or *CBL* (**Fig. 2a**). Mutations in *SH3BP1*, encoding SH3 domain–binding protein 1, were not recurrent. All *SETBP1* mutations were heterozygous and occurred within the portion of the gene encoding the SKI domain, with six identical to the *de novo* recurrent mutations reported in SGS and five identical to the mutation encoding the p.Asp868Asn alteration (**Fig. 1b**). RT-PCR analysis showed that the wild-type and mutant alleles of *SETBP1* were equally expressed (**Supplementary Fig. 8**). Similarly, 8 of the 11 *JAK3* mutations in 10 cases were the well-described activating mutation (encoding a p.Arg657Gln alteration) found in various hematological malignancies, including Down syndrome–associated acute megakaryoblastic leukemia[19–23], ALL[24,25] and natural killer (NK)/T cell lymphoma[26], and the remaining 3 were also within the portions of the gene encoding the pseudokinase or kinase domain, suggestive of gain of function.

Deep sequencing of the relevant mutant alleles enabled an accurate estimation of allele frequencies for individual mutations (**Supplementary Fig. 9**). *SETBP1* and *JAK3* mutations showed lower allele frequencies (but not with statistical significance for *SETBP1*) than did the corresponding RAS pathway mutations (**Supplementary Fig. 10a**), indicating that the former mutations represent secondary genetic hits that contributed to clonal evolution after the main tumor population was established (**Supplementary Fig. 10b**). Individuals with secondary mutations had shorter lengths of survival compared to those without mutations: 5-year overall survival (hazards ratio (HR) = 1.90, 95% CI = 0.87–4.19). In addition, none of the individuals with JMML who survived without hematopoietic stem cell transplantation (HSCT; $n = 26$) harbored any of the secondary mutations, and individuals with secondary mutations showed significantly inferior 5-year transplant-free survival (HR = 2.18, 95% CI = 1.18–4.02) (**Fig. 2b–d** and **Table 2**).

JMML is characterized by a paucity of gene mutations. The average number of mutations per sample (0.85; range of 0–4) was unexpectedly low compared to those reported in other human cancers (**Supplementary Fig. 11**); excluding common RAS pathway mutations, only 5 mutations were detected in 3 of the 13 discovery cases. This small number of mutations is only comparable to the figure reported for retinoblastoma (mean of 3.3 per case; range of 0–5) (ref. 27) and is in stark contrast to the abundance of gene mutations in chronic myelomonocytic leukemia (CMML) in adult cases, where the mean number of non-silent mutations was 12.4 per sample, of which 3.1 represented known driver changes (ref. 17 and K.Y., M.S., Y.S., D. Nowak, Y. Nagata *et al.*, unpublished data), underscoring the distinct pathogenesis in these two neoplasms that show indistinguishable morphology. The impact of germline events is underscored by the fact that 6 of the 13 discovery cases harbored germline RAS pathway mutations and an additional case without known RAS pathway mutations showed constitutive abnormalities similar to Noonan syndrome. Despite the central role of RAS pathway mutations, a small subset of cases had no documented RAS pathway mutations, even after whole-exome analysis in the two RAS pathway mutation–negative cases, raising the possibility that the latter cases represent a genetically distinct myeloproliferative neoplasm in childhood.

Another key finding in the current study is the discovery of secondary mutations that involve *SETBP1* and *JAK3*. Detected only in a subpopulation of leukemic cells, most of these mutations are thought to be involved in the progression rather than the establishment of JMML and were associated with poor clinical outcome. *SETBP1* is a newly identified proto-oncogene, and identical mutations in this gene have recently been reported in 15–25% of adult cases with atypical chronic myeloid leukemia (CML)[10], CMML and secondary AML[28]. Affecting one of three highly conserved amino acid positions, *SETBP1* mutations have been shown to abolish the binding of an E3 ubiquitin ligase (β-TrCP1) to SETBP1, which prevents ubiquitination and subsequent degradation, leading to gain of function through the consequent increase in SETBP1 protein amounts[10,28]. Although the precise leukemogenic mechanisms of *SETBP1* mutations are still unclear, we have shown that mutant *SETBP1* alleles confer self-renewal capability to myeloid progenitors *in vitro*, and *SETBP1* mutations in adult leukemia were associated with increases in *HOXA9* and *HOXA10* expression[28]. Recurrent *JAK3* mutations in JMML are also noteworthy. The JAK-STAT pathway is a key component of normal hematopoiesis[29]. As in other hematopoietic malignancies[20], the p.Arg657Gln alteration represents the most frequent change in JMML. This alteration confers interleukin (IL)-3 independence to Ba/F3 cells and induces STAT5 phosphorylation[20]. Targeting the JAK-STAT pathway with a pan-JAK inhibitor such as CP-690550 (ref. 30) could be a promising therapeutic possibility for patients with *JAK3*-mutated JMML.

In conclusion, our whole-exome sequencing analysis identified the spectrum of gene mutations in JMML. Together with the high frequency of RAS pathway mutations, the paucity of non–RAS pathway mutations is a prominent feature of JMML. Mutations of *SETBP1* and *JAK3* were common recurrent secondary events presumed to be involved in tumor progression and were associated with poor clinical outcomes. Our findings provide an important clue to understanding the pathogenesis of JMML that may help in the development of novel diagnostics and therapeutics for this leukemia.

URLs. Genomon, http://genomon.hgc.jp/exome/en/; BioCarta, http://www.biocarta.com/; dbSNP131, http://www.ncbi.nlm.nih.gov/projects/SNP/; RefSeq database, http://www.ncbi.nlm.nih.gov/RefSeq/.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession code. We deposited whole-genome and whole-exome sequence data in the European Genome-phenome Archive under accession EGAS00001000521.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.S., Y.O., H. Muramatsu, K.Y., M.T., A.K. and M.S. designed and performed the research, analyzed the data and wrote the manuscript. Y.S., K.C., H.T. and S.M. performed bioinformatics analyses of the resequencing data. X.W. and Y.X. performed Sanger sequencing. S.D., A.H., K.N., Y.T. and N.Y. collected specimens and performed the research. H. Makishima and J.P.M. designed the research and analyzed the data. S.O. and S.K. led the entire project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. We studied 92 children (61 boys and 31 girls) with JMML, including 7 individuals with NS/MPD, who were diagnosed as having JMML in institutions throughout Japan. Written informed consent was obtained from subjects' parents before sample collection. This study was approved by the ethics committees of the Nagoya University Graduate School of Medicine and the University of Tokyo in accordance with the Declaration of Helsinki. Diagnosis with JMML was made on the basis of internationally accepted criteria[1]. Characteristics of the 92 JMML cases are summarized in **Table 2**. The median age at diagnosis was 16 months (range of 1–160 months). Karyotypic abnormalities were detected in 16 subjects, including in 8 with monosomy 7. Fifty-six of the 92 subjects (61%) received allogeneic HSCT.

Sample preparation. Genomic DNA was extracted using the QIAamp DNA Blood Mini kit and the QIAamp DNA Investigator kit (Qiagen) according to the manufacturer's instructions. The T Cell Activation/Expansion kit, human (Miltenyi Biotec) was used for the expansion of $CD3^+$ T cells from subjects' peripheral blood or bone marrow mononuclear cells[3].

Whole-exome sequencing. Exome capture from paired tumor-reference DNA was performed using SureSelect Human All Exon V3 (Agilent Technologies), covering 50 Mb of coding exons, according to the manufacturer's protocol. Enriched exome fragments were subjected to massively parallel sequencing using the HiSeq 2000 platform (Illumina). Candidate somatic mutations were detected through our in-house pipeline (Genomon) as previously described[17].

Detection of mutations from whole-exome sequencing data. Detection of candidate somatic mutations was performed according to previously described algorithms with minor modifications[17]. Briefly, the number of reads containing single-nucleotide variations (SNVs) and indels in both tumor and reference samples was determined using SAMtools[31], and the null hypothesis of equal allele frequencies in tumor and reference samples was tested using the two-tailed Fisher's exact test. A variant was adopted as a candidate somatic mutation if it had $P < 0.01$, if it was observed in bidirectional reads (in both plus and minus strands of the reference sequence) and if its allele frequency was less than 0.25 in the corresponding reference sample. For the detection of germline mutations in RAS pathway genes, SNVs and indels having allele frequencies of more than 0.25 (SNVs) and 0.10 (indels) were interrogated for 46 genes, which consisted of known JMML-related RAS pathway genes and genes registered in the pathway databases ('Ras signaling pathway' in BioCarta and 'signaling to RAS' in Reactome[32]). For variant calls in tumor samples for which the paired normal reference was not available, candidate variants in the RAS pathway were detected at an allele frequency of >0.10. Finally, the list of candidate somatic and/or germline mutations was generated by excluding synonymous SNVs and other variants registered in either dbSNP131 or an in-house SNP database constructed from 180 individual samples. All candidates were validated by Sanger sequencing as previously described.

Estimation of tumor content. The tumor content of bone marrow specimens was estimated from the allele frequency of the somatic mutations identified by deep sequencing. For homozygous mutations, as indicated by an allele frequency of >0.75, the tumor content (F_{tumor}) was calculated from the observed frequency ($F_{observed}$) of the mutation according to the following equation: $F_{tumor} = 2 \times F_{observed} - 1$. For heterozygous mutations, the tumor content was calculated by doubling the allele frequency.

Power analysis of whole-exome sequencing. The power of detecting somatic mutations at each nucleotide position in whole-exome sequencing was estimated by Monte-Carlo simulation (n = 1,000) on the basis of the observed mean depth of coverage for each exon in germline and tumor samples and the observed tumor content for each sample, which were estimated using the allele frequencies of the observed mutations. For the samples with no observed somatic mutations, the average tumor content of the informative samples was employed. Simulations were performed across a total of 192,424 exons.

Copy number analysis in whole-exome sequencing data. To detect copy number lesions at a single-exon level, the mean coverage of each exon normalized by the mean depth of coverage of the entire sample was compared with that of 12 unrelated normal DNA samples. Exons showing normalized coverage greater than 3 s.d. from the mean coverage of the reference samples were called as candidates for copy number alterations. All candidate exons of RAS pathway genes were visually inspected using the Integrative Genomics Viewer[33] and were validated by Sanger sequencing of corresponding putative breakpoint-containing fragments.

