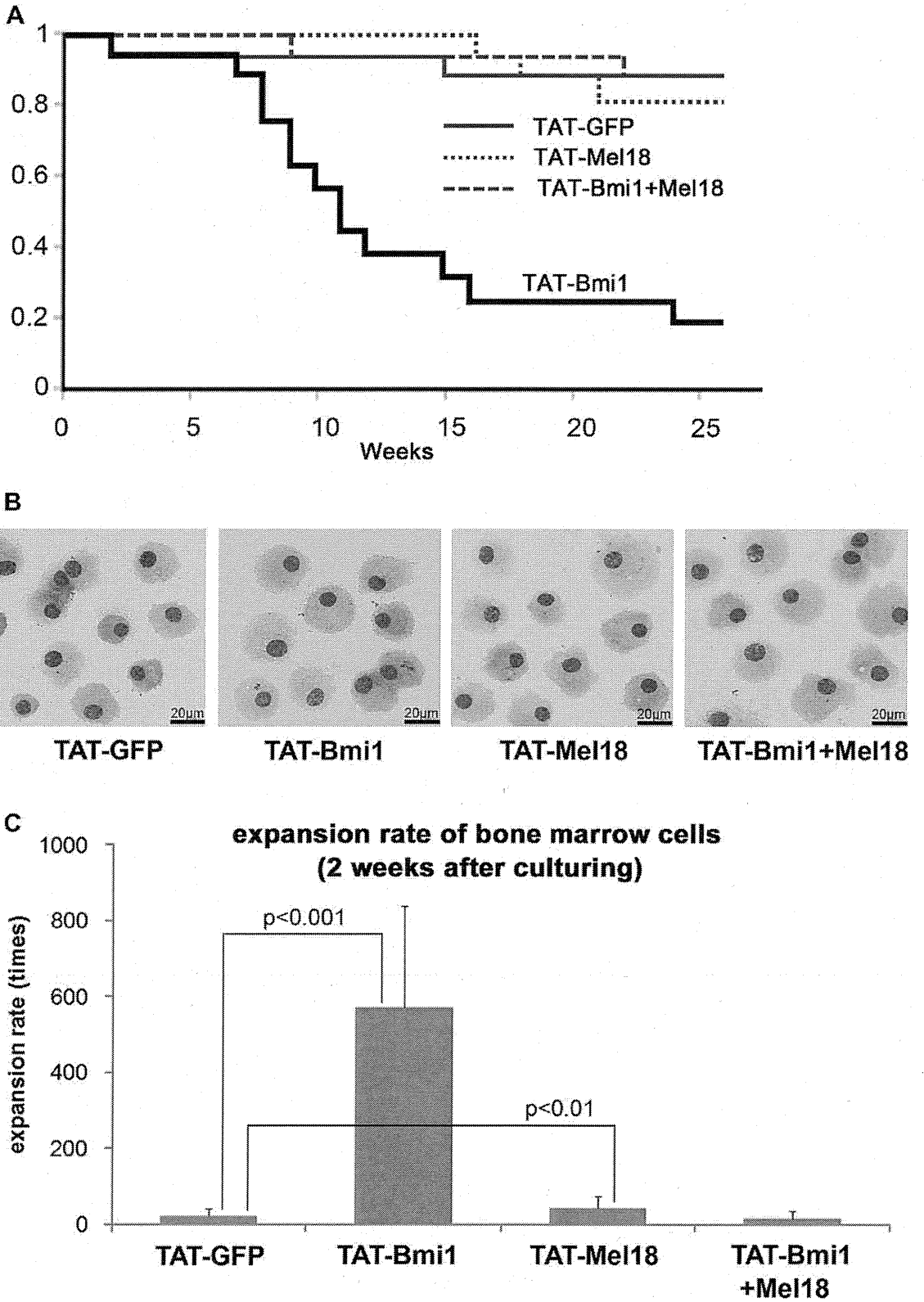

Figure 4. Long-term observation of bone marrow (BM) transplantation assay and tumorigenesis. (**A**) Approximately 80% of mice transplanted with TAT-Bmi1–transduced BM cells died at 8 to 24 weeks after transplantation. (**B**) BM cells were exposed to TAT-Polycomb for 48 h, and the cells were cultured for 8 weeks in the presence of cytokines. However, we did not observe any morphological malignancies in the cells. (**C**) However, during cell culture for 8 weeks, the number of Bmi1-exposed cells was expanded by approximately 500 times the original number of cells.

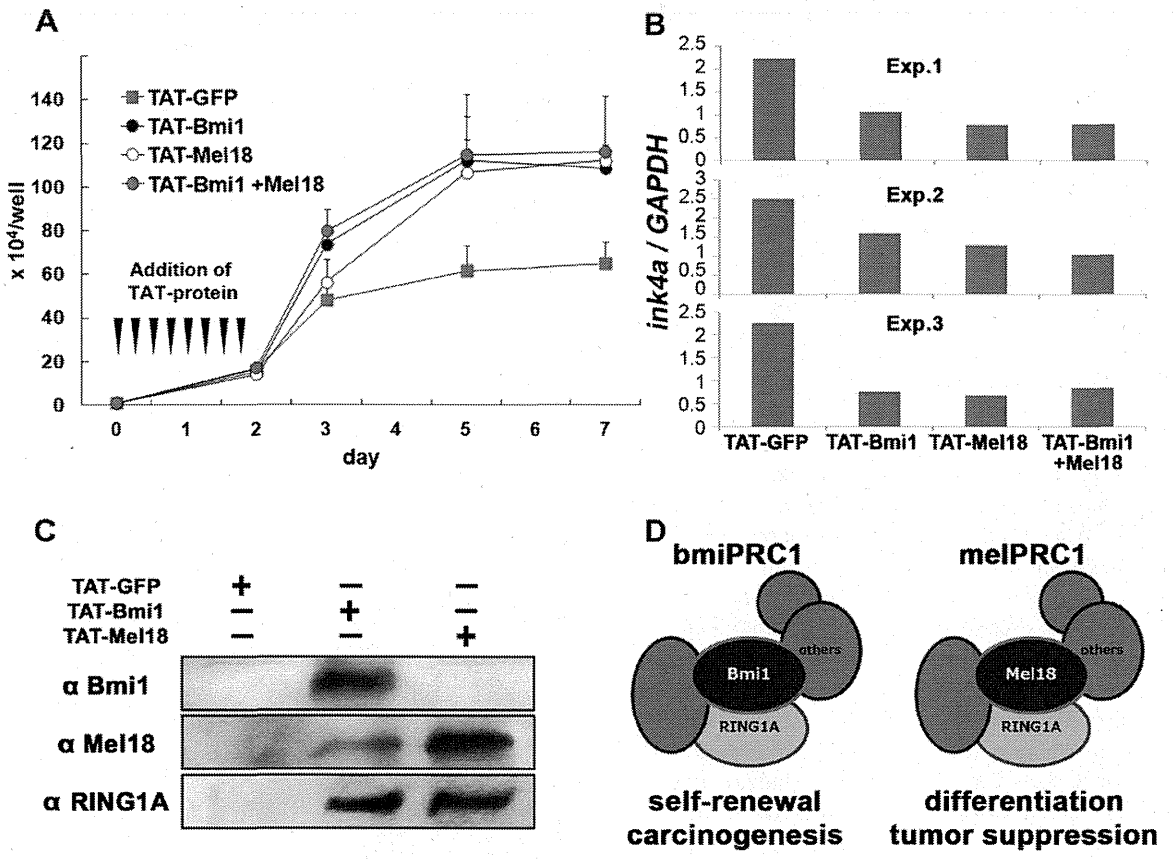

Figure 5. Regulation of target gene expression by TAT-Polycomb proteins and the components of PRC1. (**A**) We added each TAT-fusion protein to MEL cells every 6 h for 48 h and examined cell proliferation. Cell proliferation increased following the addition of either Bmi1 or Mel18. (**B**) The expression of these genes was inhibited in cells exposed to TAT-Polycomb. (**C**) A pull-down assay using HisTag was performed to analyze whether Polycomb-group complexes include both Bmi1 and Mel18. In the pull-down assay of MEL cells exposed to TAT-Bmi1, Bmi1, and RINGA1A were detected together with a small quantity of Mel18. In contrast, Bmi1 was not detected at all in the pull-down assay of MEL cells exposed to TAT-Mel18. (**D**) Results are summarized in the schema.

