Ref. 32	8 (NK cells)	Inhibitory	n.d.
BS-3	19	recurrent respiratory infections; hypogamma (3 yr)	Yes	c.263A>C p.Q88P	Ref. 25	1 (NK cells)	Inhibitory	n.d.

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UPN: unique patient number in the HLH registry. Patients UPN98 (HLH-SM), UPN199 (HLH-PZ), UPN222 (HLH-OT) have been previously reported in Ref. 27. Patients identified as BS have been diagnosed at University of Brescia and they have been already described in previous papers: BS-1 corresponds to patient B reported in Ref. 10; BS-2 corresponds to case 1, and BS-3 to case 2 reported in Ref. 25.

HLH, hemophagocytic lymphohistiocytosis; hypogamma, hypogammaglobulinemia; IM, infectious mononucleosis; SCT, stem cell transplant; n.d., not determined. Values in parenthesis indicate age of presentation of each clinical feature (yr, years).

[*] Cases received as DNA only for mutation analysis.

[¶] Brother of two males with the same *SH2D1A* mutation who died of EBV infection and Burkitt lymphoma.

[§] Intracytoplasmic expression by flow cytometry analyzing activated lymphocytes; values indicate the MRFI (see “Materials and methods”). In 16 healthy controls, the normal range was 5÷36 with a mean±SD of 14±8.

[#] 2B4 function was assessed in polyclonal activated NK cells using ^{51}Cr-release or CD107a assays. Information on UPN218, UPN221 refer to data obtained in the past, when these NK cells were still available.

[^] Percentage of iNKT in PBL