Targeted deep sequencing. Deep sequencing of the targeted genes was performed essentially as described in the 'deep sequencing of pooled target exons' section in ref. 17, except that target DNA was not pooled. Briefly, all exons of *PTPN11*, *NF1*, *KRAS*, *NRAS*, *CBL*, *SETBP1*, *JAK3* and *SH3BP1* were PCR amplified with Quick Taq HS DyeMix (TOYOBO) and the PrimeSTAR GXL DNA Polymerase kit (Takara Bio) using primers including the NotI restriction site (**Supplementary Table 3**). The PCR products from an individual sample were combined and purified with the QIAquick PCR Purification kit (Qiagen) for subsequent digestion with NotI (Fermentas). Digested PCR product was purified, concatenated with T4 DNA ligase (Takara Bio) and sonicated to generate fragments with an average size of 150 bp using Covaris. Fragments were processed for sequencing according to a modified Illumina paired-end library protocol, and sequences were read by a HiSeq 2000 instrument using a 100-bp paired-end read protocol.

Variant calls in targeted deep sequencing. Data processing and variant calling were performed with modifications to the protocol described in a previous publication[17]. Each read was aligned to the set of targeted sequences from PCR amplification, with BLAT[34] instead of Burrows-Wheeler Aligner (BWA)[35] used with the -fine option. Mapping information in the .psl format was converted to the .sam format with paired-read information. Of the successfully mapped reads, reads were excluded from further analysis if they mapped to multiple sites, mapped with more than four mismatched bases or had more than ten soft-clipped bases. Next, the Estimation_CRME script was run to eliminate strand-specific errors and exclude PCR-derived errors. A strand-specific mismatch ratio was calculated for each nucleotide variant for both strands using the bases from read cycles 11 to 50 on the next-generation sequencer. By excluding the top five cycles showing the highest mismatch rates, strand-specific mismatch rates were recalculated, and the smaller value between both strands was adopted as a nominal mismatch ratio for that variant. After excluding variants found in dbSNP131 or the in-house SNP database, non-silent variants having a mismatch ratio of greater than 0.05 were called as candidates, unless they had median values of the mismatch ratio at the relevant nucleotide positions in the 92 samples of greater than 0.01, as such variants were likely to be caused by systematic PCR problems. Finally, candidates with mismatch ratios of >0.15 were further validated by Sanger sequencing.

Annotation of the detected mutations. Detected mutations were annotated using ANNOVAR[36]. The positions of the mutations were based on the following RefSeq transcript sequences: NM_002834.3 for *PTPN11*, NM_000267.3 for *NF1*, NM_002524.4 for *NRAS*, NM_004985.3 for *KRAS*, NM_005188.3 for *CBL*, NM_015559.2 for *SETBP1* and NM_000215.3 for *JAK3*. The effect of the mutations on protein function was assessed by SIFT[37], PolyPhen-2 (ref. 38) and MutationTaster[39].

Whole-genome sequencing. Paired tumor-reference DNA samples were sequenced with the HiSeq 2000 platform according to the manufacturer's instructions to obtain 30× read coverage for reference samples and 40× coverage for tumor samples. Obtained FASTQ sequences were aligned to the human reference genome (hg19) using BWA[35] 0.5.8 with default parameters. Alignment of pairs of sequences, at least one of which was not mapped or was considered to have possible mapping problems (with mapping quality of less than 40, insertions or deletions, soft-clipped sequence of more than 10% of the length of the original sequence, irregular paired-read orientation or mate distance of greater than 2,000 bp), was attempted with BLAT[34] using default parameters, except for stepSize = 5 and repMatch = 2,253. Mapping statistics were calculated by counting the bases at each genomic position with SAMtools[31]. For variant calling, variant and reference bases with base quality of >30 were counted in both germline and tumor samples, and the Fisher's

exact test was applied. Variants with P of <0.01 were called. Variants having allele frequency of >0.25 in the germline sample were excluded. Variants found in 12 unrelated germline samples with an allele frequency of >0.01 on average were also excluded owing to the high probability that they represented false positive calls. Copy number estimation was performed by calculating the averaged ratio of read depths in germline and tumor samples in 10,000-base bins. An allele-specific copy number plot was generated by measuring the allele frequency of the tumor sample at the positions in which more than 25% of the allele mismatch was observed in germline samples. For the detection of chromosomal structural variations, soft-clipped sequences that could be mapped to a unique genomic position were selected. Structural variation candidates that had more than four supporting read pairs in total and at least one read pair from each side of the breakpoint were called. Contig sequences were generated by assembling the reads within 200 bp of the breakpoint with CAP3 (ref. 40), and structural variations having the contig sequence that could be aligned to the alternate assembly of the hg19 genome with more than 93% identity were excluded as false positives. Structural variations with read depth of greater than 150 on at least one side of the breakpoint were considered to be mapped to a repeat element and were also excluded. For detection of viruses, unmapped sequences were aligned to the collection of all viral genomes in the RefSeq database using BLAT. A virus was considered to be detected if its genome was covered by mean read coverage of >1.

cDNA sequencing. Total RNA was extracted using the RNeasy Mini kit (Qiagen) and was reverse transcribed with the ThermoScript RT-PCR system (Life Technologies). Target sequences were PCR amplified with the PrimeSTAR GXL DNA Polymerase kit using the primers listed in **Supplementary Table 3** and were sequenced.

Statistical analysis. For comparison of the frequency of mutations or other clinical features between disease groups, categorical variables were analyzed using the Fisher's exact test, and continuous variables were tested using the Mann-Whitney U test. Overall survival and transplantation-free survival were estimated by the Kaplan-Meier method. Hazard ratios for survival with 95% CIs were estimated according to the Cox proportional hazards model, and difference in survival was tested by log-rank test. STATA version 12.0 (StataCorp) was used for all statistical calculations.

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BRIEF REPORT
Intermittent X-Linked Thrombocytopenia With a Novel WAS Gene Mutation

Taizo Wada, MD, PhD,[1]* Masatsune Itoh, MD, PhD,[2] Hideaki Maeba, MD,[1] Tomoko Toma, MD, PhD,[1] Yo Niida, MD, PhD,[3] Yutaka Saikawa, MD, PhD,[2] and Akihiro Yachie, MD, PhD[1]

X-linked thrombocytopenia (XLT) is caused by mutations in the *WAS* gene and characterized by thrombocytopenia with minimal or no immunodeficiency. Patients with XLT usually exhibit persistent thrombocytopenia, and intermittent thrombocytopenia has been described only in two families. Here, we report a patient with intermittent XLT carrying a novel missense mutation (Ala56Thr). He showed residual expression of Wiskott–Aldrich syndrome protein in the lymphocytes and platelets. There appeared to be an association between normal platelet numbers and a post infectious state. Our findings further support the importance of analysis of Wiskott–Aldrich syndrome protein in male patients who exhibit fluctuating courses of thrombocytopenia. Pediatr Blood Cancer

Key words: intermittent thrombocytopenia; missense mutation; WASp; Wiskott–Aldrich syndrome; X-linked thrombocytopenia

INTRODUCTION

Wiskott–Aldrich syndrome protein (WASp) is an important regulator of the actin cytoskeleton and is constitutively expressed in all nonerythroid hematopoietic cells [1]. Mutations in the *WAS* gene result in either a loss or gain of protein function. The former leads to classical Wiskott–Aldrich syndrome (WAS) or isolated X-linked thrombocytopenia (XLT) [2,3], whereas the latter leads to a distinct disease, congenital X-linked neutropenia [4]. XLT is a milder phenotype of classical WAS, that is, characterized by persistent thrombocytopenia with minimal or no signs of eczema and immunodeficiency [1]. Notarangelo et al. [5] reported two families in which affected males with unique amino acid substitutions in the *WAS* gene exhibited intermittent thrombocytopenia in the absence of other clinical features. This rare condition is considered to be the mildest consequence of WASp deficiency. Herein, we report an additional case of intermittent XLT carrying a novel missense mutation. To further characterize the disease, the fluctuating course of thrombocytopenia, its relationship to infections, and the residual expression of WASp were evaluated.

CASE REPORT

The patient is a 4-year-old male whose medical history began shortly after birth with an abnormal newborn screening test for congenital hypothyroidism. His platelet counts were found to be in the normal range until 19 days of age, when he exhibited a late rise in thyroid stimulating hormone (30.7 mIU/L) and began replacement therapy with levothyroxine. Thereafter, regular thyroid function testing offered us the opportunity to evaluate the complete blood count longitudinally, and mild thrombocytopenia was noted after one month of age (Fig. 1A). He exhibited transient facial eczema during infancy. Ten days after he suffered from an influenza A infection at 8 months of age, the platelet count was elevated to 270×10^9/L. At 9 months of age, he was hospitalized for Norovirus gastroenteritis and marked thrombocytopenia (29×10^9/L). Platelet-associated immunoglobulin G was not elevated. Absolute neutrophil and lymphocyte counts were 2.73×10^9/L and 5.38×10^9/L, respectively. Nine days later, his platelet count was elevated to 197×10^9/L. At 12 months of age, another episode of gastroenteritis due to Rotavirus occurred. The platelet number initially decreased to 12×10^9/L and, 5 days later, recovered to 205×10^9/L. Absolute neutrophil and lymphocyte counts at the initial time were 5.69×10^9/L and 0.97×10^9/L, respectively. A similar fluctuation in platelet counts was also observed after upper respiratory infection; however, infectious episodes did not necessarily result in normalization of platelet counts. No correlation was observed between platelet counts and thyroid hormones levels. The patient was found to have normal thyroid function, and levothyroxine treatment was discontinued at 3 years of age. Consistent with previous observations [5], the mean platelet volume (MPV) was persistently low, regardless of the platelet count (mean, 6.5 fl; Fig. 1A).

