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Brief report

Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

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We investigated human leukocyte antigen (HLA) expression on leukemic cells derived from patients at diagnosis and relapse after hematopoietic stem cell transplantation (HSCT) using flow cytometry with locus-specific antibodies. Two of 3 patients who relapsed after HLA-haploidentical HSCT demonstrated loss of HLA alleles in leukemic cells at relapse; on the other hand, no loss of HLA alleles was seen in 6 patients who relapsed after HLA-identical HSCT. Single-nucleotide polymorphism array analyses of sorted leukemic cells further revealed the copy number-neutral loss of heterozygosity, namely, acquired uniparental disomy on the short arm of chromosome 6, resulting in the total loss of the mismatched HLA haplotype. These results suggest that the escape from immunosurveillance by the loss of mismatched HLA alleles may be a crucial mechanism of relapse after HLA-haploidentical HSCT. Accordingly, the status of mismatched HLA on relapsed leukemic cells should be checked before donor lymphocyte infusion. (*Blood.* 2010;115(15):3158-3161)

Introduction

Human leukocyte antigen (HLA) molecules expressed on the cell surface are required in presenting antigens to T cells. The HLA class I antigens are vital in the recognition of tumor cells by tumor-specific cytotoxic T cells. The loss of HLA class I molecules on the cell surface membrane may lead to escape from T-cell immunosurveillance and the relapse of leukemia. Previously, loss of HLA class I haplotype has been described in solid tumors.[1-3] However, there are few reports concerning HLA-haplotype loss in leukemia.[4,5]

We examined HLA class I expression in leukemic blasts from patients who relapsed after hematopoietic stem cell transplantation (HSCT) to analyze whether the loss of HLA on leukemic cells was related to the relapse after HLA-identical or haploidentical HSCT.

Methods

Patients and transplantation procedure

We identified 9 children with acute leukemia who relapsed after HSCT. Their leukemic samples were cryopreserved both at the time of the initial diagnosis and of relapse. The patients' characteristics are summarized in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Three patients received HSCT from an HLA-haploidentical family donor, and the other 6 patients received HSCT from an HLA-matched donor (4 siblings and 2 unrelated donors).

Written informed consent was given by the parents according to the protocol approved by the ethics committee of Nagoya University Graduate School of Medicine in accordance with the Declaration of Helsinki.

HLA class I expression on leukemic cells

Samples were collected at diagnosis and post-transplantation relapse. HLA expression of leukemic blasts and normal cells was analyzed by flow cytometry as previously reported.[6] Anti-HLA A2-FITC (cloneBB7.2) and anti-HLA A24-FITC (clone17a10) monoclonal antibodies were purchased from Medical & Biological Laboratories; HLA-A11 (IgM), HLA-A30, HLA-31 (IgM), HLA-25, HLA-26 (IgM), HLA-Bw6 (IgG3), and HLA-Bw4 (IgG3) antibodies were purchased from One Lambda. For leukemic cell markers, CD13-PE (IgG1) were purchased from Immunotech and CD34-APC (IgG1) were purchased from BD Biosciences. Samples were analyzed with FACSCalibur cytometer and CellQuest software. The method of genomic HLA typing was previously reported.[7]

Isolation of DNA and single nucleotide polymorphism analysis

The $CD13^+/CD34^+$ leukemic blasts were sorted by flow cytometry from bone marrow cells at the time of diagnosis and of relapse. Genomic DNA was extracted from leukemic cells sorted by a fluorescence-activated cell sorter as well as from phytohemagglutinin-stimulated patient-derived T cells and subjected to single nucleotide polymorphism (SNP) array analysis using GeneChip NspI arrays (Affymetrix) according to the manufacturer's protocol. Allele-specific copy number was detected using Copy Number Analyzer for GeneChip software as previously described.[8]

Limiting dilution-based CTLp frequency assay

The frequencies of cytotoxic T-lymphocyte precursor (CTLp) specific for the recipient-mismatched HLA molecules were analyzed using a standard limiting dilution assay.[9]