Discussion

Bmi1 is involved in cell proliferation through the negative regulation of *cdkn2a* (*ink4a/arf*), which is one of the major target genes of Bmi1 and cell cycle inhibitory genes [36]. We report that the expression of *ink4a* is inhibited in MEL cells exposed to TAT-Polycomb. We do not consider that the influence of PTD-Polycomb persists for days, and suggest that cell fate was decided within 48 h of exposure to PTD-Polycomb. Despite the opposing functions of Bmi1 and Mel18 in the self-renewal of HSCs, they showed no opposing function in the expression of *ink4a*. Therefore, *ink4a* might not be involved in the regulation of HSC self-renewal by Bmi1/Mel18. However, in the present study, the growth rate differed between BM cells and MEL cells after exposure to TAT-Polycomb. *ink4a* is located downstream of both Bmi1 and Mel18. Because both inhibit cell cycle inhibitors such as *ink4a*, the finding of increase in the number of cells is expected. However, with the BM cell including stem cell, the outcomes differed between Bmi1 and Mel18. Further studies are required to determine the cell type of BM cells that increased after 2-week culture with exposure to TAT-Bmi1 and the behavior of Polycomb proteins in stem cells and during cancer cell growth.

It has been reported that *bmi1* is essential for the maintenance and self-renewal of stem cells, including hematopoietic, neural, and cancer stem cells [16–20]. Although side population cells in the stem cells of hepatocellular carcinoma expressed *bmi1* preferentially, the number of side population cells decreased after *bmi1* knockdown with RNAi. It was thought that *BMI1* contributes to tumorigenesis and prognosis, and the high expression of *BMI1* is associated with a poor prognosis [21–24].

Although we expected that HSCs could proliferate after transduction with TAT-Bmi1, we also found a risk of tumorigenesis. In this study, although transplantation of HSCs exposed to TAT-Bmi1 into irradiated mice resulted in the short-term proliferation of hematopoietic cells derived from transplanted HSCs, 80% of the transplanted mice died after 8 weeks. Although we could not analyze the BM of the dead mice, we speculate that the transplanted HSCs exposed to TAT-Bmi1 had transformed into tumors, especially leukemia. Therefore, the probability of developing

leukemia might increase after exposure to Bmi1. The BM of the surviving mice was confirmed to be normal. Interestingly, Mel18 had the potential to inhibit this tumorigenesis; therefore, Mel18 might inhibit the development of cancers involving Bmi1. It is known that *mel18* inhibits tumorigenesis [37]. The tumorigenicity of NIH3T3 cells treated with sense or antisense *mel18* has been analyzed, and it was found that treatment with antisense *mel18* reduces tumor size, probably because of the contribution of *c-myc*. Tetsu et al. proposed that *mel18* negatively regulates the cell cycle through a *c-myc/cdc25* cascade [38].

Previous studies have reported a relationship between Bmi1 and Mel18. Some studies demonstrated the opposing functions of BMI1 and MEL18 in gastric cancer [29] and medulloblastoma. In a medulloblastoma cell line, BMI1 and MEL18 were present in distinct protein complexes [28]. Moreover, we previously reported that the increased expression of *bmi1* and *mel18* is associated with the self-renewal and differentiation of HSCs [14]. One HSC that was undergoing self-renewal or differentiation expressed *bmi1* alone or *mel18* alone, respectively. These reports indicate that *bmi1* and *mel18* have opposing functions in cell proliferation and differentiation. Elderkin et al. reported that MEL18 and BMI1 proteins are components of similar but mutually exclusive PRC1-like complexes in the human 293T cell line [39]. Our results suggest that Bmi1 and Mel18 induce opposite phenomena, which is consistent with these reports. Because Bmi1 and Mel18 are present in distinct complexes, Mel18 may function as an antagonist by inhibiting Bmi1 competitively. Thus, we have demonstrated that PRC1 complexes, including Bmi1 (referred to as bmiPRC1) and Mel18 (referred to as melPRC1), regulate the self-renewal and differentiation of HSCs, respectively. To investigate the signaling pathway, Guo et al. analyzed the proliferation of human fibroblasts transduced with *Mel18* and *Bmi1* or the knockdown of these proteins with RNAi [26]. They analyzed various molecules involved in the cell cycle and concluded that Mel18 negatively regulates *Bmi1* through *c-myc*. Furthermore, in a study using a breast cancer cell line transduced with *MEL18* and *BMI1* or knockdown of these proteins with RNAi, MEL18 was shown to inhibit Akt, thereby repressing Bmi1 and tumorigenesis [27]. In a recent study, although Akt and Mel18 were in a reverse order, the helix-loop-helix differentiation and DNA binding (Id1) protein enhanced the phosphorylation of Akt and inhibited Mel18, thereby activating *c-myc* and increasing the transcription of *bmi1* [40]. However, because the upstream and downstream signaling pathways remain controversial and the microenvironment affects the regulation of HSC self-renewal, it is commonly recognized that Bmi1 and Mel18 have opposing functions and are present in distinct complexes.

Acknowledgments

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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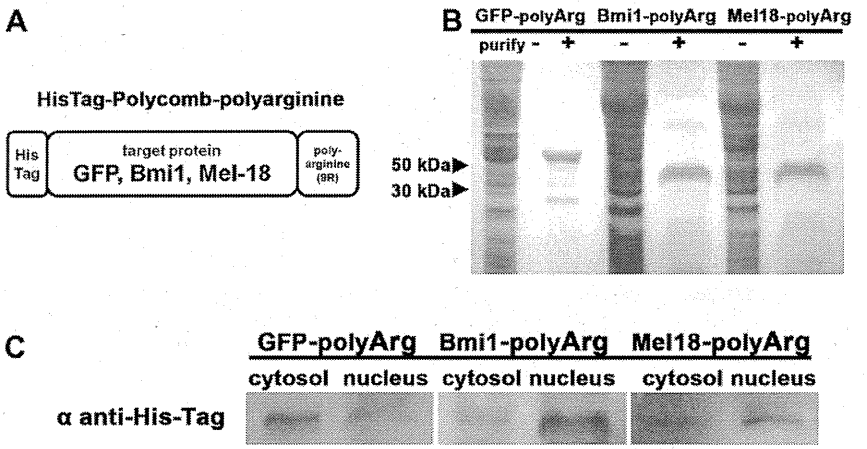
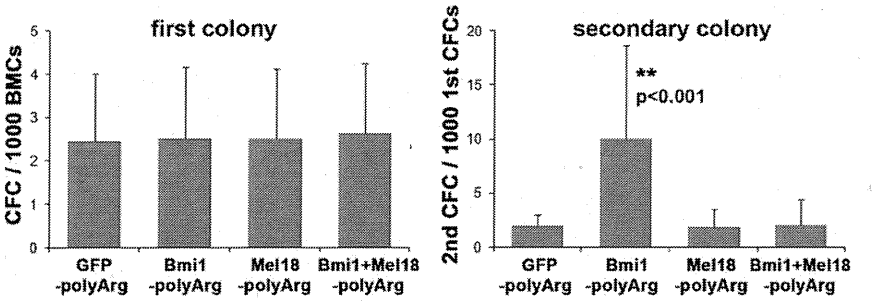


Supplementary Figure E1. Preparation of recombinant polyarginine-polycomb proteins. (**A**) *bmi1*, *mel18*, and *GFP* genes were designed to fuse a poly-arginine to the *C*-terminal of these three proteins. (**B**) The recombinant polyarginine-polycomb proteins were purified. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the polyacrylamide gel was stained with Coomassie brilliant blue. (**C**) Purified polyarginine-polycomb was added to the culture of MEL cells. The MEL cells were separated in nuclear and cytosol. GFP was detected more often in the cytosol than in the nuclear. Bmi1 and Mel18 were detected exclusively in the nuclear.



Supplementary Figure E2. In vitro colony assay of recombinant polyarginine-polycomb protein-transduced murine BM cells. Similar to the recombinant TAT-polycomb proteins, the recombinant proteins did not show any significant differences in the primary colony-forming potential. However, BM cells exposed to recombinant poly-arginine-Bmi1 proteins showed an increased number of secondary colonies. In contrast, BM cells exposed to recombinant poly-arginine-Mel18 proteins or to both proteins showed a decreased number of secondary colonies.