Immunological studies at 4 years of age revealed that proliferative response to immobilized anti-CD3 and podosome formation were detectable [6], albeit at lower levels than controls (Fig. 1B,C). The patient also exhibited a normal absolute lymphocyte count (4.76×10^9/L) and normal immunoglobulin (Ig) levels (IgG 9.23 g/L, IgA 1.74 g/L, IgM 0.66 g/L). Immunophenotypic analysis of the lymphocytes showed normal or near-normal percentage of $CD3^+$ T (61.3%; normal, 71.4 ± 5.8), $CD4^+$ T (40.9%; normal, 43.2 ± 11.5), $CD8^+$ T (14.1%; normal, 22.3 ± 6.6), $CD16^+$ NK (14.3%; normal, 6.3 ± 3.9), and $CD20^+$ B (19.9%; normal, 12.5 ± 6.7) cells. Serum antibody titers to vaccinations were detected by enzyme immunoassay in the patient: measles-specific IgG 4.9 (normal, <2.0) and rubella-specific IgG 3.1 (normal, <2.0). Serum IgG antibody against cytomegalovirus

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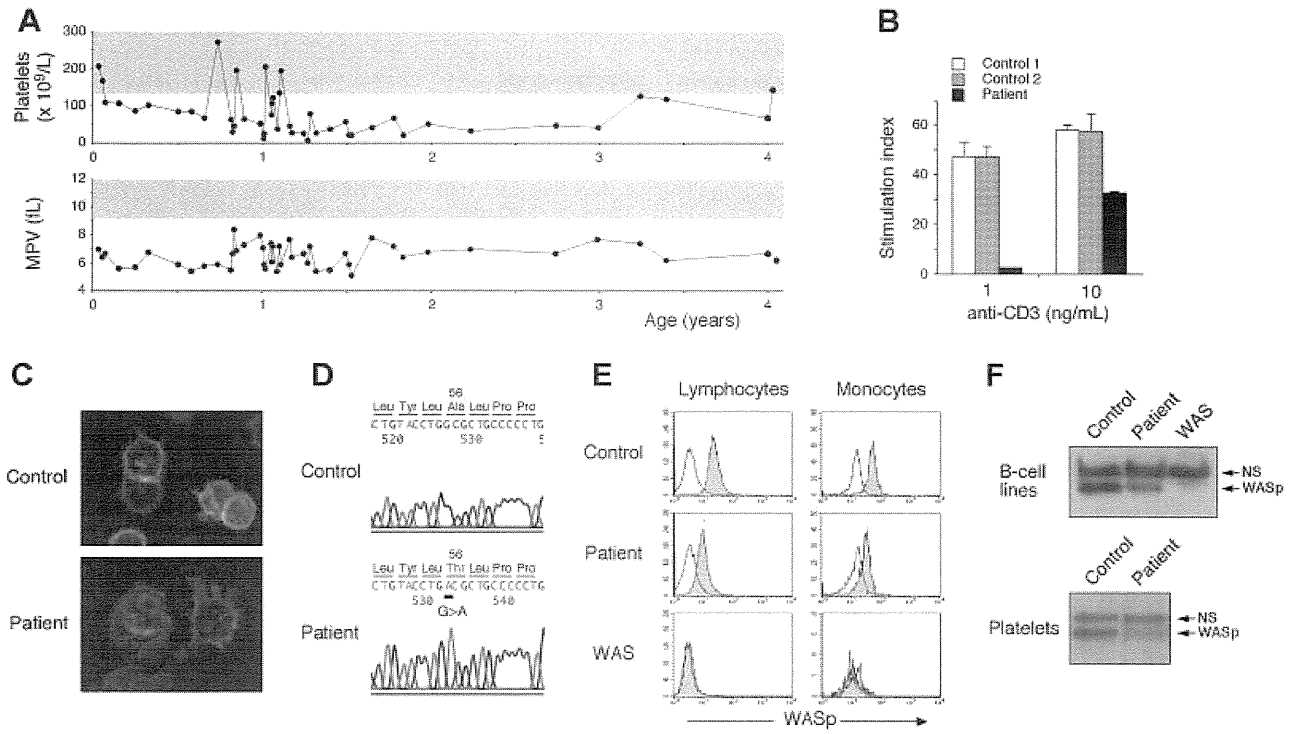

Fig. 1. Analysis of platelets and WASp. **A**: Profiles of the platelet counts and mean platelet volume (MPV). Shaded areas represent the ranges of normal values. **B**: Proliferative responses to anti-CD3. Data are presented of stimulation index (ratio between average incorporated [^{3}H]thymidine in the presence of anti-CD3 to that in the absecence of stimulation). **C**: F-actin distribution in monocyte-derived dendritic cells. Visualization of F-actin was performed with Alexa Fluor 488-conjugated phalloidin. **D**: Mutational analysis of the *WAS* gene. Direct sequencing was performed using an automated sequencer. **E**: Flow cytometric analysis of WASp expression in lymphocytes and monocytes. White histograms indicate control antibody; gray histograms represent anti-WASp monoclonal antibody. **F**: Western blot analysis of WASp expression in B-cell lines and platelets. Arrows indicate the position of the WASp and of a non-specific reactive protein (NS).

was also positive: 4.9 (normal, <2.0). Anti-A isohemagglutinin titer (IgM) was 1:64. The patient has shown no clinical evidence of immunodeficiency, autoimmunity or malignancy to date.

As part of the clinical evaluation for thrombocytopenia, mutation analysis of the *WAS* gene was performed at 10 months of age, after informed consent was obtained from the parents. The patient had a G to A substitution at nucleotide 200 in exon 2, resulting in an Ala56Thr substitution (Fig. 1D). While this missense mutation is novel, a similar missense mutation Ala56Val has been frequently reported in patients with XLT [7,8]. His mother was a heterozygous carrier of the mutation. We next analyzed WASp expression in peripheral blood mononuclear cells and platelets by flow cytometry and/or Western blot analysis (Fig. 1E,F) [9]. The lymphocytes, monocytes and platelets showed residual expression of the mutated WASp, which was consistent with the missense mutation.

DISCUSSION

In this report, we described a case of intermittent XLT with a missense mutation (Ala56Thr) resulting in residual expression of the mutated WASp. These findings are in line with the previous description of intermittent XLT, in which the missense mutations, Pro58Arg and Ile481Asn, led to substantial protein expression in both lymphocytes and platelets [5]. Studies of platelets and lymphocytes from a series of patients with diverse *WAS* mutations, however, have demonstrated that platelets of most patients were shown to lack WASp, although a complex pattern of WASp expression was found in lymphoid cells [10,11]. Further studies will be necessary to assess whether residual WASp expression in platelets could be directly associated with intermittent thrombocytopenia.

There appears to be an association between normal platelet numbers and a post infectious state in our patient. In healthy children, reactive thrombocytosis may occur following infections, in which significant elevation of thrombopoietin levels during an acute infection precedes the development of thrombocytosis [12]. Although we did not measure any growth factors related to megakaryopoiesis in our patient, the Ala56Thr missense mutation likely permitted the platelet elevation to occur following infections. Further investigations in intermittent XLT may lead to a better understanding of abnormalities in both platelet production and platelet consumption in WASp deficiency.

It may be difficult to conclusively diagnose our patient as being affected with intermittent isolated XLT because of his young age. A scoring system on a scale of 1–5 is used to estimate the severity of WAS/XLT-associated symptoms [13]. Progression from a low score representing XLT to the highest score of 5, due to either autoimmunity or malignancy, can occur at any age [8]. Despite the usefulness of the scoring system for assessment of the disease

phenotype, it cannot predict the onset of autoimmunity or malignancy. Indeed, malignancy has been reported in a patient with intermittent XLT carrying the Pro58Arg mutation [8]. Although hematopoietic stem cell transplantation is a treatment of choice for patients with classic WAS, there is a controversy in XLT due to the excellent long-term survival. However, if a human leukocyte antigen-identical donor is available, hematopoietic stem cell transplantation can be considered for patients with XLT because of their high probability of severe disease-related complications [8]. Therefore, careful follow-up is necessary for patients with WASp deficiency, including intermittent XLT. In addition, our findings further support the importance of analysis of WASp in male patients who present with fluctuating courses of thrombocytopenia, similar to idiopathic thrombocytopenic purpura.

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Sustained elevation of serum interleukin-18 and its association with hemophagocytic lymphohistiocytosis in XIAP deficiency

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ABSTRACT

X-linked lymphoproliferative syndrome (XLP) is a rare primary immunodeficiency characterized by increased vulnerability to Epstein–Barr virus infection. XLP type 1 is caused by mutations in *SH2D1A*, whereas X-linked inhibitor of apoptosis (XIAP) encoded by *XIAP/BIRC4* is mutated in XLP type 2. In XIAP deficiency, hemophagocytic lymphohistiocytosis (HLH) occurs more frequently and recurrence is common. However, the underlying mechanisms remain mostly unknown. We describe the characteristics of the cytokine profiles of serum samples from 10 XIAP-deficient patients. The concentration of interleukin (IL)-18 was strikingly elevated in the patients presented with HLH, and remained high after the recovery from HLH although levels of other pro-inflammatory cytokines approached the normal range. Longitudinal examination of two patients demonstrated marked exacerbation of IL-18 levels during every occasion of HLH. These findings may suggest the association between HLH susceptibility and high serum IL-18 levels in XIAP deficiency.

1. Introduction

X-linked lymphoproliferative syndrome (XLP) is a rare primary immunodeficiency that is characterized by an extreme susceptibility to Epstein–Barr virus infection resulting in fatal infectious mononucleosis or hemophagocytic lymphohistiocytosis (HLH), hypogammaglobulinemia, and malignant lymphoma [1–3]. Most cases are caused by mutations in the *SH2D1A* gene, which encodes the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), a cytoplasmic adaptor molecule involved in intracellular signaling downstream of the SLAM family of surface receptors (XLP type 1). SAP is expressed in T, natural killer (NK), and invariant NKT cells [1–3]. A deficiency of X-linked inhibitor of apoptosis (XIAP) caused by *XIAP/BIRC4* gene mutations has been also identified to cause XLP (XLP type 2) [4]. XIAP, a member of the inhibitor of apoptosis family of proteins, plays an antiapoptotic role as a potent inhibitor of caspases 3, 7, and 9, and also possesses E3 ubiquitin ligase function [1–3]. In contrast to SAP, XIAP is expressed ubiquitously. Patients with XIAP deficiency have been reported to develop HLH more frequently compared with SAP deficiency [5]. However, it remains unknown why a deficiency of XIAP leads to HLH and its recurrence in patients with XIAP deficiency.

HLH is a heterogeneous group of diseases that are characterized by uncontrolled proliferation of activated macrophages and T cells

Abbreviations: XLP, X-linked lymphoproliferative syndrome; SLAM, signaling lymphocyte activation molecule; SAP, SLAM-associated protein; XIAP, X-linked inhibitor of apoptosis; HLH, hemophagocytic lymphohistiocytosis; FHL, familial hemophagocytic lymphohistiocytosis; EBV, Epstein–Barr virus; sJIA, systemic juvenile idiopathic arthritis; AOSD, adult-onset Still's disease; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; ATP, adenosine triphosphate; NOD2, nucleotide-binding and oligomerization domain 2 ; NK, natural killer.

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with overproduction of pro-inflammatory cytokines [6,7]. Patients with HLH may present with fever, cytopenia, hepatosplenomegaly, liver dysfunction, coagulation abnormalities, and hemophagocytosis. [6,7] HLH is comprised of primary and secondary forms. Primary HLH includes familial HLH (FHL), which is caused by genetic defects related to granule-dependent cytotoxicity, and immunodeficiencies, such as XLP. FHL type 2 (FHL2) due to perforin deficiency is the most common form of primary HLH [6,7]. Perforin is a crucial effector molecule for cytotoxicity that is present in the granules of cytotoxic T cells and NK cells. Secondary HLH is associated with a variety of infections, autoimmune diseases and malignancies. Epstein–Barr virus (EBV)-associated HLH (EBV-HLH) is the most frequent subtype of secondary HLH in Japan [8]. Studies of cytokines from HLH patients have demonstrated elevated concentration of many pro-inflammatory cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-18 [9–11].