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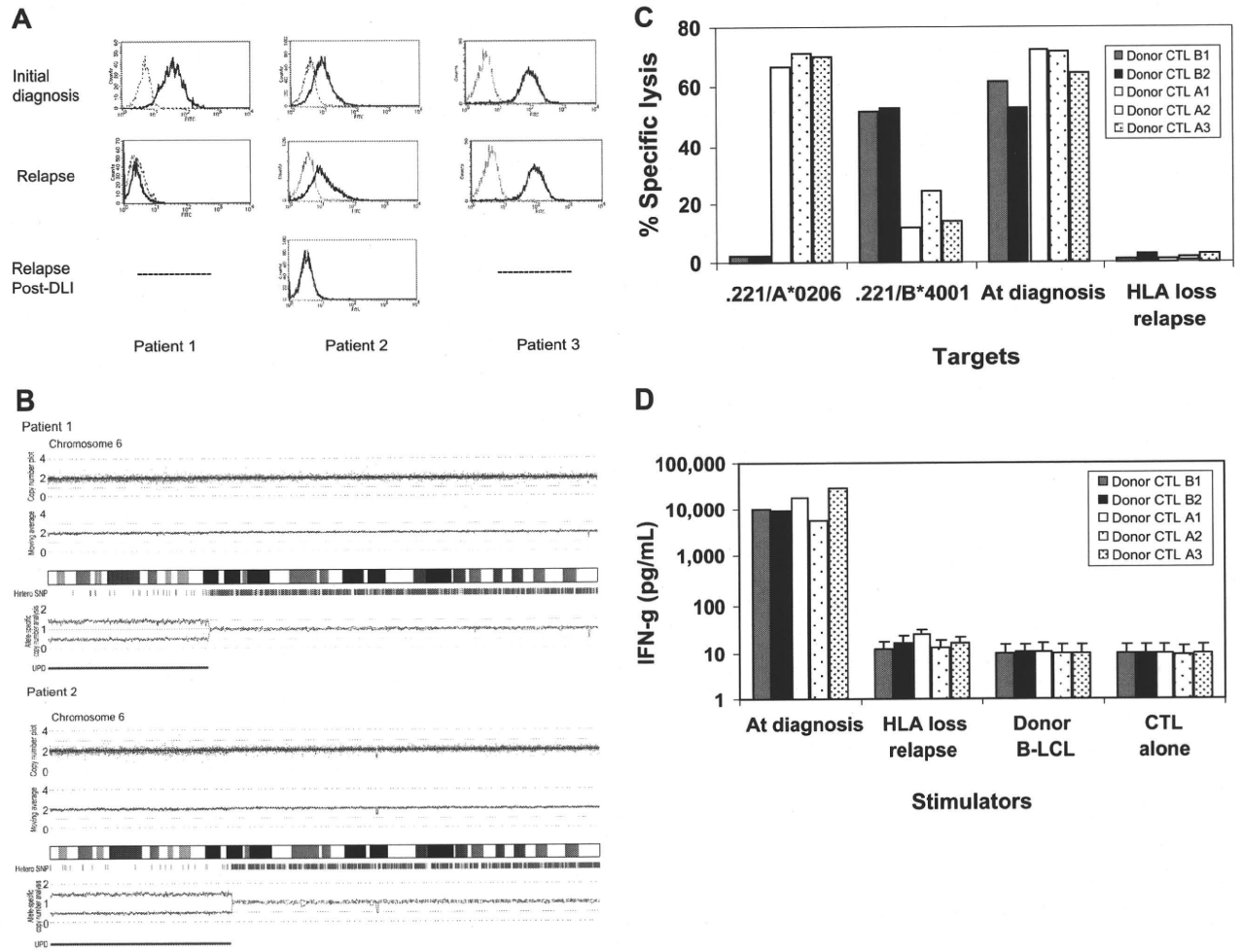

Figure 1. The loss of mismatched HLA expression on leukemic blasts caused by uniparental disomy on chromosome 6p impaired recognition and killing of donor's alloreactive cytotoxic T lymphocytes. (A) Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by $CD34^+$ and $CD13^+$, and then the surface expression of mismatched human leukocyte antigen (HLA) alleles was examined with anti–HLA-A2 antibodies. In 3 patients with acute myelogenous leukemia (AML) who experienced relapse after HLA-haploidentical HSCT, HLA-A2 expression was lost in patient 1 at relapse 15 months after HSCT and lost in patient 2 at second relapse 6 months after DLI. (B) Single nucleotide polymorphism (SNP) array analyses of sorted leukemic cells with the loss of an HLA allele revealed that the short arm of chromosome 6 shows copy number-neutral loss of heterozygosity or acquired uniparental disomy as detected by dissociated allele-specific copy number plots (red and blue lines at the bottom), resulting in the total loss of the mismatched HLA haplotype in both patient 1 and patient 2. The presence of acquired uniparental disomy is also indicated by normal total copy numbers with missing heterozygous SNPs (green bars) in the distal part of the short arm. (C) Recipient alloantigen-specific cytotoxic T-lymphocyte (CTL) clones were generated by a conventional cloning method from cytotoxicity-positive wells obtained in the limiting dilution assays using the donor $CD8^+$ cells as responders. Donor CTL clones A1, A2, and A3 were specific for HLA-A*0206. Donor CTL clones B1 and B3 were specific for HLA-B*4001, all of which recognize mismatched HLA alleles between the donor and recipient. Those 5 representative CTL clones were tested for HLA specificity and recognition of leukemic blasts obtained at the time of the initial diagnosis and at the time of HLA loss relapse after DLI by a standard ^{51}Cr-release assay at the effector/target ratio of 30:1. (D) Their interferon-γ production was also assessed against leukemic blasts collected at the time of diagnosis and at the time of HLA-loss relapse.