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CASE REPORT

Two cases of partial dominant interferon-γ receptor 1 deficiency that presented with different clinical courses of bacille Calmette–Guérin multiple osteomyelitis

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Abstract We experienced two cases of unrelated Japanese children with bacille Calmette–Guérin (BCG) multiple osteomyelitis with partial interferon (IFN)-γ receptor 1 (IFNGR1) deficiency. Heterozygous small deletions with frame shift (811 del4 and 818 del4) were detected, which were consistent with the diagnosis of partial dominant IFNGR1 deficiency. Case 1: a 2-year-old boy visited us because of limb and neck pain. He had been vaccinated with BCG at 17 months of age. Multiple destructive lesions were observed in the skull, ribs, femur, and vertebral bones. *Mycobacterium bovis* (BCG Tokyo 172 strain by RFLP technique) was detected in the bone specimen. The BCG multiple osteomyelitis was treated successfully without recurrence. Case 2: an 18-month-old girl developed multiple osteomyelitis 9 months after BCG inoculation. Radiologic images showed multiple osteolytic lesions in the skull, ribs, femur, and vertebrae. *M. bovis* (BCG Tokyo 172 strain) was detected in the cultures from a bone biopsy. Her clinical course showed recurrent osteomyelitis and lymphadenitis with no pulmonary involvement. The effects of high-dose antimycobacterial drugs and IFN-γ administration were transient, and complete remission has since been achieved by combination antimycobacterial therapy, including levofloxacin. Partial dominant IFNGR1 deficiency is a rare disorder, but it should be considered when a patient presents with multiple osteomyelitis after BCG vaccination. The cases that are resistant to conventional regimens require additional second-line antituberculous drugs, such as levofloxacin.

Keywords Interferon-γ receptor 1 deficiency · Multiple osteomyelitis · Bacille Calmette–Guérin · Mycobacterial infection · Levofloxacin

Introduction

Interleukin-12 (IL-12)- and IFN-γ (IFNG)-mediated immunity plays an important role in host defense against intracellular pathogens [1]. Mendelian susceptibility to mycobacterial disease (MSMD) is a rare disorder and sometimes lethal disease that occurs in response to poorly virulent mycobacteria, such as bacille Calmette–Guérin (BCG) and environmental nontuberculous mycobacteria (NTM). In patients with MSMD, different types of mutations in six genes—IFNGR1, IFNGR2, IL12RB1, IL12B, STAT-1, and NEMO—have been revealed [2].

Sasaki et al. [3] previously reported a partial IFNGR1 mutation in three Japanese children with BCG osteomyelitis and in the father of one of the patients. We have followed the two unrelated cases over 10 years since their onset in the same department (Koshigaya Municipal Hospital). Based on our longitudinal experience, we intend to provide important clinical information for the diagnosis and treatment of IFN-γR1 deficiency in Japan.

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Case report

Case 1

A Japanese boy became spontaneously positive to a tuberculin purified protein derivative (PPD) skin test at the age of 11 months. There was no family history of tuberculosis. A chest X-ray film showed no abnormal findings. The PPD skin test turned negative after 6 months of prophylactic treatment with isoniazid (INH). He was inoculated with BCG (Tokyo 172 strain) by the multiple puncture technique at the age of 17 months. Nine months later (at 26 months of age), he started to limp and could not move his neck. He visited Koshigaya Municipal Hospital, and multiple osteolytic lesions were observed on his skull, vertebrae (cervical and lumbar), ribs, and femur by X-ray, bone scintigram, and magnetic resonance (MR) imaging. *Mycobacterium* was detected in the bone biopsy. *Mycobacterium bovis* was identified as the BCG Tokyo 172 strain by restriction fragment length polymorphism (RFLP). The BCG osteomyelitis was treated successfully with antimycobacterial therapy with isoniazid (INH), rifampicin (RFP), and streptomycin (SM) for 1.5 years without recurrence. He is now 17 years old and has not had a mycobacterial infection since the treatment.

Case 2 (Fig. 1)

An 18-month-old girl (13 years old at present) developed left axillary lymphadenitis 2 months after BCG inoculation at the age of 8 months. Multiple skin eruptions and abscesses appeared 9 months after the vaccination. At the BCG inoculation site, there were signs of hypertrophic scar and keloid. Granuloma was also observed below the inoculation site. X-ray, skeletal scintigram, and MR imaging showed multiple osteolytic lesions in the skull, ribs, femur, and vertebrae. A bone biopsy specimen of the femur revealed granulomatous inflammation without central necrosis. *M. bovis* (BCG Tokyo 172 strain) was detected in cultures from the bone biopsy by RFLP. She was treated with INH, RFP, and SM, and showed slow improvement. Eighteen months after her initial presentation, she started to develop recurrent osteomyelitis. Additional administration of ethambutol (EB) and IFN-γ was effective but the effect was temporary. She exhibited osteomyelitis soon after discontinuation of EB and RFP. High-dose INH and EB, with the addition of clarithromycin (CAM) and IFN-γ, proved effective. Her osteomyelitis appeared to have subsided. However, later, at the age of 11 years, she experienced a third relapse of the osteomyelitis. Antimycobacterial therapy was started again, but lymphadenitis also developed on her right supraclavicle. The findings from the swollen lymph nodes were nonspecific. Additional administration of high-dose IFN-γ was partially effective against the osteomyelitis and the lymphadenitis. As the cervical lymphadenopathy appeared again, the CAM was changed to levofloxacin (LVFX). A three-drug regimen of INH, RFP, and LVFX for a period of 9 months was successful in achieving remission.

The clinical features of these two unrelated Japanese children with BCG multiple osteomyelitis are summarized in Table 1. Two-color flow cytometric analysis was performed [3] and showed significantly higher levels of IFNGR1 expression on monocytes in both cases. IL-12 and IFN-γ production was normal. Genomic DNA was obtained from peripheral blood mononuclear cells. cDNA sequences were analyzed by polymerase chain reaction. Heterozygous small deletions with frame shift (case 1, 811 del4; case 2,

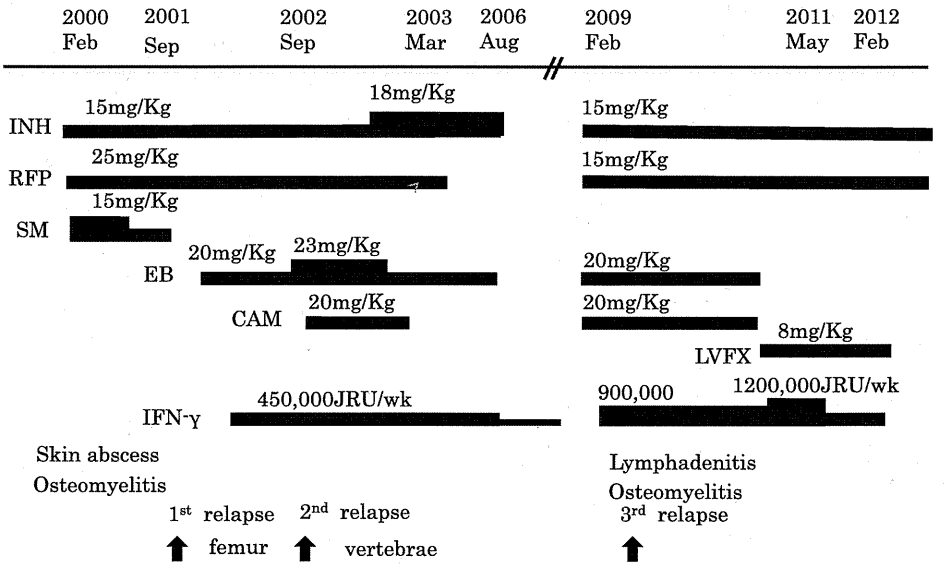

Fig. 1 Recurrent osteomyelitis and lymphadenitis in case 2. *INH* isoniazid, *RFP* rifampicin, *SM* streptomycin, *EB* ethambutol, *CAM* clarithromycin

Table 1 Immunological data at the onset of patients with bacille Calmette–Guérin (BCG) osteomyelitis

Case	1 (17 years/M)	2 (13 years/F)
BCG given at	1 year 5 months	8 months
Age at onset	2 years 2 months	1 year 5 months
Type	Multiple	Multiple, recurrent
Histology	Inflammation	Granuloma
Other organs	None	Skin, lymph node
WBCs/μl	5,300	29,600
Lymphocytes/μl	3,657	7,400
IgG, mg/dl	1,370	1,430
IgA, mg/dl	188	104
IgM, mg/dl	602	181
CD3 cells, %	40.7	56.6
CD4:CD8	3	3
CD19 cells, %	10.4	26.4
PHA response	Normal	Normal
Cytokine production IL-12/INF-γ	Normal	Normal

818 del4) were detected, which were consistent with the diagnosis of partial dominant IFNGR1 deficiency (data not shown). Sequence analysis of six coding regions was performed and showed that none of the family members of the patients had any mutations. Furthermore, neither sets of parents were consanguineous. Thus, de novo mutation had occurred in both cases 1 and 2.