IL-18 is a potent pro-inflammatory cytokine that was originally identified as an IFN-γ-inducing factor [12,13]. It has been reported that IL-18 induces either Th1 or Th2 polarization depending on the immunologic context, and promotes a variety of innate immune processes associated with infection, inflammation, and autoimmunity [12,13]. In this report, we describe the characteristic cytokine profiles of XIAP deficiency, especially IL-18, which might be associated with its HLH susceptibility.

2. Methods

2.1. Patients

We studied 10 patients with XIAP deficiency from 9 families (Table 1). Case reports of Pt2.1, Pt2.2, Pt4, Pt5, Pt8, and Pt9 have been described elsewhere [5,14,15]. Patients Pt2.1, Pt2.2, Pt4, and Pt5 were reported in a previous report as P6.1, P6.2, P1, and P3.1, respectively [14]. Patient Pt7 was a younger brother of P2.1 and P2.2 [14]. Patients Pt8 and Pt9 were reported in a previous report as Patient 3 and Patient 9, respectively [5]. All patients showed typical features of HLH, such as persistent fever, cytopenia, liver dysfunction, and hyperferritinemia during the acute phase of HLH. The age of onset was variable, ranging from early infancy to 8 years. Most patients were treated with corticosteroids with or without cyclosporine A, and showed recurrent HLH. Patient Pt3 underwent cord blood transplantation and is alive with no evidence of disease. Patients Pt8 and Pt9 underwent allogeneic bone marrow transplantation but died of complications [16]. Blood samples were collected during the acute phase of HLH and/or during a clinically stable phase. We also investigated 4 cases of SAP deficiency, 6 cases of FHL2, and 11 cases of EBV-HLH as disease controls [17]. No detectable mutations within the *SH2D1A* or *XIAP/BIRC4* genes were observed in the 5 male patients with EBV-HLH [18]. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

2.2. Analysis of XIAP mutations and protein expression

DNA was extracted from blood samples using standard methods. The *XIAP/BIRC4* gene was amplified from genomic DNA using specific primers [5,14]. Sequencing was performed on purified polymerase chain reaction products using the ABI Prism BigDye Terminator Cycle sequencing kit on an ABI 310 or 3130 automated sequencer (Applied Biosystems, Foster, CA). Flow cytometric and Western blot analysis of intracellular XIAP were performed, as described previously [5,14].

2.3. Cytokine determination

Serum concentrations of cytokines were determined using the following enzyme-linked immunosorbent assay kits: neopterin (IBL, Hamburg, Germany); IFN-γ, IL-6, and TNF-α (R&D systems, Minneapolis, MN); and IL-18 (MBL, Nagoya, Japan) [17,19]. Analysis of differences among groups was performed using the Student's *t*-test for unpaired samples. Differences with *p*-values less than 0.05 were considered significant.

Table 1
Patient characteristics.

	Pt1	Pt2.1[b]	Pt2.2[b]	Pt3	Pt4	Pt5	Pt6	Pt7	Pt8	Pt9
Nucleotide mutation	c.847C > T	c.1021_1022delAA	c.1021_1022delAA	c.664C > T	c.712C > T	c.650delG	c.1056 + 1G > A	c.1141C > T	c.1445C > G	c.310C > T
Effect	Q283X	N341fs	N341fs	R222X	R238X	W217fs	Splice defect	R381X	P482R	Q104X
Protein expression [a]	Negative	Residual	Residual	Residual	Residual	Negative	Negative	Negative	Residual	Negative
Age at onset	1 mo	17 mo	15 mo	5 mo	20 mo	2 mo	3 yr	8 mo	Infancy	8 yr
Current age	5 yr	3 yr	15 yr	2 yr	7 yr	12 yr	9 yr	10 mo	Deceased	Deceased
Family history	No	Yes	Yes	No	No	Yes	No	Yes	No	Yes
HLH	+	+	+	+	+	+	+	+	+	+
Recurrent HLH	+	+	+	+	+	+	+	–	NA	+
Fever	+	+	+	+	+	+	+	+	+	+
Splenomegaly	–	+	+	+	+	–	–	+	+	+
Cytopenia	+	+	+	–	+	+	–	+	+	+
EBV	–	+	+	–	+	+	–	–	–	–
Hypogammaglobulinemia	–	–	–	–	–	–	–	–	–	+
Colitis	–	–	–	–	+	–	–	–	NA	NA
Lymphoma	–	–	–	–	–	–	–	–	–	–
Treatment	PSL, CsA	IVIG, Dex	PSL	Dex, CsA PSL	PSL, CsA Dex tocillizumab	PSL, CsA	PSL	Dex, CsA	HLH 2004, including IT treatment	Dex rituximab
Allogeneic HSCT	–	–	–	+	–	–	–	–	+	+

HLH, hemophagocytic lymphohistiocytosis; mo, month; yr, year; NA, not applicable; EBV, Epstein–Barr virus; PSL, prednisolone; CsA, cyclosporin A; Dex, dexamethasone, IVIG, intravenous immunoglobulin; and HSCT, hematopoietic stem cell transplantation.

[a] Intracellular expression of XIAP in lymphocytes was analyzed by flow cytometry or Western blot.

[b] Sibling cases.

2.4. Cell cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from patients and controls by Ficoll-Hypaque gradient centrifugation. To stimulate monocytes, PBMCs were incubated with 100 ng/mL lipopolysaccharide (LPS; Sigma–Aldrich, St. Louis, MO) with or without 5 mM adenosine triphosphate (ATP; Sigma–Aldrich) in RPMI 1640 medium containing 10% fetal calf serum and antibiotics [20]. After 24 h, culture supernatants were collected and stored at −80 °C until cytokine assay.

3. Results

3.1. XIAP/BIRK4 mutations and protein expression

As shown in Table 1, most patients had nonsense or frameshift mutations in the *XIAP/BIRC4* gene. Patients Pt1 and Pt3 were found to carry novel nonsense mutations, c.847C > T (Q283X) and c.664C > T (R222X), respectively. Intracellular XIAP was not detected in PBMCs from Patient Pt1, whereas residual expression was demonstrated in Patient Pt3. Patient Pt6 had a novel splice site mutation, c.1056 + 1G > A, resulting in no detectable expression of XIAP.

3.2. Cytokine profiles

The concentration of IL-18 was markedly elevated in XIAP-deficient patients who presented with HLH (mean, 86,500 pg/mL; Fig. 1A). Children who were affected with SAP deficiency, FHL2 or EBV-HLH exhibited much less elevation of IL-18 with mean concentrations of 6600 pg/mL (range, 6200–7000 pg/mL), 3680 pg/mL (range, 1300–10,500 pg/mL) and 4256 pg/mL (range, 3050–6950 pg/mL), respectively (Fig. 1A) [17]. Other pro-inflammatory cytokines such as IL-6, neopterin, IFN-γ, and TNF-α were elevated in patients with XIAP deficiency, however, levels of these cytokines were comparable to those of patients with SAP deficiency, FHL2, or EBV-HLH (Fig. 1A). The levels of soluble IL-2 receptor were also elevated in patients with XIAP deficiency (mean, 2910 IU/mL).

As expected, after the recovery from HLH, the concentration of each pro-inflammatory cytokine declined in patients with SAP deficiency, FHL2 and EBV-HLH. However, as XIAP-deficient patients recovered from HLH, serum IL-6, neopterin, IFN-γ, and TNF-α levels approached the normal range but the levels of IL-18 remained high in these patients (mean, 4090 pg/mL; Fig. 1A). Longitudinal examination of the cytokine profiles in Patients Pt1 and Pt2.1 clearly demonstrated sustained elevation of IL-18 and its marked exacerbation on every occasion of recurrent HLH (Fig. 1B).

3.3. Secretion of IL-18 from PBMCs after in vitro stimulation

To assess whether hypersecretion of IL-18 was observed from monocytes after inflammasome stimulation, PBMCs from normal individuals and 3 patients with XIAP deficiency (Pt1, Pt2.1, and Pt2.2) were cultured for 24 h with LPS and ATP. Consistent with previous observations [20], IL-18 was efficiently secreted in normal PBMCs after stimulation with LPS plus ATP (Fig. 2), although TNF-α and IL-1β were produced in response to stimulation with only LPS (data not shown). There was little production of IL-18 in PBMCs from patients with XIAP deficiency after stimulation with LPS as well as no stimulation, suggesting no spontaneous production of IL-18. More importantly, levels of IL-18 secretion after stimulation with LPS plus ATP in PBMCs from the patients were comparable to those of normal controls (Fig. 2).

4. Discussion

XIAP deficiency is characterized by a high incidence of HLH [1–3]. Most patients suffer from recurrent HLH, although the reason for the increase in HLH susceptibility remains unknown. To further characterize the disease, we investigated the cytokine profiles during the acute phase of HLH and during a clinically stable phase from 10 patients with XIAP deficiency and compared them to patients with SAP deficiency, FHL2 (perforin deficiency), and EBV-HLH.

Hypersecretion of pro-inflammatory cytokines from activated T cells and macrophages has been considered to account for the severe systemic symptoms of HLH [6,7]. Indeed, during the acute phase of HLH, patients exhibited hypercytokinemia, i.e., IL-6, IL-18, neopterin, IFN-γ, and TNF-α, regardless of underlying genetic disease. However, compared with our previous studies of cytokines in other forms of HLH [17], the concentration of IL-18 was strikingly elevated during HLH and remained high after the recovery from HLH in XIAP-deficient patients. Patients Pt2.2 and Pt5, both of whom had disease for longer than 10 years, also showed high IL-18 levels during the clinically stable phase, indicating that this characteristic appears to be consistent and stable in the patients. No correlation was observed between the concentration of IL-18 and the levels of residual XIAP protein expression. In addition, the level of IL-18 declined to within the normal range (142 pg/mL) in Patient Pt3 only after successful stem cell transplantation. Taken together, these findings may indicate the association between HLH susceptibility in XIAP deficiency and high serum levels of IL-18 and the possible role of serum IL-18 as a marker of disease activity.

Similar cytokine profiles have recently been described in patients with systemic juvenile idiopathic arthritis (sJIA) and with adult-onset Still's disease (AOSD), which also showed persistent high levels of serum IL-18 [19,21–26]. sJIA is a systemic inflammatory disease classified within the spectrum of JIA and is characterized by fever, rash and arthritis [12]. AOSD is likely to be the adult counterpart of sJIA [12]. In both diseases, macrophage activation syndrome, a form of HLH associated with rheumatic diseases, occurs frequently [12]. The concentration of IL-18 was further elevated in patients with sJIA/AOSD who presented with macrophage activation syndrome [19]. These profiles are quite similar to those of XIAP deficiency. Although the etiology of sJIA/AOSD remains unknown, these disorders might share a common final pathway of abnormal IL-18 secretion. On the other hand, studies of NK cells from patients with sJIA have demonstrated that a defect in IL-18 receptor β phosphorylation was involved in impaired NK cell function in sJIA [27]. Further studies are necessary to assess whether similar defects could exist and contribute to the disease pathogenesis in patients with XIAP deficiency. Moreover, it is tempting to speculate that XIAP-deficient patients might be misdiagnosed with sJIA/AOSD. In fact, our Patient Pt1 was initially given the diagnosis of sJIA despite the absence of arthritis (case 4 in Ref. [7]). Although arthritis may not be prominent, particularly early in the course of sJIA [28], Patient Pt1 has shown no clinical or laboratory evidence of arthritis to date.