Cytotoxic assay of CTL clones against leukemic blasts and a mismatched HLA cDNA-transfected B-lymphoblastoid cell line

The remaining cells of several cytotoxicity-positive wells used for the CTLp assay for the donor were used to obtain allo-HLA–restricted CTLs. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,11]

The HLA class I–deficient 721.221 B-lymphoblastoid cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 1mM sodium pyruvate. Retroviral transduction was conducted as previously described.[12]

The cytotoxicity of CTL clones against target cells was analyzed by conventional chromium 51 (^{51}Cr) release assay as previously reported.[13]

CTL clones (10^4 cells/well) were mixed with the indicated stimulator cells (10^4 cells/well) in 96-well, round-bottom polypropylene plates and spun at 1200*g* for 3 minutes before overnight incubation in 200 μL of RPMI 1640 medium supplemented with 10% fetal bovine serum. On the next day, 50 μL of supernatant was collected and interferon-γ was measured by enzyme-linked immunosorbent assay with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich).

Results and discussion

Three children with high-risk acute myelogenous leukemia (AML) received haploidentical grafts from their parents but relapsed 8, 14, and 15 months after HSCT. Patient 2 received 3 courses of donor lymphocyte infusion (DLI) for relapsed leukemia after haploidentical HSCT. After the third unmanipulated DLI (10^7 $CD3^+$/kg), she experienced acute grade-III graft-versus-host disease and achieved complete remission. However, she experienced a second relapse 6 months later. To monitor residual disease in those patients, we used flow cytometric analysis with antibodies specific for the mismatched HLA alleles between the donor and patient. Surprisingly, we found total loss of

HLA-A2 expression on $CD13^+/CD34^+$ leukemic cells from bone marrow in 2 of 3 patients who underwent HLA-haploidentical HSCT, whereas microscopic analysis showed relapse (Figure 1A). To test whether HLA class I molecules could be up-regulated, samples were cultured for 48 hours in medium supplemented with tumor necrosis factor-α or interferon-γ and measured again; however, no restoration was observed (data not shown).

Next, to examine the potential loss of genes encoding the undetectable HLA alleles, we sorted $CD13^+/CD34^+$ leukemic blasts and performed DNA genotyping. We found that, in addition to the HLA-A locus, the HLA-B, -C, and -DR loci were not encoded; only the mismatched haplotype was lost in both patients (supplemental Table 2). We then questioned whether this phenomenon would also occur in HLA-matched HSCT settings using anti-HLA class I antibodies. We did not observe any loss of HLA class I expression in any of the patients at the time of relapse (supplemental Figure 1). These results suggest that loss of HLA class I haplotype at the time of posttransplantation relapse is uncommon in HLA-matched HSCT.

To elucidate the mechanism of the loss of the mismatched HLA haplotype, we performed an SNP array analysis of genomic DNA extracted from leukemic blasts at the time of diagnosis and of relapse. Genomic DNA from patient-derived T cells was used as a reference. Leukemic cells at the time of relapse showed copy number-neutral loss of heterozygosity or an acquired uniparental disomy (UPD) of the short arm of chromosome 6 encompassing the HLA locus, whereas no allelic imbalance was identified at the time of diagnosis (Figure 1B). Loss of one allele from one parent and duplication of the remaining allele from the other parent led to UPD.[14]

In patient 2, we examined whether the number of CTLp had changed during the posttransplantation course. Limiting dilution analysis with a split-well ^{51}Cr-release assay was carried out to compare the CTLp frequencies specific for the mismatched antigens between the recipient and donor. Interestingly, the CTLp frequencies were recovered after DLI (Table 1). Restoration of CTLp after 3 DLIs could eradicate such leukemic cells, lasting for 6 months thereafter.

Next, we generated allo-HLA–restricted CTLs from $CD8^+$ cells obtained at day 520 in patient 2 and tested with the 721.221 B-lymphoblastoid cell line transfected with 1 of 3 mismatched HLA alleles (Figure 1C-D).

Despite high transplantation-related mortality resulting from severe graft-versus-host disease and posttransplantation infections, haploidentical HSCT has been widely used with the expectation of a strong graft-versus-leukemia effect.[15] However, our observation provides a possible limitation of this strategy. Indeed, 2 of 3 patients showed genomic loss of the recipient-specific HLA-haplotype, which led to escape from the graft-versus-leukemia effect and relapse of the disease.

Vago et al also reported a similar observation in 5 of 17 (29.4%) patients whose disease relapsed after haploidentical HSCT.[16] Relapsed leukemic cells may possess genomic instability that elicits genetic diversity.[17] Immunologic pressure by alloreaction to major HLA antigens may select leukemic variants of HLA class I loss, which results in the survival and proliferation of these variants.

In haploidentical HSCT, the importance of natural killer (NK)–cell alloreactivity is emphasized to achieve the graft-versus-leukemia effect.[18,19] HLA loss on leukemic blasts may in turn enhance the NK-cell alloreactivity. Our 2 patients with HLA loss had a group 1 homozygous HLA-C locus that is a suppressive killer immunoglobulin-like receptor (KIR) for NK cells and a KIR-matched donor (supplemental Table 2). Because UPD does not change the total copy number of the gene, donor NK cells should have been suppressed even after UPD occurred in these patients. Interestingly, the remaining patient who experienced relapse without HLA loss after HLA-haploidentical HSCT had a KIR-mismatched donor, so alloreactive NK cells were possibly enhanced to kill leukemic blasts with HLA loss.