Discussion

Bacille Calmette–Guérin vaccines are safe in immunocompetent hosts, and Japanese BCG substrain Tokyo 172 is the safest BCG in the world [4]. Complications of BCG vaccination can be severe and life threatening in infants with immunodeficiency. Systemic adverse reactions to BCG vaccine, including osteomyelitis and disseminated BCG infection, are rare. Toida and Nakata [5] reviewed severe adverse reactions to BCG from 1951 to 2004 in Japan and identified 39 cases (incidence rate, 0.0182 cases per 100,000 vaccinations). Thirteen cases exhibited primary immunodeficiency; 5 of these exhibited chronic granulomatous diseases, 4 exhibited severe combined immunodeficiency, and 4 exhibited IFNGR1 deficiency. Unidentified defects in cellular immunity were observed in 6 cases. The 6 fatal cases had cellular immunodeficiencies. Bone and joint involvement was observed in 27 cases, 15 cases with multiple lesions and 12 cases with single site lesions.

Hoshina et al. [6] analyzed the clinical characteristics and the genetic background of 46 patients with MSMD in Japan from 1999 to 2009, and found that 6 had mutations in the IFN-γR1 gene. All the cases of IFN-γR1 deficiency exhibited multiple osteomyelitis, and disseminated mycobacterial infection recurred in 5 patients. All the patients exhibited the partial dominant type, and 4 of them had 818 del4. Two of the patients were from the same family, and therefore autosomal dominant inheritance was suspected. The 4 others were considered to have occurred spontaneously. In Taiwan, 3 patients from two unrelated families were identified with a hotspot IFNGR1 deletion mutation (818 del4) and exhibited chronic granulomatous disease-like features, presenting as cutaneous granuloma and multiple osteomyelitis infected with NTM [7]. Fewer patients of Asian origin have been reported with partial dominant IFNGR1 deficiency compared with those of Western countries [8]. The clinical phenotype of partial dominant IFNGR1 deficiency is milder than that of complete deficiency. In this type, BCG and NTM are the major pathogens. Complete IFN-γ receptor deficiency is associated with the early onset of severe disease caused by BCG or NTM, whereas the other genetic forms are associated with a milder course of mycobacterial infection [8].

Patients with partial IFGR1 deficiency usually respond well to antibiotic treatment, and for those who do not respond well, additional IFN-γ therapy has been shown to be effective [9]. There is no single standard regimen for the treatment of children with BCG osteomyelitis. *M. bovis* is resistant to pyrazinamides because of the expression of a pyrazinamidase. Case 1 was successfully treated with a long-term combination therapy of INH, RFP, and SM. However, in case 2, conventional therapy was inadequate to fight the infection. Additional administration of EB and relatively low dose IFN-γ was not able to control the intractable osteomyelitis. As NTM infection was also possible, high-dose EB, INH, and CAM were administered. The regimen was effective but temporary. Combination therapy, including LVFX and high-dose INF-γ, was the most successful strategy. Treatment with second-line antituberculous drugs, such as fluoroquinolone, and two first-line drugs (RFP and EB), may be more effective than RFP and EB alone against multidrug-resistant *M. bovis* [10]. LVFX plays an important role as a substitute agent for those patients who are intolerant of first-line antituberculous agents.

IFN-γ receptor deficiency is a rare disorder that should be considered when patients exhibit BCG lymphadenitis and disseminated osteomyelitis. Multifocal mycobacterial osteomyelitis without other organ involvement is only seen in dominant partial IFNGR1 deficiency [6, 8]. This type of immunodeficiency tends to exhibit recurrent mycobacterial infection and resistance to conventional antimycobacterial therapy. LVFX is likely an effective option for cases with the partial dominant type that are resistant.

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In the United States the regulation of nonstandardized AEs presented some similarities with our approach. AEs were classified into 4 categories according to scientific data supporting their use in diagnosis and treatment, and the extracts were regularly evaluated by the regulatory agencies. The last update was conducted between 2003 and 2011, and the process was recently reviewed by Slater et al.[1] It was shown that for nearly half of nonstandardized AEs there were, in fact, little or no data to support their effectiveness. We had similar results: 66 of 84 AEs were validated for diagnosis, but only for 29 of 66 was there at least 1 published piece of data to support their effectiveness for immunotherapy (Table I). Among those 66 authorized AEs, approximately one third are standardized. There is no consensus about the standardization methods, and the European approaches present some differences compared with the US approach (see Table E1 in this article's Online Repository at www.jacionline.org). Briefly, in-house reference preparation (IHRP) AEs are standardized *in vivo* and *in vitro*. Each manufacturer has its own IHRP, and there is no national standard. Batch-to-batch standardization is performed *in vitro* through a comparison of the AEs with the IHRP.[9]

In the future, the NPP list will be updated every 5 years, and requests for MA will be made and processed for standardized AEs produced industrially and frequently used for immunotherapy.

In conclusion, for the first time in Europe, this work guarantees that available AEs are clinically relevant and safe. Moreover, it guarantees that all AEs comply with recent European guidelines on APs, including rare allergens for which it is not possible to obtain large clinical studies requested for MA. The process involved all the representatives of allergists and manufacturers and is still ongoing.

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Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles

To the Editor:

Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency associated with hypogammaglobulinemia and other various clinical manifestations. CVID was originally reported to be a disease primarily caused by defective B-cell function, with defective terminal B-cell differentiation rendering B cells unable to produce immunoglobulin. However, combined immunodeficiency (CID) involving both defective B and T cells is often misdiagnosed as CVID.[1] Indeed, one study reported that $CD4^+$ T-cell numbers were decreased in 29% of 473 patients with CVID[2]; similarly, another study found that naive T-cell numbers were markedly reduced in 44% (11/25) of patients with CVID.[3] These observations indicated that a subgroup of patients with clinically diagnosed CVID is T-cell deficient. Consistently, some patients with CVID have complications that might be related to T-cell deficiency, including opportunistic infections, autoimmune diseases, and malignancies, which is similar to that observed in patients with CID.[1,4] Therefore identifying novel markers to better classify CVID and distinguish CID from CVID will be required to best manage medical treatment for CVID.

We recently performed real-time PCR-based quantification of T-cell receptor excision circles (TREC) and signal joint immunoglobulin κ-deleting recombination excision circles (KREC) for mass screening of severe combined immunodeficiency (SCID)[5] and B-lymphocyte deficiency[6] in neonates. TREC and KREC are associated with T-cell and B-cell neogenesis, respectively.[7] Here we retrospectively report that TREC and KREC are useful for classifying patients with clinically diagnosed CVID.

Hypogammaglobulinemic patients (n = 113) were referred to our hospital for immunodeficiency from 2005-2011, and the following patients were excluded from the CVID pool by estimating their SCID genes based on clinical manifestations and lymphocyte subset analysis: 18 patients with SCID diagnoses; 14 patients less than 2 years of age (transient infantile hypogammaglobulinemia); 10 patients with IgM levels of greater than 100 mg/dL (hyper-IgM syndrome); 26 patients with diseases other than CVID caused by known gene alterations (10 with X-linked agammaglobulinemia and 11 with hyper-IgM syndrome

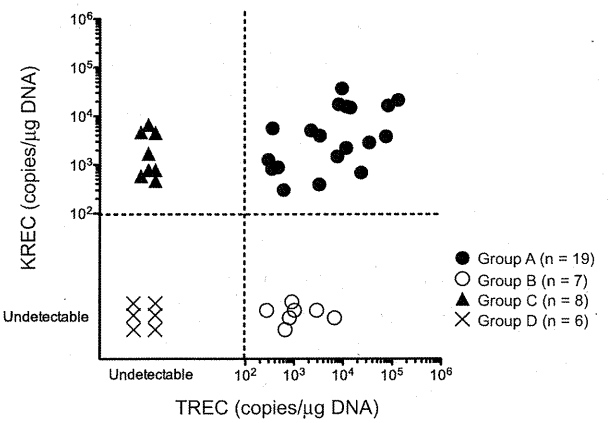

FIG 1. Quantifying TREC and KREC classifies patients with CVID into 4 groups. Patients with CVID were classified as follows: TREC(+)/KREC(+), group A (19 patients); TREC(+)/KREC(−), group B (7 patients); TREC(−)/KREC(+), group C (8 patients); and TREC(−)/KREC(−), group D (6 patients). *Undetectable*, Less than 100 copies/μg DNA.

[*CD40L* or *AICDA* mutated]), (2 with DiGeorge syndrome, and 3 with *FOXP3*, *IKBKG*, or *6p* deletions); and 5 patients with drug-induced hypogammaglobulinemia. The remaining 40 patients with decreased IgG (≥2 SDs below the mean for age), IgM, and/or IgA levels, as well as absent isohemagglutinins, poor response to vaccines, or both were included in this study as patients with CVID and analyzed for TREC/KREC levels, retrospectively.