It is well known that IL-18 is produced mainly by monocytes/macrophages in response to a variety of stimuli, and that IL-18 is synthesized as a precursor molecule, which is cleaved by the caspase-1 within the inflammasome [12,13]. However, the mechanism underlying the sustained elevation of IL-18 in XIAP deficiency is unclear at present. The cellular source of the IL-18 also remains to be elucidated. Our preliminary experiments failed to demonstrate that PBMCs from XIAP-deficient patients secreted larger amounts of IL-18 upon stimulation

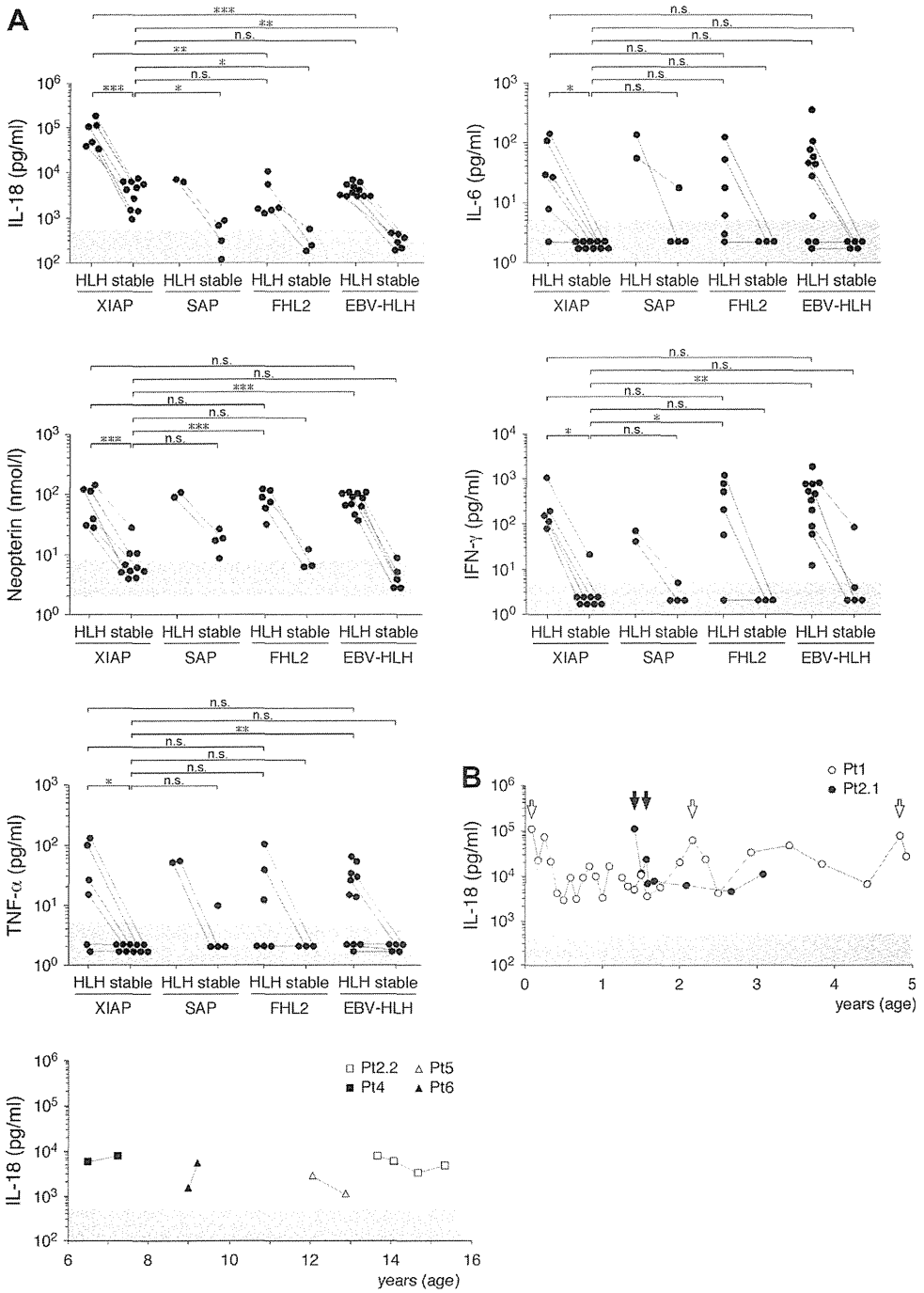

Fig. 1. Cytokine profiles. (A) Serum concentrations of interleukin (IL)-18, IL-6 and neopterin were measured in patients with X-linked inhibitor of apoptosis (XIAP) deficiency, with signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) deficiency, with familial hemophagocytic lymphohistiocytosis type 2 (FHL2) due to perforin deficiency, and with Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH). Shaded areas represent the ranges of the normal values. (B) Longitudinal examination of IL-18 levels. Arrows indicate episodes of HLH. HLH, hemophagocytic lymphohistiocytosis; n.s., not significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

with LPS and ATP, compared with normal controls. However, recent evidence suggests that XIAP mediates signaling of nucleotide-binding and oligomerization domain 2 (NOD2) in inflammation and innate immunity [29–31]. Because NOD2 can activate caspase-1, it is possible that XIAP is involved in inflammasome-mediated IL-18 production.

It is unknown why a deficiency of XIAP leads to the development of HLH [1–3]. Deficiency of XIAP has been observed to result in increased sensitivity of T cells to undergo reactivation-induced cell death [4,5]. Therefore XIAP may function to prevent HLH by inhibiting the apoptosis of cells that mediate the clearance of pathogens. Decreased numbers of NKT cells may also contribute to the development of HLH similar to SAP deficiency (XLP type 1), although there is some controversy over the numbers of such populations [32]. In addition, the loss of XIAP may influence immune cell activation resulting in alterations in pro-inflammatory cytokine production and cell survival in murine studies [33–35]. Because hypersecretion of IL-18 has been reported to play

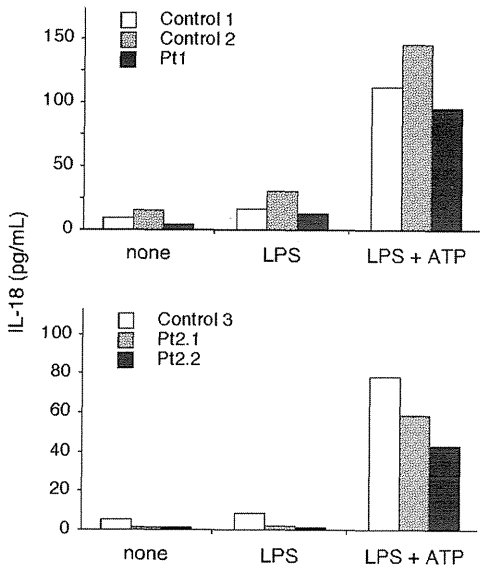

Fig. 2. IL-18 secretion after in vitro stimulation. Peripheral blood mononuclear cells were cultured with lipopolysaccharide (LPS) and adenosine triphosphate (ATP) for 24 h. The levels of IL-18 in culture supernatants were measured by enzyme-linked immunosorbent assay.

important roles in the pathogenesis of HLH [36], sustained elevation of IL-18 and its marked exacerbation on HLH in patients with XIAP deficiency would be an additional possibility to explain HLH susceptibility in this disease.

In summary, our studies demonstrate markedly elevated serum levels of IL-18 that are associated with HLH in XIAP deficiency. Characterization of the mechanism underlying the hypersecretion of IL-18 could provide important insights into the pathogenesis of the disease.

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Down-regulation of CD5 expression on activated CD8$^+$ T cells in familial hemophagocytic lymphohistiocytosis with perforin gene mutations

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ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is characterized by uncontrolled activation of T cells and macrophages with overproduction of cytokines. Familial HLH type 2 (FHL2) is the most common form of primary HLH and is caused by mutations in *PRF1*. We have recently described a significant increase in the subpopulation of CD8$^+$ T cells with clonal expansion and CD5 down-regulation in Epstein-Barr virus associated-HLH, which represented a valuable tool for its diagnosis. However, this unusual phenotype of CD8$^+$ T cells has not been investigated fully in patients with FHL2. We performed immunophenotypic analysis of peripheral blood and measured serum pro-inflammatory cytokines in five patients with FHL2. All patients showed significantly increased subpopulations of activated CD8$^+$ T cells with down-regulation of CD5, which were negligible among normal controls. Analysis of T-cell receptor Vβ repertoire suggested the reactive and oligoclonal expansion of these cells. The proportion of the subset declined after successful treatment concomitant with reduction in the serum levels of cytokines in all patients except one who continued to have a high proportion of the subset and died. These findings suggest that down-regulation of CD5 on activated CD8$^+$ T cells may serve as a useful marker of dysregulated T cell activation and proliferation in FHL2.

1. Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal disease that is characterized by marked systemic inflammation and unregulated activation of macrophages and T cells [1,2]. Patients with HLH may present with fever, cytopenia, hepatosplenomegaly, liver dysfunction, coagulation abnormalities, and hemophagocytosis [1,2]. HLH is comprised of primary and secondary forms. Primary HLH includes familial HLH (FHL), which is caused by genetic defects related to granule-dependent cytotoxicity, and immunodeficiencies, such as X-linked lymphoproliferative syndrome. Mutations in the *PRF1*, *UNC13D*, *STX11*, and *STXBP2* genes cause FHL type 2 (FHL2), FHL3, FHL4, and FHL5, respectively. Perforin is a crucial effector molecule for cytotoxicity that is present in the granules of cytotoxic T lymphocytes and natural killer (NK) cells. FHL2 (perforin deficiency) accounts for more than half of the FHL cases in Japan [3]. Secondary HLH is associated with a variety of infections, autoimmune diseases and malignancies. Epstein-Barr virus (EBV)-associated HLH (EBV–HLH) is the most frequent subtype of HLH in Japan [4]. Establishing a diagnosis of HLH may be difficult when based solely on clinical and laboratory findings, because those findings are often present in severely ill patients. It is also difficult to differentiate between primary and secondary HLH and diagnose a specific subtype of HLH during the acute phase of HLH.