Although one limitation of our study is an insufficient number of cases, our results combined with those in a recent report[16] suggest that leukemic cells occasionally escape from immunosurveillance through the loss of the mismatched HLA haplotype by the mechanism of UPD after haploidentical HSCT. DLI for relapsed AML is less effective than that for chronic myelogenous leukemia after HLA-matched HSCT.[20] However, DLI is effective even for the relapse of AML after haploidentical HSCT.[21] Evaluation of loss or down-regulation of HLA on relapsed leukemic blasts after HLA-haploidentical HSCT should be considered because DLI would probably be ineffective in patients whose leukemic cells lose HLA class I antigen.

Table 1. The CTLp frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Samples	Maximum $CD8^+$ input*	No. of growing wells†	CTLp frequency^{-1} (95% confidence interval)
Donor	33 300	8	8.6×10^5 (1.49×10^6-5.0×10^5)
Day 100	35 500	0	UD
Day 180	17 700	0	UD
Day 300‡	86 000	0	UD
Day 520§	95 000	7	4.3×10^5 (7.2×10^5-2.5×10^5)

Purified $CD8^+$ T cells from the peripheral blood mononuclear cells obtained after transplantation from patient 2 and her donor were cultured at 2- or 3-fold serial dilutions with 33 Gy-irradiated 3×10^4 leukemic blasts cryopreserved at the time of initial diagnosis in 96-well, round-bottom plates in advanced RPMI 1640 medium supplemented with 4% pooled human serum, interleukin-6 (IL-6), and IL-7 (10 ng/mL; both from R&D Systems). The IL-2 (50 U/mL) was added on day 7 with a half medium change. For each dilution, there were at least 12 replicates. On day 14 of culture, a split-well analysis was performed for recipient-specific cytotoxicity against ^{51}Cr-radiolabeled recipient T-cell blasts, donor T-cell blasts, and leukemic blasts harvested at the time of initial diagnosis and at the time of relapse after DLI if indicated. The supernatants were measured in a γ counter after 4-hour incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was more than 3 SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated using L-Calc software (StemCell Technologies). The CTLp frequencies reactive with recipient T-cell blasts in $CD8^+$ T cells obtained around days 100, 180, and 300 (4 months before relapse) were undetectable, whereas the CTLp frequency obtained at day 520 (1 month after the third DLI or 2 weeks after remission confirmed by bone marrow aspirate) was close to the CTLp frequency in the donor $CD8^+$ cells. Complete remission and more than 99% donor chimerism were confirmed on those days

CTLp indicates CTL precursor; and UD, undetermined because no growing wells are present.

*Number of input $CD8^+$ T cells seeded at the highest number per well.

†Number of wells out of 12 wells that received the highest $CD8^+$ cells and showed detectable growth.

‡Corresponds to 4 months before relapse.

§Corresponds to 1 month after the third DLI or 2 weeks after complete remission was confirmed by bone marrow aspirate.

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Authorship

Contribution: I.B.V. performed experiments and wrote the manuscript; Y.T. designed the research, analyzed data, and wrote the manuscript; Y.A., H.S., M.K., and S.O. performed experiments, analyzed data, and wrote the manuscript; S.K. supervised this work and wrote the manuscript; and all other authors were responsible for clinical work and critically reviewed the manuscript.

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Autoimmune lymphoproliferative syndrome-like disease with somatic KRAS mutation

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Autoimmune Lymphoproliferative Syndrome Like Disease With Somatic *KRAS* Mutation

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Abstract

Autoimmune lymphoproliferative syndrome (ALPS) is classically defined as a disease with defective FAS-mediated apoptosis (Type I–III). Germline *NRAS* mutation was recently identified in Type IV ALPS. We report two cases with ALPS like disease with somatic *KRAS* mutation. Both of the cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia (JMML), and are likely to be defined as a new disease entity of RAS associated ALPS like disease (RALD).

Introduction

ALPS is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway[1,2], currently categorized as Type Ia, germline *TNFRSF6/FAS* mutation; Type Ib, germline *FAS ligand* mutation; Type Is, somatic *TNFRSF6/FAS* mutation; and Type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases such as immune cytopenia and hyper-γ-globulinemia. An additional subclassification has been proposed that includes Types III and IV, whereby Type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis, and Type IV as one showing germline *NRAS* mutation[3]. Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to IL-2 depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS associated leukoproliferative disease [4].

JMML is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. About 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells including mutations of *NF1*, *RAS* family[5], *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood (PB) mononuclear cells (MNC) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of CD34 positive BM-MNC[6].

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous (CFC) syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7],

among these tumors the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present two cases with autoimmune cytopenia and remarkable lymphadenopathy and hepatosplenomegaly, both of which were identified as having a somatic KRAS G13D mutation without any clinical features of germline *RAS* mutation such as CFC or Noonan syndrome.