Ages of patients with CVID ranged from 2 to 52 years (median age, 15.5 years). The sex ratio of the patients was 21 male/19 female patients. Serum IgG, IgA, and IgM levels were 370 ± 33 mg/dL (0-716 mg/dL), 30 ± 7 mg/dL (1-196 mg/dL), and 40 ± 6 mg/dL (2-213 mg/dL), respectively. TREC and KREC quantification was performed by using DNA samples extracted from peripheral blood, as reported previously.[5,6] Clinical symptoms were then assessed retrospectively. The study protocol was approved by the National Defense Medical College Institutional Review Board, and written informed consent was obtained from adult patients or parents of minor patients in accordance with the Declaration of Helsinki.

Based on TREC and KREC copy numbers, the 40 patients with CVID were classified into 4 groups (groups A, B, C, and D; Fig 1). Comparing lymphocyte subsets, $CD3^+$ T-cell numbers were similar among groups A, B, and D but were significantly lower in group C ($P < .05$; group A, 1806 ± 204 cells/μL; group B, 1665 ± 430 cells/μL; group C, 517 ± 124 cells/μL; and group D, 1425 ± 724 cells/μL; $P = .0019$, Tukey multiple comparison test based on 1-way ANOVA). $CD3^+CD4^+CD45RO^+$ memory T-lymphocyte percentages in groups B, C, and D were significantly higher than those in group A ($P < .0001$; group A, 37% ± 16%; group B, 67% ± 13% [$P = .0006$]; group C, 92% ± 8.2% [$P < .0001$]; and group D: 83% ± 14% [$P < .0001$]; see Fig E1 in this article's Online Repository at www.jacionline.org); additionally, the percentages of these cells in groups C and D were higher than in group B ($P = .0115$). These results indicate that group C and D patients have markedly decreased $CD4^+CD45RA^+$ naive T-cell counts than group A patients and that counts in group B are also significantly decreased, although less so than in groups C or D, which is consistent with a report showing lower TREC copy numbers in $CD4^+CD45RO^+$ cells. Some patients in groups B, C, and D exhibited normal $CD4^+CD45RO^+$ percentages, although TREC levels, KREC levels, or both decreased. This discrepancy indicates that TREC/KREC levels could be independent markers to determine the patient's immunologic status in addition to $CD4^+CD45RA^+$; the reasons underlying the discrepancy between $CD4^+CD45RA^+$ and TREC/KREC levels remain unsolved.

$CD19^+$ B-cell numbers in group A were significantly higher ($P < .05$) than those in groups B and D (group A, 269 ± 65 cells/μL; group B, 35 ± 16 cells/μL; group C, 60 ± 11 cells/μL; and group D, 29 ± 16 cells/μL; $P = .0001$). However, B-cell subpopulations, including $CD27^-$, IgD^+CD27^+, and IgD^-CD27^+ cells, were not significantly different among the groups. Standardizing KREC copy numbers for each patient by dividing their $CD19^+$ by their $CD27^+$ percentages revealed the same patient classification as that shown in Fig 1 (data not shown), indicating that the original classification was independent of $CD19^+$ B-cell or $CD27^+$ memory B-cell percentages.

Because TREC and KREC levels decrease with age (see Fig E2 in this article's Online Repository at www.jacionline.org)[5,6] and age distribution was wide in this study, we compared patients' ages among groups at the time of analysis to determine whether classification was associated with age. TREC/KREC-based classification was independent of both age and sex because age distribution was not significantly different among groups ($P > .05$; group A, 12.7 ± 2.3 years [2-30 years]; group B, 23.4 ± 4.2 years [6-39 years]; group C, 21.5 ± 6.1 years [4-52 years]; and group D, 25.5 ± 4.4 years [15-46 years]; data not shown) nor was male/female sex ratio (overall, 21/19; group A, 10/9; group B, 2/5; group C, 5/3; and group D, 4/2; $P = .4916$, χ^2 test; data not shown).

We next evaluated whether any correlation existed between TREC/KREC-based classification and clinical symptoms in each patient group. All patients in the study had been treated with intravenous immunoglobulin (IVIG) substitution at the time of analysis. We found that the cumulative events of complications (opportunistic infections, autoimmune diseases, and malignancies) per 10 patient-years were highest in group D (0.98 events/10 patient-years), followed by group C (0.63 events/10 patient-years), group B (0.30 events/10 patient-years), and group A (0.04 events/10 patient-years), where events in groups D and C were significantly higher than group A (group A vs group D, $P = .0022$; group A vs group C, $P = .0092$; group A vs group B, $P = .0692$; Fig 2). Furthermore, we found similar results when evaluating only patients 19 years old or older for group D (1.01 events/10 patient-years), group C (0.56 events/10 patient-years), group B (0.32 events/10 patient-years), and group A (0.06 events/10 patient-years; group A vs group D, $P = .0074$; group A vs group C, $P = .0407$; group A vs group B, $P = .1492$; data not shown). Categorizing patients by using several different previously reported CVID classifications (focused primarily on separating patients based on levels of circulating B-cell subsets), we found that no classification scheme showed any significant event increases in any particular group (see Fig E3 in this article's Online Repository at www.jacionline.org). Assessing longitudinal cumulative opportunistic infection incidence among the groups, group D and C values were significantly higher than in group A (see Fig E4, A, in this article's Online Repository at www.jacionline.org; $P = .0059$). Autoimmune and malignant diseases ($P = .5168$ and $P = .6900$, respectively) were observed in groups B and D but not in group A (see Fig E4, *B* and *C*). Cumulative events were significantly different between groups ($P = .0313$, log-rank test; group A, 5.3% and 5.3%; group B, 14.3% and

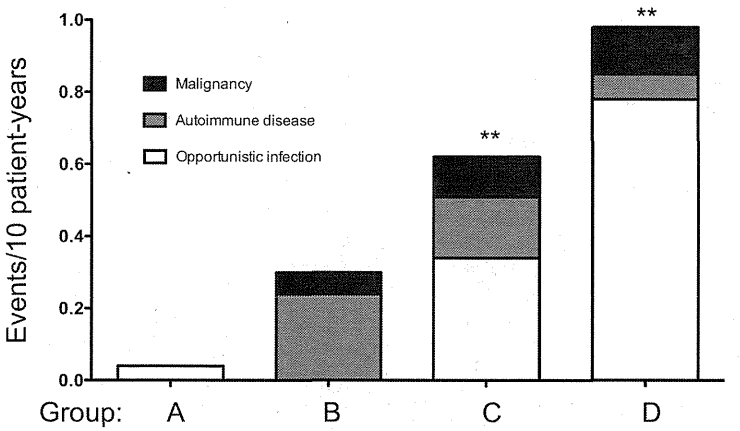

FIG 2. Cumulative incidence of complication events per 10 patient-years differs among groups. Opportunistic infections, autoimmune diseases, and malignancies were evaluated for each patient group. Complication incidences in group D (0.98 events/10 patient-years), group C (0.63 events/10 patient-years), and group B (0.30 events/10 patient-years) were higher than in group A (0.04 events/10 patient-years). Group A versus group D: $^{**}P = .0022$; group A versus C: $^{**}P = .0092$; group A vs group B: $P = .0692$.

57.1%; group C, 27.1% and 63.5%; and group D, 33.3% and 83.3% at 10 and 30 years of age, respectively; see Fig E4, *D*). One patient in group D died of *Pneumocystis jirovecii* pneumonia, and 2 other patients in the same group received hematopoietic stem cell transplantation after complications caused by EBV-related lymphoproliferative disorder.

Assessing these data, TREC/KREC-based classification matches clinical outcomes. Because group D patients exhibited the most frequent complications (opportunistic infections, autoimmune diseases, and malignancies), they could receive a diagnosis of CID based on these symptoms. If they are indeed determined to have CID, then TREC/KREC analysis is helpful to distinguish between CID and CVID. Their TREC(−)/KREC(−) phenotype might relate to defective V(D)J recombination in T- and B-cell development[8] because patients with B-negative SCID (*RAG1*, *RAG2*, *Artemis*, and *LIG4*), as well as patients with ataxia-telangiectasia (AT) and Nijmegen breakage syndrome (NBS; see Fig E5 in this article's Online Repository at www.jacionline.org),[5,6] were also negative for both TREC and KREC; it is intriguing to speculate that an unknown V(D)J recombination gene or genes is responsible. As for treatment, hematopoietic stem cell transplantation should be considered the preferred treatment to "cure" group D patients, as reported in patients with severe CVID/CID, because event-free survival is poor.[9]

In contrast to group D patients, TREC(+)/KREC(+) group A patients treated with IVIG substitution therapy remained healthy. One possible explanation is that these patients harbor defects only in terminal B-cell differentiation, but not in T cells, and represent typical patients with CVID, as originally reported.

Group C patients had a high frequency of both opportunistic infections and malignancies, suggesting that these TREC(−) patients have T-cell defects. Although group C patients had a similar TREC/KREC pattern to patients with SCID with B cells (*IL2RG* and *JAK3*; see Fig E5, *A*), they do not fulfill the European Society for Immunodeficiencies criteria for SCID, and no mutation was identified in the SCID genes estimated from clinical manifestation and lymphocyte subset analysis. However, from our data, they would likely benefit from undergoing similar treatment to patients with SCID or CID to prevent these complications.