Abbreviations: EBV, Epstein-Barr virus; FHL, familial hemophagocytic lymphohistiocytosis; HLA, human leukocyte antigen; HLH, hemophagocytic lymphohistiocytosis; IM, infectious mononucleosis; mAb, monoclonal antibody; NK, natural killer; PE, phycoerythrin; PBMCs, peripheral blood mononuclear cells; TCR, T-cell receptor.

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We have recently reported the clonal proliferation of activated $CD8^+$ T cells with down-regulation of CD5 in patients with EBV–HLH [5]. This unique immunophenotype of $CD8^+$ T cells could be a valuable tool for the diagnosis of EBV–HLH [5]. However, the immunophenotypic features of T cells in other subtypes of HLH have not been fully characterized. Human CD5 is a membrane glycoprotein that belongs to the scavenger receptor cysteine-rich family of receptors [6–9]. It is expressed on thymocytes, mature peripheral T cells and a small population of B cells, and is involved in the modulation of antigen-specific receptor-mediated activation and differentiation signals [6–9]. It has recently been reported that CD5 is recruited and colocalized with CD3 at the immunological synapse and inhibits T-cell receptor (TCR) signaling in T cells without interfering with immunological synapse formation [10]. Although an expanded subpopulation of $CD8^+$ T cells lacking expression of CD5 has been reported in a single case of FHL2 [11], the nature of $CD8^+$ T cells with down-regulation of CD5 in FLH remains to be elucidated. In this report, we describe the down-regulation of CD5 on activated $CD8^+$ T cells in patients with FHL2 and discuss the relationship between down-regulation of CD5 and systemic inflammation.

2. Materials and methods

2.1. Patients

We studied five patients with FHL2 from five families, all of whom were born to non-consanguineous Japanese parents. Table 1 presents the clinical and laboratory data of the patients. All patients showed typical features of HLH, such as persistent fever, hepatosplenomegaly, cytopenia, liver dysfunction, and hypercytokinemia, i.e., neopterin, interferon-γ, and interleukin-6 at the onset of FHL2. Defective NK cell activity was a universal feature. In patient P3, HLH was triggered by a primary EBV infection, in which the major cellular target of EBV infection, as assessed by *in situ* hybridization for EBV-encoded small RNA1 was B cells (data not shown) but not $CD8^+$ T cells, resulting in marked lymphocytosis with atypical lymphocytes. The disease onset was during early infancy in all patients except for patient P4. Patient P2 did not respond to the HLH-2004 treatment protocol [12], and died at 12 days of age. Patients P1, P3, P4 and P5 underwent stem cell transplantation at the ages of 5 months, 2 years, 5 months and 5 months, respectively [13]. Patients P1 and P3 are alive with no

Table 1
Patient characteristics.

	P1	P2	P3	P4	P5	Normal range
Onset age	1 month	1 day	2 yr	3 months	1 month	
Fever	+	+	+	+	+	
Hepatosplenomegaly	+	+	+	+	–	
Hemophagocytosis*	+	NA	+	+	NA	
Stem cell transplantation	+	–	+	+	+	
Outcome	Alive	Deceased	Alive	Deceased	Alive	
Laboratory findings						
NK cell activity (%)	3.8	2.0	0.0	0.0	0.0	18–40
WBC (/μL)	3100	16,600	51,000	3330	5700	5000–19,500
Neutrophils (/μL)	580	13,600	4290	830	470	3150–6200
Lymphocytes (/μL)	2290	1660	40,800	2060	4380	1500–3000
Hemoglobin (g/dL)	9.2	18.6	8.0	7.8	6.4	9.0–14.0
Platelets (×103/μL)	86	75	50	18	81	150–350
Triglycerides (mg/dL)	356	105	600	129	158	30–149
Fibrinogen (mg/dL)	147	117	NA	59	NA	183–381
Ferritin (ng/mL)	9983	532	2400	1038	427	4.6–204.0
sIL-2R (IU/mL)	3306	11,209	31,000	18,355	4180	220–530
Lymphocyte subsets						
$CD3^+$ (%)	69.2	87.6	92.6	77.0	66.4	64.4–80.2
$CD4^+$ (%)	34.4	40.2	33.5	20.8	29.5	47.3–58.9
$CD8^+$ (%)	31.0	42.1	56.8	53.7	25.1	10.3–24.3
Serum cytokines						
Neopterin (nmol/L)	60	90	125	120	78	2–8
IFN-γ (pg/mL)	37	510	57	1200	205	<5
IL-6 (pg/mL)	122	18	<5	52	<5	<5

NK, natural killer; WBC, white blood cells; sIL-2R, soluble interleukin-2 receptor; IFN-γ, interferon-γ; IL-6, interleukin-6; NA, not available.
* Hemophagocytosis in bone marrow.

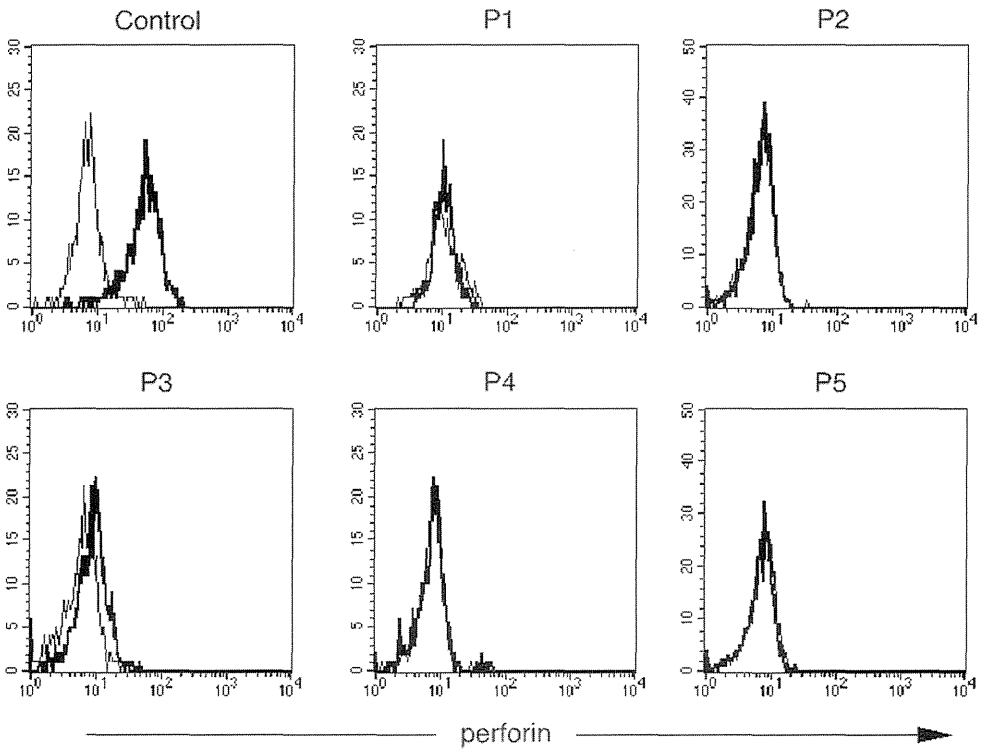

Fig. 1. Perforin expression. Intracellular expression of perforin in $CD3^-$ $CD56^+$ NK cells. Thin lines indicate control antibody; thick lines represent monoclonal antibody specific for perforin.

Table 2
PRF1 mutations.

	Nucleotide mutation	Predicted effect
P1	c.1090_1091delCT	p.Leu364fs
	c.1562A>G	p.Tyr521Cys
P2	c.996_1000delGCCCG	p.Pro333fs
	c.1090_1091delCT	p.Leu364fs
P3	c.1246C>T	p.Gly416X
	c.1349C>T	p.Thr450Met
P4	c.1090_1091delCT*	p.Leu364fs
P5	c.1090_1091delCT*	p.Leu364fs

* Homozygous mutation.

evidence of disease, but patient P4 died of acute graft-versus-host disease 5 months later. In patient P5, the transplantation was performed recently. We also investigated 10 cases of EBV–HLH and 13 cases of infectious mononucleosis (IM) as disease controls [5,14]. All patients with EBV–HLH showed typical clinical features of HLH and exhibited high viral copy numbers. It was noted that $CD8^+$ T cells were the major cellular targets of EBV in all EBV–HLH patients by *in situ* hybridization for EBV-encoded small RNA1 in the available samples. No detectable mutations within the *SH2D1A* or *XIAP/BIRC4* genes were observed in the five male patients with EBV–HLH [5]. The diagnosis of IM was clinically determined. All patients with IM exhibited self-limited disease. Primary EBV infection was serologically confirmed for all cases of EBV–HLH and IM. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

2.2. Flow cytometry

For the analysis of perforin expression, peripheral blood mononuclear cells (PBMCs) were isolated from patients and controls by Ficoll-Hypaque gradient centrifugation. PBMCs were stained with fluorescein isothiocyanate-conjugated anti-CD56 and peridinin chlorophyll protein-conjugated anti-CD3 monoclonal antibodies (mAbs). After washing, cells were fixed and permeabilized with Cytofix/Cytoperm Plus kit and incubated with phycoerythrin (PE)-conjugated anti-perforin mAb or control Ab (BD, San Diego, CA, USA). Perforin expression was evaluated in $CD3^-$ $CD56^+$ NK cells with a FACSCalibur flow cytometer using CellQuest software (BD Bioscience, Tokyo, Japan). For the analysis of CD5 expression, whole blood was stained with fluorescein isothiocyanate-conjugated anti-CD5, PE-conjugated anti-human leukocyte antigen (HLA)-DR (both from BD), and R-PE-Cy5-conjugated anti-CD4 or anti-CD8 (Dako, Glostrup, Denmark) mAbs. CD5 expression on $CD8^+$ T cells was considered negative if it was similar to levels of the NK cells and most of the B cells that do not express CD5 [5]. Analysis of differences among the data groups was performed using Student's unpaired *t*-test. *p*-Values less than 0.05 were considered significant. Flow cytometric analysis of the TCR Vβ repertoire was performed as previously described [15].

2.3. Cytokine determination

Serum or plasma concentrations of cytokines were determined using the following enzyme-linked immunosorbent assay kits: interferon-γ and interleukin-6 (R&D systems, Minneapolis, MN, USA); and neopterin (IBL, Hamburg, Germany) [14].

2.4. Mutation analysis

DNA was extracted from blood samples using standard methods. The *PRF1* gene was amplified from genomic DNA using specific primers [16]. Sequencing was performed on purified polymerase chain reaction products using the ABI Prism BigDye Terminator

Cycle sequencing kit on an ABI 3130 automated sequencer (Applied Biosystems, Foster, CA, USA).