Patients and Methods

All studies were approved by the ethical board of Tokyo Medical and Dental University.

Case 1

A 9-month-old boy had enormous bilateral cervical lymphadenopathy and hepatosplenomegaly (Supplemental data 1 Fig. 1a, b). Blood test revealed presence of hemolytic anemia and autoimmune thrombocytopenia. hyper-γ-globulinemia with various auto-antibodies was also noted. ALPS and JMML were nominated as the diseases to be differentially diagnosed. Detailed clinical history and laboratory data are provided as Supplemental data 1. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in results and discussion section.

Case 2

A 5-month-old girl had a fever, massive hepatosplenomegaly (Supplemental data 1 Fig. 1d). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper-γ-globulinemia and auto-antibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNC showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive

monocytosis or increased HbF. Detailed clinical history and laboratory data is provided as Supplemental data 1.

Detailed methods for experiments are described in Supplemental data 2

Results and Discussion

Case 1 showed a high likelihood of being a case of ALPS according to the symptoms and clinical data presented (Supplemental data 1, Table 1) except for number of Double-negative T (DNT) cells which was only 1.4% of TCRαβ cells (Fig. 1a). JMML was also nominated as a disease to be differentiated, because remarkable hepatosplenomegaly with thrombocythemia and moderate monocytosis was noted. However, no hypersensitivity to GM-CSF as determined by colony formation assay for BM-MNC (data not shown) or phospho STAT5 staining (data not shown) was observed. DNA sequence for JMML associated genes such as *NRAS, KRAS, HRAS, PTPN11* and *CBL* was determined, and KRAS G13D mutation was identified (Fig. 1b). The mutation was seen exclusively in the hematopoietic cell lineage and no mutation was seen in the oral mucosa or nail-derived DNA. Granulocytes, monocytes, T cells, and B cells were all positive for KRAS G13D mutation (data not shown). The proportion of mutated cells in each hematopoietic lineage was quantitated by mutation allele specific quantitative PCR methods, which revealed mutated allele was almost equally present in granulocytes, T cells and B cells (Fig 1c). CD34-positive hematopoietic stem cells (HSC) was also positive for KRAS G13D mutation, and 60% of CFU-GM colonies developed from isolated CD34 cells carried KRAS G13D mutation (data not shown). These observations suggest that the mutation occurred at the HSC level, and HSCs consists of wild type and mutant HSCs.

NRAS mutated Type IV ALPS was previously characterized by apoptosis

resistance of T-cells in IL-2 depeletion[3]. Then, activated T cells were subjected to an apoptosis assay by FAS stimulation or IL-2 depletion. Remarkable resistance to IL-2 depletion but not to FAS-dependent apoptosis (Fig. 1d and e) was seen. This was in contrast to T cells from FAS mutated ALPS type 1a which showed remarkable resistance to FAS dependent apoptosis and normal apoptosis induction by IL-2 withdrawal (Fig. 1d and e). Western blotting analysis of activated T cells or Epstein-Barr virus-transformed B cells showed reduced expression of Bim (Fig. 1f).

In case 2, autoimmune phenotype and hepatosplenomegaly were remarkable as shown in Supplementary data 1. The patient was initially diagnosed as Evans Syndrome based on presence of hemolytic anemia and autoimmune thrombocytopenia. DNT cells were 1.1% of TCRαβ cells in the peripheral blood, which did not meet with the criteria of ALPS. Although spontaneous colony formation was shown in PB- and BM-MNC, and GM-CSF hypersensitivity was demonstrated in BM-MNC derived CD34 positive cell (Supplemental data 1 Table2), she showed no massive monocytosis or increased HbF. Thus the diagnosis was less likely to be ALPS or JMML. DNA sequencing of JMML related genes such as *NRAS, KRAS, HRAS, PTPN11*, and *CBL* identified somatic but not germline KRAS G13D mutation (Fig. 1b). KRAS G13D mutation was detected in granulocytes and T cells. Mutation was not identified in B cells by conventional DNA sequencing (data not shown). Mutant allele specific quantitative PCR revealed mutated allele was almost equally present in granulocytes and T cells, but barely in B cells (Fig. 1c). Activated T cells showed resistance to IL-2 depletion but not to FAS-dependent apoptosis (Fig. 1d and e). Both of our cases were characterized by strong autoimmunity, immune cytopenia and lymphadenopathy or hepatosplenomegaly with partial similarity with ALPS or JMML. However, they did not meet with the well defied diagnostic

criteria of ALPS[2] or JMML[6]. It is interesting that Case 2 presented GM-CSF hypersensitivity, which is one of the hallmarks of JMML. Given the strict clinical and laboratory criteria of JMML and ALPS, our two cases should be defined as a new disease entity, like RAS associated ALPS like disease (RALD). Recently defined NRAS mutated ALPS type IV may also be included in a similar disease entity.