Although opportunistic infections were rare in group B patients, autoimmune diseases were often observed. This is consistent with this group being TREC(+)/KREC(−) and the idea that balance between T and B cells is important to prevent autoimmune diseases in patients with CVID.[1] Intriguingly, a group of patients with AT and NBS were also TREC(+)/KREC(−) (see Fig E4, *B*), which is similar to group B patients. Additionally, $CD45RA^{+}CD4^{+}$ naive T-cell numbers were reduced in most group B patients, which is similar to the phenotype exhibited by patients with AT and NBS. This finding raises the possibility that although some group B patients are also T-cell deficient, as well as B-cell deficient, and should be treated similarly to patients with CID, other patients have only B-cell deficiency and are effectively treated with IVIG substitution therapy.

By analyzing a large CVID patient cohort, the overall survival rate of patients with more than 1 complication was worse than that for patients without other complications.[4] Our findings indicate that low TREC levels, KREC levels, or both are useful markers that correlate well with the overall survival rate in patients with CVID. Therefore we conclude that TREC and KREC are useful markers to assess the clinical severity and pathogenesis of each patient with CVID and to distinguish CID from CVID. Thus patient classification based on TREC/KREC levels would provide a helpful tool for deciding on an effective treatment plan for each patient with CVID.

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Homing frequency of human T cells inferred from peripheral blood depletion kinetics after sphingosine-1-phosphate receptor blockade

To the Editor:

Naive and central memory (CM) T cells home through lymph nodes (LNs), whereas T cells with an effector memory (EM) phenotype preferentially screen peripheral tissues in search of cognate antigen.[1] LN entry and egress are distinct and highly regulated processes mediated by an orchestrated interplay of chemokines/chemokine receptors and adhesion molecules.[2] Interaction of peripheral node addressins with L-selectin on T cells allows tethering/rolling along high endothelial venules (HEVs).[2] Interaction of the chemokine receptor CCR7 with its ligands CCL19/CCL21 and CXCR4 with CXCL12 then mediates firm adhesion to HEVs through high-affinity interactions of lymphocyte function–associated antigen 1 and intercellular adhesion molecule 1, permitting transmigration of T cells across the HEV cell layer.[2] Within the LNs, T-cell migration is directed through T-cell zones toward the cortical sinuses.[3] A sphingosine-1-phosphate (S1P) gradient established across the endothelial cells of the cortical sinuses is directing LN egress of T cells through efferent lymph back to the peripheral blood circulation.[4] Acting as a functional antagonist on the S1P receptor, the pharmacologic compound fingolimod, which has shown efficacy in the treatment of multiple sclerosis (MS), blocks this egress.[4,5] As a consequence, in fingolimod-treated subjects naive and CM T cells are trapped in LNs and reduced in the blood circulation.[6]

Here, by studying depletion kinetics of T cells in the blood of *de novo* fingolimod-exposed subjects in combination with *in vitro* migration experiments, homing frequencies and LN access hierarchy between T-cell subsets were derived indirectly. First, we defined the effect of *de novo* fingolimod exposure on the number of circulating $CD4^+$ and $CD8^+$ phenotypic T-cell subsets in patients with MS during a 6-hour observation period (hourly measurements, 1 time before and 6 times after drug exposure) by using flow cytometry (detailed information on patients and methods is provided in the Methods section and Table E1 in this article's Online Repository at www.jacionline.org). In fingolimod-treated subjects, 6 hours after the first drug dose, numbers of $CD4^+$ T-cell subsets with an LN homing phenotype (ie, naive and CM T cells) were significantly reduced (Fig 1, *A* [representative example; absolute cell counts], and Fig 1, *B* [pooled data; proportional change]). Intriguingly, the kinetics of reduction differed between phenotypic naive (CD62 ligand [CD62L]–positive $CD45RA^+$) and CM ($CD62L^+CD45RA^-$) $CD4^+$ T cells. Specifically, compared with baseline measurements, naive $CD4^+$ T-cell counts started to decrease earlier than CM $CD4^+$ T-cell counts (2 vs 5 hours after fingolimod exposure; Fig 1, *B*). In $CD8^+$ T cells, contrasting $CD4^+$ T cells, only naive ($CD62L^+CD45RA^+$) $CD8^+$ T-cell counts decreased significantly (after 3 vs 2 hours in naive $CD4^+$ T cells) after the first dose of fingolimod (Fig 1, *C* [representative example; absolute cell counts], and Fig 1, *D* [pooled data; proportional change]).

On the basis of these *ex vivo* depletion kinetics, *in vitro* chemotaxis experiments were performed, as described in the Methods section in this article's Online Repository. In a transwell system spontaneous migration of bulk $CD4^+$ and $CD8^+$ T cells was comparably low in healthy control subjects and untreated patients with MS (and was further decreased in the presence of fingolimod; see Fig E1 in this article's Online Repository at www.jacionline.org). Gradients of CXCL12, CCL19, and CCL21 mediated a clear increase in migration of bulk $CD4^+$ and $CD8^+$ T cells from healthy control subjects and untreated patients with MS, which was not significantly influenced by fingolimod (see Fig E1). Dot plot distribution (as a percentage) of migrated versus nonmigrated, phenotypic naive, CM, EM, and (for $CD8^+$ T cells) CD45RA re-expressing EM cells (EMRA) was then compared between control cells (spontaneous migration) and cells that migrated toward CXCL12, CCL19, or CCL21. An example of CXCL12-mediated changes in the

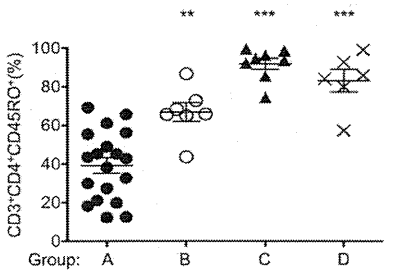
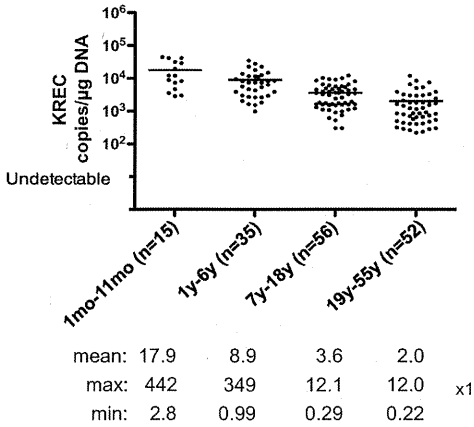


FIG E1. $CD45RO^+CD3^+CD4^+$ T-cell frequency within $CD4^+CD3^+$ lymphocytes was analyzed among groups. $CD45RO^+CD3^+CD4^+$ lymphocyte counts were significantly higher in groups B, C, and D compared with those in group A ($P < .0001$). Group A: 37% ± 16%; group B: 67% ± 13% (**$P < .01$); group C: 92% ± 8.2% (***$P < .001$); and group D: 83% ± 14% (***$P < .001$).



mean:	17.9	8.9	3.6	2.0
max:	442	349	12.1	12.0
min:	2.8	0.99	0.29	0.22

x10^3 copies/µg DNA

FIG E2. KREC levels were analyzed in genomic DNA samples extracted from peripheral blood of control subjects at different age groups (n = 158; age range, 1 month to 55 years). KREC levels were significantly higher in infants (17.9 ± 3.9 × 10^3 copies/µg DNA) compared with other children's age groups (8.9 ± 1.3 × 10^3 copies/µg DNA in the 1- to 6-year-old group and 3.6 ± 3.8 × 10^3 copies/µg DNA in the 7- to 18-year-old group) and adults (2.0 ± 3.3 × 10^3 copies/µg DNA; $P < .0001$).