2.5. Cell cultures

To stimulate T cells, PBMCs were incubated for 72 h with 5 μg/mL phytohemagglutinin, with 1 μg/mL anti-CD3 (OKT3) plus 5 μg/mL anti-CD28 mAb, or with 20 ng/mL phorbol myristate acetate plus 250 ng/mL ionomycin in RPMI 1640 medium containing 10% fetal calf serum and antibiotics [17].

3. Results

3.1. Perforin expression and PRF1 mutations

We first analyzed perforin expression by flow cytometry. As shown in Fig. 1, perforin was not detectable in NK cells from patients P1, P2, P4 or P5. NK cells from patient P3 showed residual expression of the mutated perforin, which might be associated with the later age of onset, compared with the other patients. Table 2 presents the sequencing data for each patient. Patient P1 was a compound heterozygote bearing Leu364fs and Tyr521Cys mutations in *PRF1*. The effect of the novel missense mutation Tyr521Cys was evaluated using a web-based analysis tool, and was found to be deleterious on the basis of the SFIT program [18]. Two distinct frameshift mutations, Pro333fs and Leu364fs, were demonstrated in patient P2. The former is a novel deletion mutation. The 1090_1091delCT (Leu364fs) mutation which was found in patients P1, P2, P4 and P5 has been repeatedly reported in Japanese patients with FHL2 [3]. All patients had at least one nonsense or frameshift mutation.

3.2. Increased subpopulation of activated CD8$^+$ T cells with down-regulation of CD5

Immunophenotypic analysis of the lymphocytes demonstrated an increased percentage of CD8$^+$ T cells expressing the activation marker HLA-DR in the acute phase of the disease (Table 1, Fig. 2A). Compared with CD8$^+$ T cells, CD4$^+$ T cells were less activated, except for patient P3 who had primary EBV infection. More importantly, the activated CD8$^+$ T cells exhibited down-regulation of CD5, which was normally expressed on both CD4$^+$ and CD8$^+$ T cells from normal individuals (Fig. 2A, B). In contrast to the CD8$^+$ T cells, CD4$^+$ T cells from the patients with FHL2 exhibited normal expression of CD5. Although patients with IM have been reported to exhibit marked immune responses to regulate EBV-infected B cells and an increased subpopulation of highly activated CD8$^+$ T cells, down-regulation of CD5 was not detected in these CD8$^+$ T cells (Fig. 2B) [5].

The percentage of this unique subset (CD5$^-$ HLA-DR$^+$ CD8$^+$ T cells) decreased after treatment with the HLH-2004 protocol in patient P1 (22.1–3.9%), P4 (35.2–0.4%) and P5 (20.3–0.8%) concomitant with the relief of clinical symptoms and serum levels of neopterin and IFN-γ (Fig. 2C). We did not perform serial analysis in patient P3 due to the availability of sample. In contrast, patient P2 did not respond well to the treatment and continued to exhibit a high percentage of the subset (58.3–63.0%) and died at 12 days of age. CD107 cytotoxicity assay was not performed in our patients.

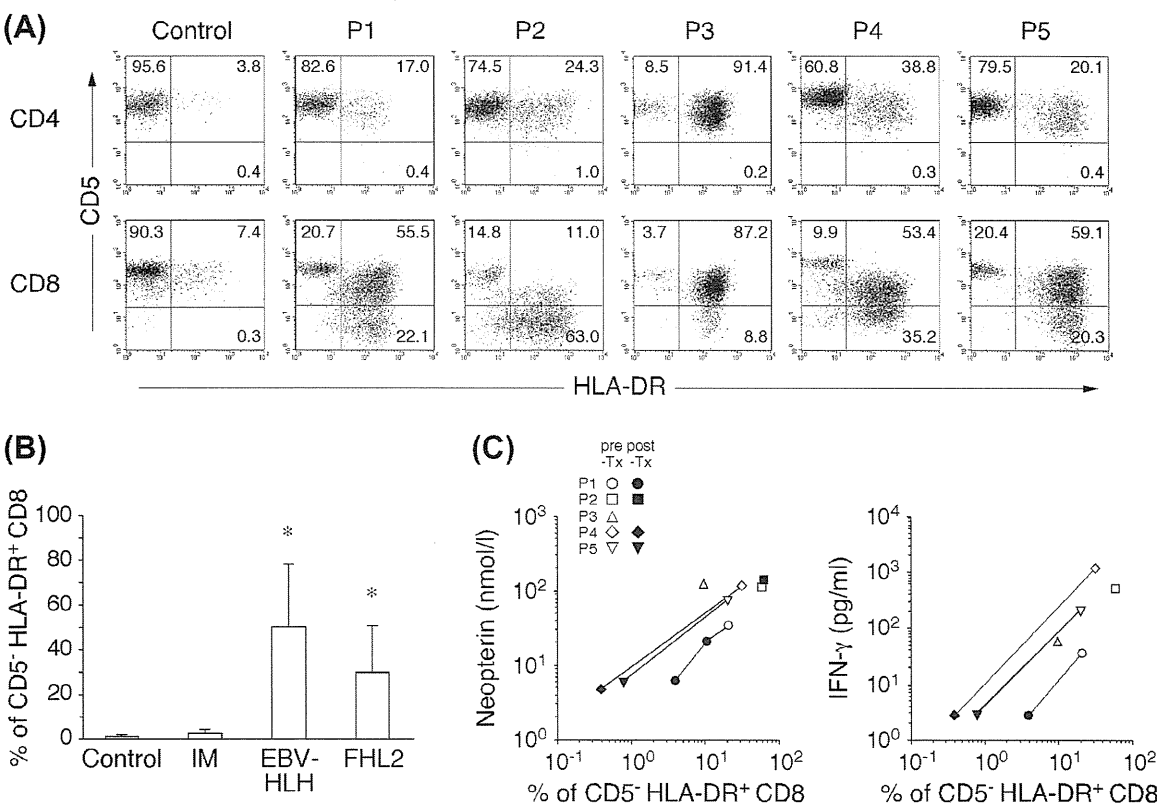

Fig. 2. Expression of CD5 by CD8$^+$ T cells (A) Expression of CD5 and HLA-DR on the CD4$^+$ and CD8$^+$ T cells. The percentage of cells gated in each region is shown. (B) The frequency of CD5$^-$ HLA-DR$^+$ CD8$^+$ T cells. Shown are the percentages of CD5$^-$ HLA-DR$^+$ cells among CD8$^+$ T cells in controls (n = 10), IM (n = 13), EBV–HLH (n = 10), and FHL2 (n = 5). Bars represent the standard deviation. *p < 0.01. (C) Correlation between the percentages of CD5$^-$ HLA-DR$^+$ CD8$^+$ T cells and serum levels of neopterin and IFN-γ. Open symbols indicate data of pre-treatment (Tx) time points; solid symbols represent those of post-Tx time points. EBV, Epstein-Barr virus; FHL2, familial hemophagocytic lymphohistiocytosis type 2; IM, infectious mononucleosis; HLH, hemophagocytic lymphohistiocytosis; IFN-γ, interferon-γ.

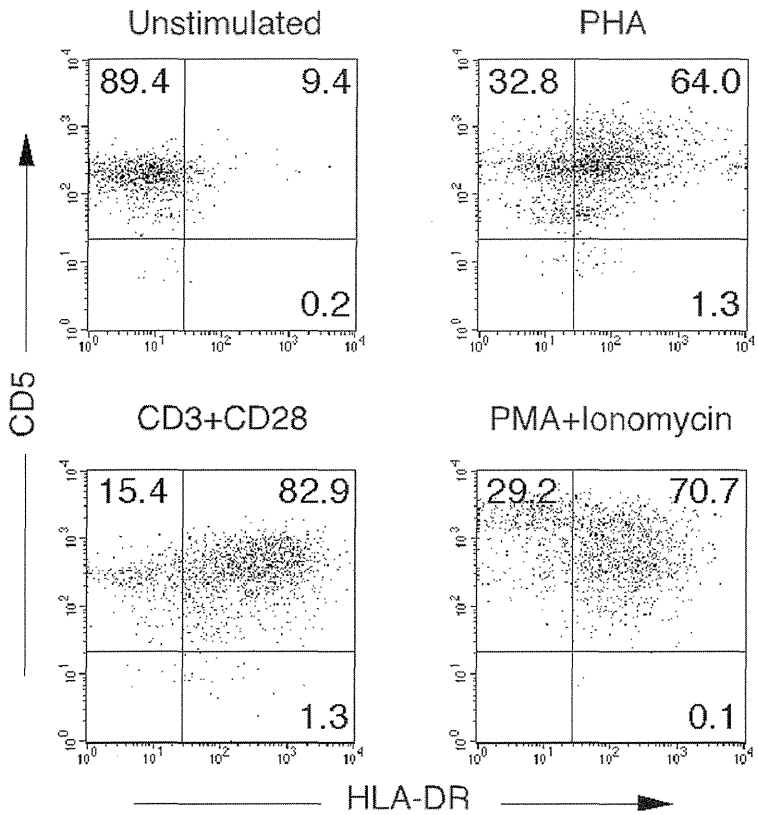

Fig. 3. Expression of CD5 after *in vitro* stimulation. PBMCs from normal controls were cultured for 72 h with phytohemagglutinin (PHA), with anti-CD3 (OKT3) plus anti-CD28 mAb, or with phorbol myristate acetate (PMA) plus ionomycin. Expression of CD5 and HLA-DR on $CD8^+$ T cells is shown.

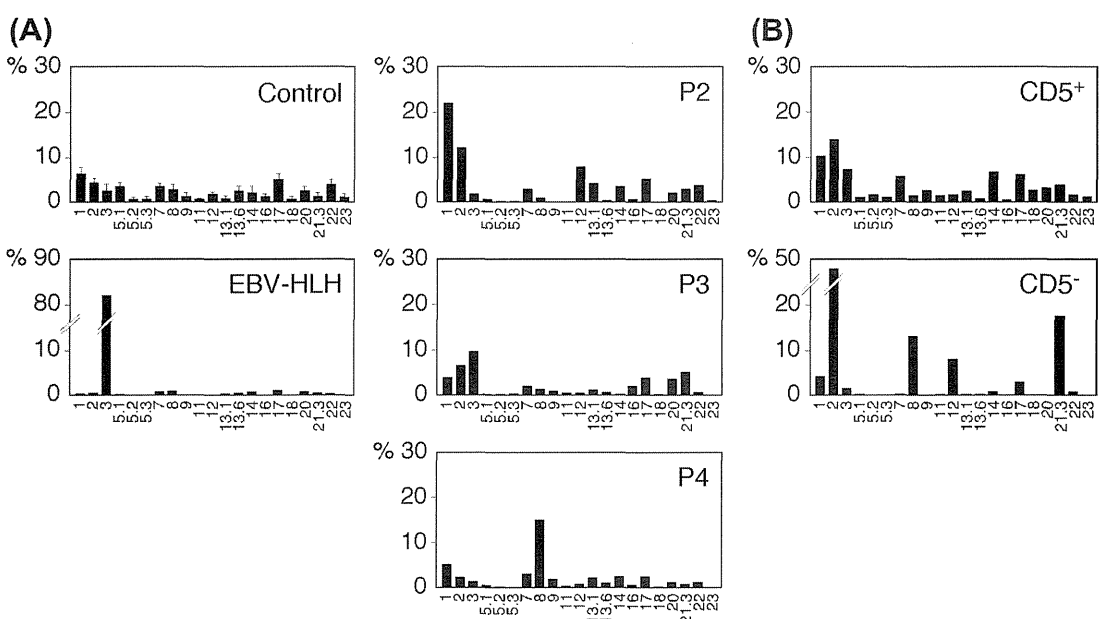

Fig. 4. TCR Vβ repertoire. (A) Peripheral blood samples were stained with monoclonal antibodies (mAbs) for individual TCR Vβ together with anti-CD4 and anti-CD8 mAbs. The percentage of TCR Vβ expression within the $CD8^+$ T cells was shown. (B) Peripheral blood samples were stained with mAbs for individual TCR Vβ subfamilies together with anti-CD8 and anti-CD5 mAbs. The percentage of TCR Vβ expression within the $CD5^+$ $CD8^+$ and $CD5^-$ $CD8^+$ T cells from patient P2 at 12 days of age was shown.