There are several cases of JMML reported simultaneously having clinical and laboratory findings compatible with autoimmune disease[8,9]. Autoimmune syndromes are occasionally seen in patients with myelodysplastic syndromes, including chronic myelomonocytic leukemia[10]. These previous findings may suggest a close relationship of autoimmune disease and JMML. Since KRAS G13D has been identified in JMML[11-13], it is tempting to speculate that KRAS G13D mutation is involved in JMML as well as RAS associated ALPS like disease (RALD). It should be noted in JMML, erythroid cells reportedly carry mutant RAS, while B and T cell involvement was variable[13]. In both of our cases, myeloid cells and T cells carried mutant RAS, while B cells were affected variably. These findings would support a hypothesis that the clinical and hematological features are related to the differentiation stages of hematopoietic stem cells where RAS mutation is acquired. JMML-like myelo-monocytic proliferation may predict an involvement of RAS mutation in myeloid stem/precursor cell level whereas ALPS-like phenotype may predict that of stem/precursor cells of lymphoid lineage, especially of T cells. Under the light of subtle differences between the two cases presented, their hematological and clinical features may reflect the characteristics of the stem cell level where *KRAS* mutation is acquired. Involvement of the precursors with higher propensity toward lymphoid lineage may lead to autoimmune phenotypes, while involvement of those with propensity toward the myeloid lineage may lead to GM-CSF hypersensitivity while still

sharing some overlapping autoimmune characteristics.

One may argue from the other points of view with regard to the clinicopathological features of these disorders. First, transformation in a fetal HSC might be obligatory for the development of JMML[14] and that in HSC later in life may not have the same consequences. Second, certain KRAS mutations may be more potent than the others. Codon 13 mutations are generally less deleterious biochemically than codon 12 substitutions, and patients with JMML with codon 13 mutations have been reported to show spontaneous hematologic improvement[12,15]. Thus further studies are needed to reveal in-depth clinicopathological characteristics in this type of lympho-myelo proliferative disorders.

KRAS mutation may initiate the oncogenic pathway as one of the first genetic hits, but is insufficient to cause frank malignancy by itself[16,17]. Considering recent findings that additional mutations of the genes involved in DNA repair, cell cycle arrest, and apoptosis are required for full malignant transformation, one can argue that RAS associated ALPS like disease (RALD) patients will also develop malignancies during the course of the diseases. Occasional association of myeloid blast crisis in JMML and that of lymphoid malignancies in ALPS will support this notion. Thus the two patients are now being followed up carefully. It was recently revealed that half of the patients diagnosed with Evans syndrome, an autoimmune disease presenting with hemolytic anemia and thrombocytopenia, meet the criteria for ALPS diagnosis[18,19]. In this study, FAS-mediated apoptosis analysis was utilized for the screening. Considering the cases we presented, it will be intriguing to re-evaluate Evans syndrome by IL-2 depletion-dependent apoptosis assay focusing on the overlapping autoimmunity with RAS associated ALPS like disease (RALD).

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Author ship

MT and SM designed entire experiments and wrote this manuscript. KS, NM, and MT treat those patients, and designed clinical laboratory test. JP performed experiments described in Fig.1b-f. KM, HM, and SD performed colony and mutational analysis. MN, TM, KK, SK, YK and AT supervised clinical and immunological experiments, or coordinated clinical information.

Conflict of interest disclosure

The authors declare no conflict of interest.

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Figure Legends

Figure 1

a. Flow cytometric analysis of DNT cells. CD8 and CD4 double staining was performed in TCRαβ-expressing cells.

b. Electropherogram showing KRAS G13D mutation in BM-MNC in case 1 (left panel) and case 2 (right panel).