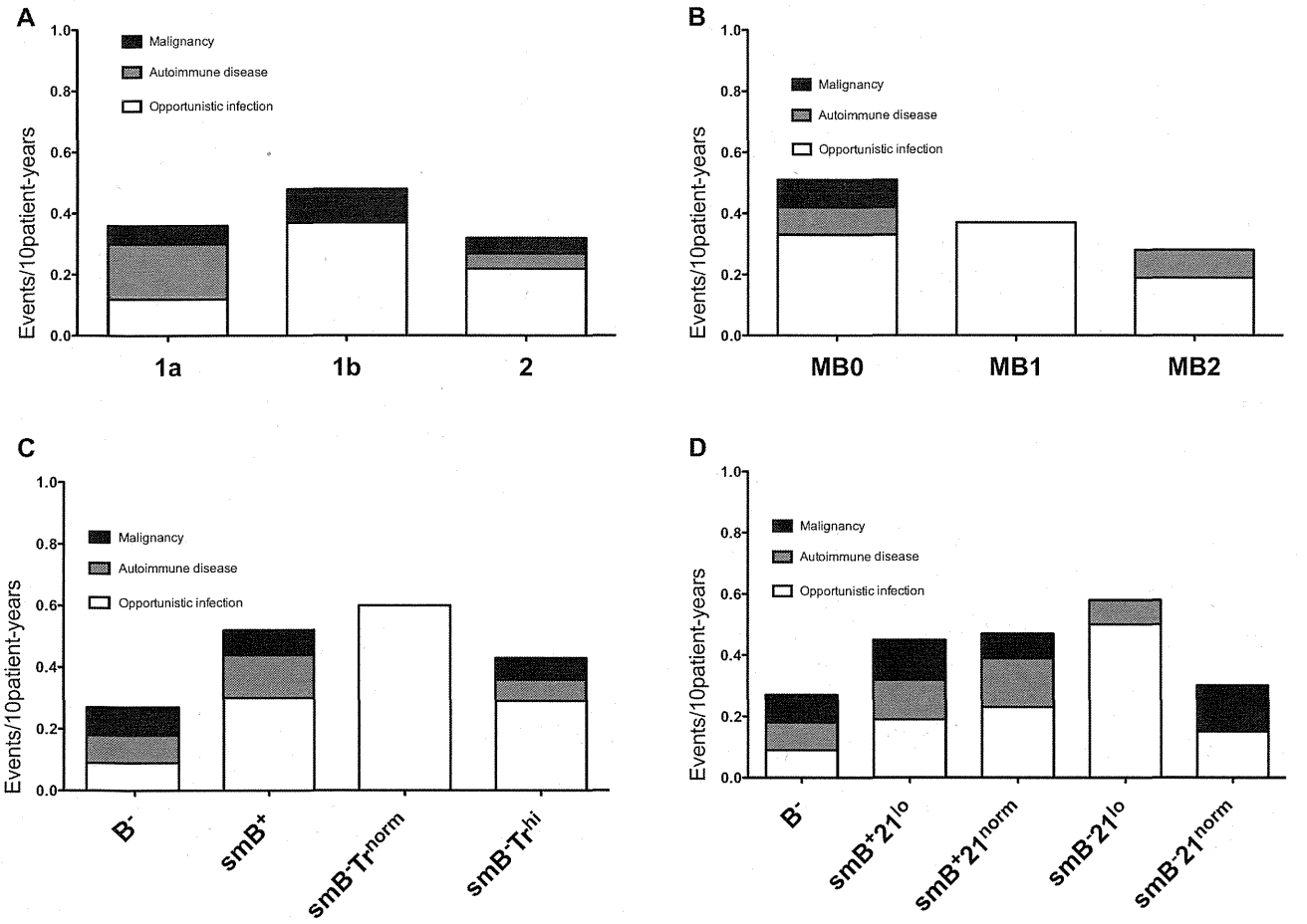


FIG E3. Patients were classified in the following way and analyzed for cumulative incidence of complications: **A,** Freiburg; **B,** Paris; and **C,** EUROclass classifications, according to $CD38^{hi}IgM^{hi}$ transitional B cells (Fig E3, *A-C*) or $CD21^{low}$ B cells **(D)**. Five patients were excluded from the Freiburg and Paris classifications because of decreased B-cell numbers (<1%). Additionally, we excluded 4 patients in the Freiburg classification, 1 patient in the Paris classification, and 4 patients in the EUROclass classification for transitional B cells and 8 in the EUROclass classification for $CD21^{low}$ B cells because of lack of data. The following cumulative events/10 patient-years were found. Freiburg classification: 1a, 0.36; 1b, 0.48; 2, 0.32. Paris classification: MB0, 0.50; MB1, 0.37; MB2, 0.28. EUROclass classification according to transitional B cells: B^-, 0.27; smB^+, 0.52; smB^-Tr^{norm}, 0.60; smB^-Tr^{high}, 0.43. EUROclass classification according to $CD21^{lo}$ B cells: B^-, 0.27; smB^+21^{lo}, 0.45; smB^+21^{norm}, 0.47; smB^-21^{lo}, 0.58; smB^-21^{norm}, 0.30. No classification showed any significantly increased events in any particular group according to calculated *P* values, as follows—Freiburg classification: 1a vs 2 = .898, 1b vs 2 = .479, 1a vs 1b = .838; Paris classification: MB0 vs MB2 = .179, MB1 vs MB2 = .654, MB0 vs MB1 = .764; EUROclass classification according to transitional B cells: B^- vs smB^+ = .298, smB^-Tr^{norm} vs smB^+ = .809, smB^-Tr^{hi} vs smB^+ = .702, smB^-Tr^{hi} vs smB^-Tr^{norm} = .641, smB^-Tr^{norm} vs B^- = .329, smB^-Tr^{hi} vs B^- = .508; EUROclass classification according to $CD21^{lo}$ B cells: B^- vs smB^+21^{norm} = .443, smB^+21^{lo} vs smB^+21^{norm} = .930, smB^-21^{lo} vs smB^+21^{norm} = .695, smB^-21^{norm} vs smB^+21^{norm} = .575, B^- vs smB^-21^{norm} = .926, smB^+21^{lo} vs smB^-21^{norm} = .609, smB^-21^{lo} vs smB^-21^{norm} = .399, B^- vs smB^+21^{lo} = 0.474, B^- vs smB^-21^{lo} = 0.270, smB^+21^{lo} vs smB^-21^{lo} = 0.618.

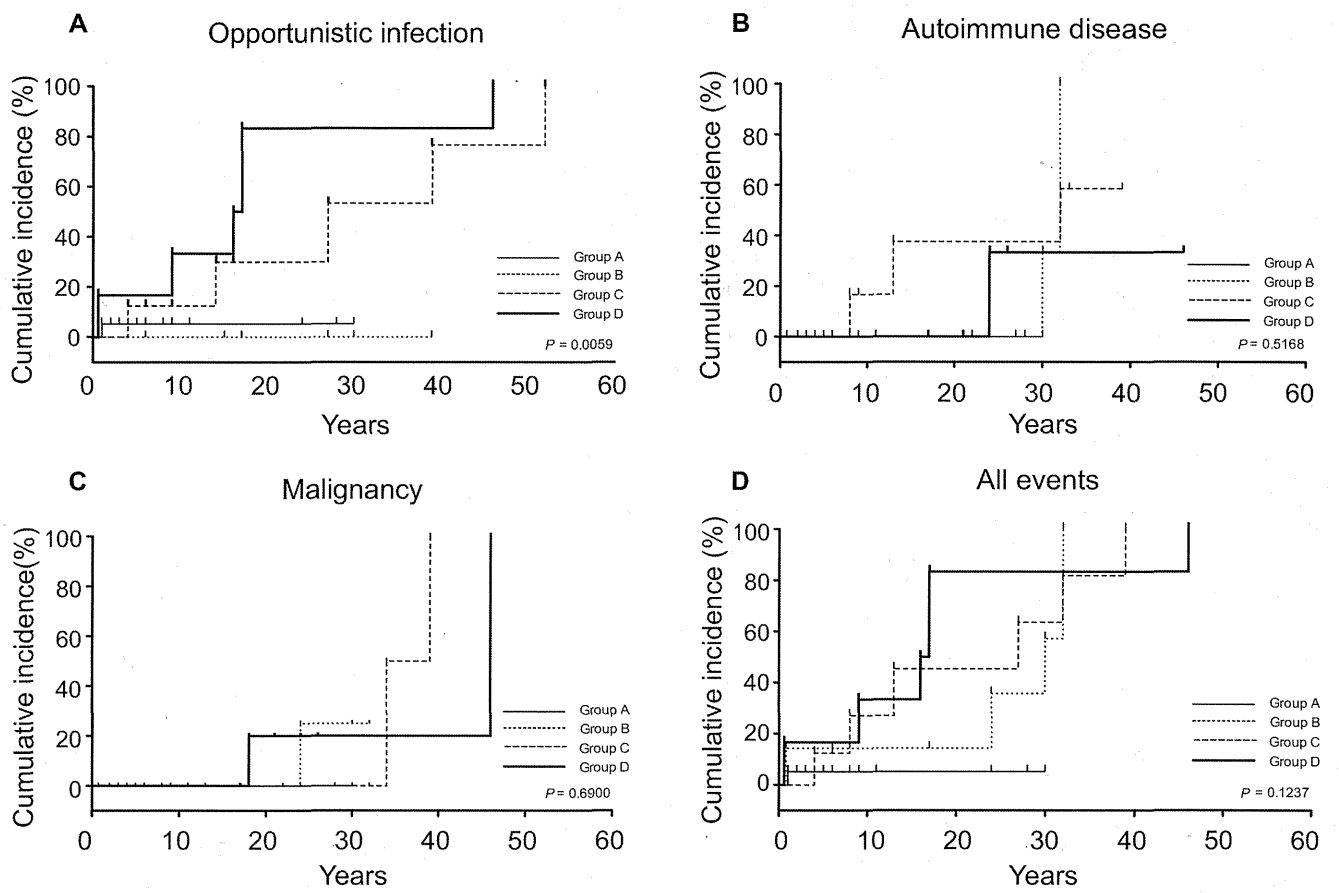


FIG E4. Comparing longitudinal cumulative incidence of complication events among groups. Cumulative incidence was estimated separately and longitudinally by using the Kaplan-Meier method and statistically compared between groups by using the log-rank test. The cumulative incidence of opportunistic infections **(A)**, autoimmune diseases **(B)**, malignancies **(C)**, and all events **(D)** is shown.