3.3. CD5 expression on normal T cells after in vitro stimulation

To assess whether down-regulation of CD5 was induced on normal T cells after simulation, PBMCs from three normal individuals were cultured for 72 h with phytohemagglutinin, anti-CD3 plus anti-CD28 mAbs, or phorbol myristate acetate plus ionomycin. Although normal $CD8^+$ T cells expressed activation marker HLA-DR after any of the stimulation, CD5 was not down-regulated (Fig. 3). We were not able to analyze expression of CD5 after *in vitro* stimulation using PBMCs from patients with FHL2 because

no appropriate samples were available, except for from patient P4. No down-regulation of CD5 was observed on the stimulated $CD8^+$ T cells from patient P4 (data not shown).

3.4. TCR Vβ repertoire of $CD8^+$ T cells

We have previously demonstrated that activated and EBV-infected $CD8^+$ T cells with down-regulation of CD5 proliferated clonally in patients with EBV–HLH [5]. To assess clonality of the $CD8^+$ T cells from patients with FHL2, we investigated the diversity of the TCR Vβ repertoire in $CD8^+$ T cells by flow cytometry (Fig. 4A). Although a massive expansion of a specific TCR Vβ has often been demonstrated for $CD8^+$ T cells from patients with EBV–HLH, $CD8^+$ T cells from patients with FHL2 exhibited oligoclonal or polyclonal proliferation. Oligoclonal expansion was more prominent in $CD5^-$ $CD8^+$ T cells than in $CD5^+$ $CD8^+$ T cells from patient P2 at 12 days of age (Fig. 4B).

4. Discussion

HLH is a heterogeneous group of diseases that are characterized by uncontrolled proliferation of activated macrophages and T cells with overproduction of pro-inflammatory cytokines [1,2]. Activated $CD8^+$ T cells are frequently observed during the acute phase of HLH. We have recently described the clinical significance of down-regulation of CD5 on activated and clonally-expanded $CD8^+$ T cells that were predominantly infected by EBV in patients with EBV–HLH [5]. However, down-regulation of CD5 is likely a general consequence of the dysregulated proliferation of $CD8^+$ T cells. Increased subpopulations of $CD5^-$ $CD8^+$ T cells have been reported in patients with allogeneic bone marrow transplantation, human immunodeficiency virus-1 infection, acute herpes virus infections and peripheral T-cell neoplasma [19–22]. In addition, a 17-day-old patient with FHL2 has been reported to exhibit uncontrolled proliferation of $CD5^-$ $CD8^+$ T cells that showed massive infiltration into the liver [11]. We therefore investigated whether this unusual subset of $CD5^-$ $CD8^+$ T cells was generally present in FHL2, which represents most common form of FHL.

In the present study, we demonstrated a significant increase in the subpopulation of $CD5^-$ $CD8^+$ T cells in all patients with FHL2, compared with control participants. These cells expressed HLA-DR antigen, indicating an activated phenotype. In contrast to EBV–HLH in which expanded $CD8^+$ T cells often reacted with a specific TCR Vβ mAb reflecting clonal proliferation of EBV-infected cells [5], $CD8^+$ T cells from patients with FHL2 exhibited much milder restriction in TCR Vβ repertoire suggesting reactive proliferation. This unique subset of $CD5^-$ HLA-DR^+ $CD8^+$ T cells in FHL2 was detectable only in the acute phase of HLH in which patients exhibited hypercytokinemia, and declined progressively after successful treatment concomitant with the levels of serum ferritin, soluble IL-2 receptor and pro-inflammatory cytokines. Thus, serial analysis of CD5 expression and activation markers on $CD8^+$ T cells may represent an additional valuable tool for the follow-up of patients with FHL2. Moreover, this profile might provide us with clinical clues to suspect FHL2 during the initial flow cytometric assessment of lymphocyte subsets with a small amount of peripheral blood.

The mechanism underlying down-regulation of CD5 on activated $CD8^+$ T cells from patients with FHL2, as well as with EBV–HLH, remains to be elucidated. It is also unknown whether $CD8^+$ T cells from other HLH cases exhibit similar profiles during the acute phase. Failure of the down-regulation of CD5 on normal $CD8^+$ T cells after *in vitro* stimulation suggests that this profile indicates the highly dysregulated activation and proliferation of $CD8^+$ T cells *in vivo* in the setting of HLH. On the other hand, studies of T cells from CD5-deficient mice have reported hyperresponsiveness of $CD5^-$ T cells to TCR stimulation, suggesting that down-regulation of CD5 might contribute to the uncontrolled proliferation of $CD8^+$ T cells in FHL2. Further studies are necessary to address these issues. Because a variety of primary and secondary causes of HLH lead to similar clinical and biological features, and HLH can range from a self-limited episode to a rapidly fatal course [11], it is necessary to identify a parameter that can predict severe cases of HLH, for which the timely initiation of life-saving therapy would be required. Further investigations are underway to determine whether down-regulation of CD5 on activated $CD8^+$ T cells could distinguish severe cases of HLH from other inflammatory conditions, including mild cases of HLH.

In summary, our studies demonstrate the increased subpopulation of activated $CD8^+$ T cells with down-regulation of CD5 in the acute phase of FHL2, and point to an additional aspect of the immune dysregulation in this disease.

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Soluble ST2 as a marker of disease activity in systemic juvenile idiopathic arthritis

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ABSTRACT

To assess the role of interleukin (IL)-33 and ST2, the receptor for IL-33, in the pathogenesis of systemic juvenile idiopathic arthritis (s-JIA), we sequentially measured the serum levels of IL-33 and soluble ST2 (sST2) in patients with s-JIA and determined their correlation with measures of disease activity and severity. Twenty-four patients with s-JIA, 5 with rheumatoid factor positive polyarticular JIA (RF + poly-JIA), and 20 age-matched healthy controls (HCs) were analyzed. IL-33 and sST2 levels were quantified in serum by enzyme-linked immunosorbent assays. Serum IL-33 levels in most patients with active s-JIA were below the lowest detection limit. Serum IL-33 levels in patients with RF + poly-JIA were significantly higher than those in patients with s-JIA and HC. Serum sST2 levels in patients during the active phase of s-JIA were much higher than those in patients with poly-JIA and HC. Serum sST2 levels in patients with s-JIA were significantly elevated even in the inactive phase, when other clinical parameters were normalized. Serum sST2 levels correlated positively with the clinical parameters of disease activity. These findings indicate that ST2 may be an important mediator in s-JIA. Serum sST2 levels in patients with s-JIA correlated with disease activity, suggesting a potential role as a promising indicator of disease activity.

1. Introduction

Juvenile idiopathic arthritis (JIA) is a heterogeneous and multifactorial autoimmune disease characterized by chronic joint inflammation in children with onset ages younger than 16 years. It is the most common chronic rheumatic disease of childhood and an important cause of short-term and long-term disability. JIA has different subtypes that are defined based on the number of joints involved in the first 6 months of disease and the extra-articular involvement [1]. The subtypes include oligoarticular JIA (<5 joints), polyarticular JIA (poly-JIA) (⩾5 joints) and systemic JIA (s-JIA). s-JIA is defined by arthritis with spiking fever persisting for more than 2 weeks and at least one of the following clinical features of systemic inflammation: skin rash, lymphadenopathy, hepatosplenomegaly or serositis (pleuritis or pericarditis) [1]. Markedly distinct clinical and laboratory features of poly-JIA and s-JIA indicate their distinct pathogenesis and immunologic abnormality. The pathogenesis of JIA is not fully understood but likely includes genetic and environmental factors which show some commonality to various adult rheumatic diseases e.g. rheumatoid factor positive (RF+)poly-JIA and adult RA, s-JIA and adult Still's disease [2,3]. High numbers of autoreactive T cells within the joint of poly-JIA patients indicate an antigen-driven activation of the adaptive immune system [4]. However, the typical clinical signs of s-JIA are rather associated with granulocytosis, thrombocytosis and increase of acute-phase reactants in the peripheral blood, indicating an uncontrolled activation of the innate immune system. Recent studies have shown that inflammatory cytokines, including interleukin (IL)-1, IL-6, and IL-18, play pathogenic roles in the disease processes of s-JIA [5]. Furthermore, the treatment with biologic therapies to block these cytokines has a dramatic effect for s-JIA patients [5]. These findings indicate that autoinflammatory mechanisms seem to play a major role in the pathogenesis of s-JIA.

Macrophage activation syndrome (MAS) is a severe, potentially life-threatening complication of s-JIA. It is clinically characterized by fever, hepatosplenomegaly, lymphadenopathy, profound depression of all three blood cell lines, deranged liver function, intravascular coagulation, and central nervous system dysfunction [6]. The excessive activation and proliferation of T lymphocytes and macrophages are observed in MAS. Massive hypercytokinemia is strongly associated with the pathogenesis of MAS [6].

Interleukin-33 (IL-33) is a novel IL-1 family cytokine that plays a major role in inflammatory, infectious, and autoimmune diseases [7,8]. IL-33 was identified as the ligand for the orphan receptor, ST2 [9]. ST2 molecule is a member of the IL-1 receptor family that exists in two forms: a transmembrane full-length form and a soluble,

Abbreviations: s-JIA, systemic juvenile idiopathic arthritis; MAS, macrophage activation syndrome; sST2, solubleST2; IL, interleukin; LPS, lipopolysaccharide; RF, rheumatoid factor.

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