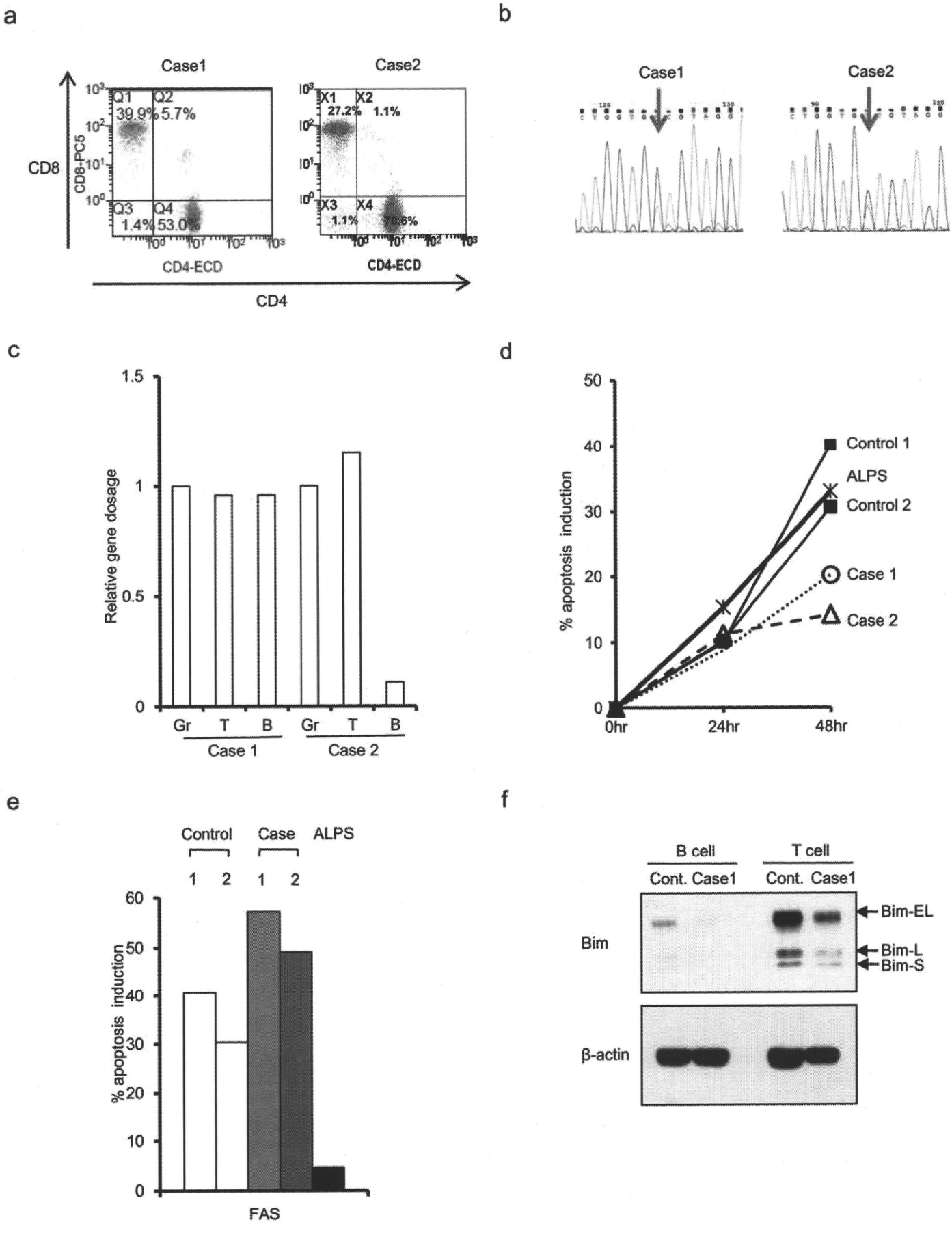
c. Gene dosage of mutated allele in granulocyte (Gr), T cell (T) and B cell (B). Relative gene dosage was estimated by a mutant allele specific PCR method in case 1 and 2 using albumin gene as internal control.

d. Apoptosis assay using activated T cells. Apoptosis percent was measured by flow cytometry with Annexin V staining 24 and 48 hr after IL-2 depletion

e. Apoptosis percent was measured 24hr after addition of anti-FAS CH11 antibody (final 100ng/ml)

f. Western blotting analysis of Bim expression.

Figure 1


a
Case1
Case2
CD8
CD8-PC5
Q1
39.9%
Q2
5.7%
Q3
1.4%
Q4
53.0%
X1
27.2%
X2
1.1%
X3
1.1%
X4
70.6%
CD4-ECD
CD4
b
Case1
Case2
c
Relative gene dosage
1.5
1
0.5
0
Gr
T
B
Gr
T
B
Case 1
Case 2
d
% apoptosis induction
50
40
30
20
10
0
0hr
24hr
48hr
Control 1
ALPS
Control 2
Case 1
Case 2
e
Control
Case
ALPS
1
2
1
2
% apoptosis induction
60
50
40
30
20
10
0
FAS
f
B cell
T cell
Cont.
Case1
Cont.
Case1
Bim
Bim-EL
Bim-L
Bim-S
β-actin

LETTERS

IκBζ regulates T_H17 development by cooperating with ROR nuclear receptors

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Interleukin (IL)-17-producing helper T (T_H17) cells are a distinct T-cell subset characterized by its pathological role in autoimmune diseases[1–3]. IL-6 and transforming growth factor-β (TGF-β) induce T_H17 development, in which the orphan nuclear receptors, RORγt and RORα, have an indispensable role[4–6]. However, in the absence of IL-6 and TGF-β, the ectopic expression of RORγt or RORα leads to only a modest IL-17 production[5,7,8]. Here we identify a nuclear IκB family member, IκBζ (encoded by the *Nfkbiz* gene), as a transcription factor required for T_H17 development in mice. The ectopic expression of IκBζ in naive $CD4^+$ T cells together with RORγt or RORα potently induces T_H17 development, even in the absence of IL-6 and TGF-β. Notably, $Nfkbiz^{-/-}$ mice have a defect in T_H17 development and a resistance to experimental autoimmune encephalomyelitis (EAE). The T-cell-intrinsic function of IκBζ was clearly demonstrated by the resistance to EAE of the $Rag2^{-/-}$ mice into which $Nfkbiz^{-/-}$ $CD4^+$ T cells were transferred. In cooperation with RORγt and RORα, IκBζ enhances *Il17a* expression by binding directly to the regulatory region of the *Il17a* gene. This study provides evidence for the transcriptional mechanisms underlying T_H17 development and points to a molecular basis for a novel therapeutic strategy against autoimmune disease.