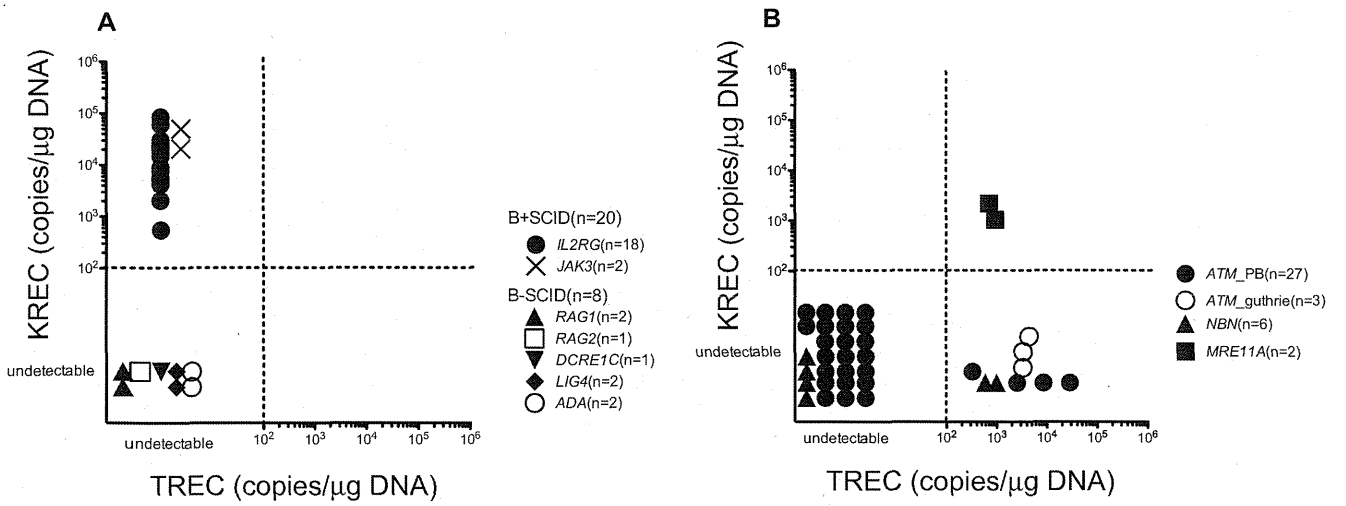


FIG E5. TREC and KREC quantification classifies patients with SCID, AT, NBS, or ataxia-telangiectasia–like disease *(ATLD)* into 4 groups. **A,** Patients with B^+SCID (n = 20) were classified as group C, and patients with B^-SCID (n = 8) were classified as group D; these patients were included in the previous studies.[5,6] **B,** Although most patients with AT (n = 23) and patients with NBS (n = 4) were classified as group D, TRECs were detected in peripheral blood samples (n = 4 in patients with AT and n = 2 in patients with NBS) and neonatal Guthrie cards (n = 3) of some patients with AT, who were classified as group B. Patients with ATLD with *MRE11A* mutations were classified as group A.

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Neonatal Herpes Encephalitis Caused by a Virologically Confirmed Acyclovir-Resistant Herpes Simplex Virus 1 Strain

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CASE REPORT

Neonatal Herpes Encephalitis Caused by a Virologically Confirmed Acyclovir-Resistant Herpes Simplex Virus 1 Strain

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A neonate with herpes simplex virus 1 encephalitis was treated with intravenous acyclovir. During the course of therapy, the infection became intractable to the treatment and a mutation in the viral thymidine kinase gene (nucleotide G375T, amino acid Q125H) developed. This mutation was demonstrated *in vitro* to confer acyclovir resistance.

CASE REPORT

A 13-day-old boy was admitted to National Defense Medical College Hospital due to lethargy and failure to thrive. He was born at 39 weeks 5 days of gestation and 2,558 g birth weight to a healthy 35-year-old mother (gravida 2, para 2). Group B streptococcus (GBS) was detected from the mother's vagina in the third trimester, but the baby's bacterial culture tests performed at birth, including throat, skin, and blood analyses, were negative for GBS. The mother did not have a history of genital herpes. Her herpes simplex virus 1 (HSV-1) and HSV-2 serostatus was not examined, and her history of acyclovir (ACV) use was not clear. Furthermore, the genital swab culture examination for HSV was not performed. On admission, physical examination of the boy revealed skin blisters on the forehead and upper lip. A swab from the blister showed positive and negative reactions for the specific antigens of HSV-1 and HSV-2, respectively, in a direct immunofluorescent antibody assay (Denka Seiken Co. Ltd., Tokyo, Japan) performed by a qualified clinical laboratory (SRL Inc., Tokyo, Japan). A serum sample collected on admission showed positive and negative reactions in the enzyme-linked immunosorbent assay for detection of anti-HSV IgM and IgG antibody (SRL Inc.), respectively. A lumbar puncture revealed pleocytosis (547 cells/μl) and an elevated protein level (168 mg/dl) in the cerebrospinal fluid (CSF). The CSF was also positive for HSV-1 DNA, which was determined by a previously reported method (1) in PCR testing (SRL Inc.). The boy was diagnosed as having neonatal herpes encephalitis (NHE), and intravenous high-dose ACV (60 mg/kg/day) treatment was initiated. His general status improved with resolution of the skin lesions within a few days of the beginning of treatment. However, the viral load in the CSF determined by TaqMan-based quantitative real-time PCR (SRL Inc.), which dropped temporarily, increased again after 4 weeks from the initiation of ACV treatment (Fig. 1A) without obvious deterioration in clinical symptoms. Because the standard dose of ACV was given and drugs which have antagonistic effects for ACV were not used, we assumed that an ACV-resistant HSV-1 strain had developed. The ACV concentration in the CSF was not measured. Foscarnet, an antiviral drug recommended for treatment of ACV-resistant HSV infections (2), was not immediately available. Therefore, vidarabine (15 mg/kg/day) was added to the therapeutic regimen from the fifth week of the treatment course. Subsequently, HSV-DNA in the CSF decreased to a level that was finally undetectable; hence, the antiviral drug treatment was terminated. Because virus isolation from the CSF of the patient was unsuccessful, as is common in herpes encephalitis cases (3), we could not perform a plaque reduction assay to test the *in vitro* inhibition concentration of ACV. Neuroimaging showed residual necrotic changes of the bilateral temporal lobes. Unfortunately, neurodevelopmental sequelae remained in this patient.

To reveal the mechanism of the clinical ACV resistance, sequencing analysis of the viral thymidine kinase (*vTK*) gene was conducted using the CSF samples collected at two different time points. As denoted by the arrows in Fig. 1A, sample 1 and sample 2 were collected before the initiation of and at the 5th week of ACV treatment, respectively. Full-length *vTK* genes were successfully amplified from both samples by a previously reported nested PCR method (4). By direct sequencing, one nucleotide substitution, G375T, leading to a Q125H amino acid substitution was detected. CSF sample 2 contained a mixture of *vTK* genes with and without this mutation (Fig. 1B). To examine whether or not this mutation induced HSV-1 ACV resistance, further analysis was conducted.

The analysis was performed according to a method developed by our group (4). The concept for the novel assay system is to assess the sensitivity of the HSV-1 to ACV and other *vTK*-associated drugs by measuring the replication capacity of the *vTK*-deficient and highly ACV-resistant HSV-1 TAR strain (5) in 293T cells expressed with the recombinant *vTK* protein of the HSV-1 strain of interest. In this study, *vTK* expression plasmid vectors were constructed using pTARGET (Promega, Madison, WI). A *vTK* expression plasmid without the G375T mutation, which was inserted with the *vTK* PCR product from sample 1, was constructed

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