IκBζ is a nuclear protein homologous to Bcl3, which interacts with the NF-κB subunit via the ankyrin repeat domain (ARD)[9,10]. In macrophages, IκBζ induced by Toll-like receptor (TLR) stimulation is essential for the induction of a subset of secondary response genes, including *Il6* (refs 11, 12). However, the function of IκBζ in other cell types has not been elucidated.

We explored the expression of IκBζ in helper T-cell subsets and found that IκBζ was most highly expressed in T_H17 cells (Fig. 1a). Thus, we evaluated the involvement of IκBζ in EAE, which is a model of T_H17 cell-mediated autoimmune disease[1]. After myelin oligodendrocyte glycoprotein (MOG) immunization, wild-type mice developed severe paralytic symptoms, whereas $Nfkbiz^{-/-}$ mice exhibited almost no neuronal deficit (Fig. 1b). Histopathological analyses showed inflammatory cell infiltration and demyelination in the spinal cord of the wild-type mice, but not the $Nfkbiz^{-/-}$ mice (Fig. 1c). IL-17 production was reduced in the spleen and lymph node cells from immunized $Nfkbiz^{-/-}$ mice, but interferon-γ (IFN-γ) production was normal (Fig. 1d and Supplementary Fig. 1). These results indicate that $Nfkbiz^{-/-}$ mice have a defect in T_H17 development.

IκBζ expression was also detected in dendritic cells (Fig. 1a), which produce the inflammatory cytokines required for T_H17 development[1,13]. To exclude the possibility that impaired T_H17 development in $Nfkbiz^{-/-}$ mice is caused by a defect in dendritic cells, we evaluated inflammatory cytokine expression in dendritic cells. We confirmed normal production of tumour necrosis factor-α (TNF-α) and expression of CD40 and CD86 in $Nfkbiz^{-/-}$ dendritic cells after TLR stimulation (Supplementary Fig. 2 and data not shown). TLR ligand-induced production of IL-6 was partially suppressed in $Nfkbiz^{-/-}$ dendritic cells (Supplementary Fig. 2), but a co-culture of naive $CD4^+$ T cells and dendritic cells indicated that $Nfkbiz^{-/-}$ dendritic cells were able to support T_H17 development normally (Fig. 1e, f). However, T_H17 development from $Nfkbiz^{-/-}$ naive $CD4^+$ T cells was markedly impaired regardless of the origin of the co-cultured dendritic cells (Fig. 1e, f).

To demonstrate the $CD4^+$ T-cell-intrinsic function of IκBζ *in vivo*, we transferred wild-type or $Nfkbiz^{-/-}$ $CD4^+$ T cells into $Rag2^{-/-}$ mice (Supplementary Fig. 3), and immunized them with MOG peptide. The mice transferred with wild-type $CD4^+$ T cells developed severe EAE, whereas the mice transferred with $Nfkbiz^{-/-}$ $CD4^+$ T cells had only minimal symptoms (Fig. 1g, h). We observed no significant difference in proliferation between wild-type and $Nfkbiz^{-/-}$ $CD4^+$ T cells (Supplementary Fig. 4). The frequencies of IFN-γ^-IL-17^+ and IFN-γ^+IL-17^+ T cells, but not IFN-γ^+IL-17^- T cells, were much lower in the mice that received $Nfkbiz^{-/-}$ $CD4^+$ T cells than wild-type $CD4^+$ T cells, even at early time points after MOG immunization (Fig. 1i). Collectively, $Nfkbiz^{-/-}$ $CD4^+$ T cells have an intrinsic defect in their ability to differentiate into T_H17 cells, resulting in a low sensitivity to EAE.

When activated with anti-CD3 and anti-CD28 under T_H1- and T_H2-polarizing conditions, $Nfkbiz^{-/-}$ naive $CD4^+$ T cells normally produced IFN-γ and IL-4, respectively (Fig. 2a). On activation by the combination of IL-6 and TGF-β (the T_H17-polarizing conditions), IL-17 production in $Nfkbiz^{-/-}$ T cells was significantly reduced compared with wild-type T cells, even when we added IL-23, IL-1α, TNF-α or replaced IL-6 with IL-21 (Fig. 2a, b). The expression of *Il17f*, *Il21*, *Il23r* and *Il22* messenger RNA was much lower in $Nfkbiz^{-/-}$ T cells than in wild-type T cells (Fig. 2c). The expression of *Rorc* (encoding RORγ) and *Rora* (encoding RORα) mRNA was comparable between wild-type and $Nfkbiz^{-/-}$ T cells (Fig. 2d). The mRNA expression of *Runx1*, the aryl hydrocarbon receptor (*Ahr*), *Irf4*, *Socs3* and *Batf*,

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