NF-κB activity compared to cells transfected with WT-NEMO, while co-transfection with WT and +64-NEMO (WT/+64) resulted in approximately 50% of WT activity. Considering that +64-NEMO is not expressed at the protein level, the 50% NF-κB activity observed here is likely derived from half the amount of WT-NEMO plasmid. On the other hand, +171-NEMO is thought to have residual activity, even after co-transfection with WT-NEMO. This result suggests that these mutants do not seem to exert a dominant-negative effect against WT-NEMO-mediated NF-κB activation. However, we could not completely rule out the negative effect caused by the other abnormally spliced variants, since we examined only two representative variants.

The Functional Activity via NEMO Is Impaired in the Patient

To analyze the functional impairment caused by the NEMO mutation, we examined the CD23, CD56, and CD95 expression on $CD19^+$ B cells, markers of activated B cells, in response to CD40L stimulation. As shown in Fig. 4a, CD54 and CD95 expressions were reduced compared to healthy controls, and CD23 expression was not detected in the patient's B cells, suggesting that activation of B cells was not completely abrogated in the patient, but instead $CD19^+$ B cells from the patient showed weak levels of activation. Therefore, the patient's cells showed partial, but not complete, impairment following CD40L stimulation. Next, we tested TNF-α production in response to LPS stimulation in peripheral blood $CD14^+$ monocytes. As shown in Fig. 4b, $CD14^+$ monocytes from the patient showed a lower level of TNF-α production compared with those from healthy controls. To further clarify the functional defects, we assessed NF-κB DNA-binding ability in response to IL-1β stimulation using EBV-B cells. As shown in Fig. 4c, NF-κB DNA-binding ability was severely impaired, but not abolished, in the patient. Thus, similar to other patients with XL-ED-ID, the patient's cells also showed impairment in response to various stimuli which induce IKK activation.

Memory B Cells Are Decreased in the Patient

The number of $CD27^+$ memory B cells within the $CD19^+$ B cell population was decreased in the patient (6.0%) in comparison to the number observed in healthy controls (30.4±17.8%, $n=10$). A reduced number of $CD27^+$ memory B cells has also been reported in patients with X-linked anhidrotic ectodermal dysplasia with hyper-IgM syndrome (HED-ID) caused by NEMO impairment [18, 19] as well as in a patient with a 5′ untranslated region (UTR) mutation of *IKBKG*, with high levels of IgA [20]. However, as far as we know, a reduction in the memory B cell compartment has not yet been reported in patients with ED-ID. B cells in patients with defect in NF-κB are unable to undergo somatic hypermutation and class switch recombination, resulting in a loss of memory B cells [19, 21, 22]. Although we need to test other patients with ED-ID to confirm this memory B cell phenotype, the diminished memory B cell population may become a common finding not only in patients with HED-ID, but also in patients with an impairment of NEMO.

The Increase in $CD4^+$ T Cell Proliferation Is Impaired for Measles and Mumps Infections

The patient developed measles in spite of having a history of measles vaccination. Furthermore, although specific antibodies against measles and mumps virus were not detected, specific antibodies against CMV, Epstein–Barr virus, Varicella zoster virus, and rubella virus were normal. To clarify the mechanism underlying the impairment of specific antibody production against measles and mumps viruses, we tested the specific T cell response against these viral infections. We analyzed $CD4^+$ T cells using a CFSE proliferation assay according to the method described in a previous report [23]. $CD4^+$ T cells from the patient were unable to proliferate in response to measles lysate and mumps lysate (Fig. 5a, b). On the other hand, they proliferated well in response to PHA and rubella lysate. CFSE is a commonly used and useful tool for analyzing specific T cell response against *Candida*, CMV, measles viruses, and others, and these results suggest that the specific T cell response against measles and mumps virus is impaired in the patient [14, 15, 23]. These findings were compatible with patient's laboratory findings of the impairment of a specific antibody production against measles virus and mumps virus, in spite of having received these vaccinations and having a prior measles infection.

Discussion

We identified a novel hemizygous splice-site mutation in *IKBKG* in a Japanese boy with XL-ED-ID. Both the WT and various abnormally spliced forms of NEMO mRNA were observed in the patient's cells. There are two possibilities which may account for this finding. One is leakage through the splice-site mutation, the other is mosaicism. Leakage through the splice-site mutation has also been described in many human diseases [24–26], including in a patient with a NEMO abnormality who had a splice-site mutation, 1056−1 G>A [27]. Similar to what was observed in our current study, the ratio of WT to mutant NEMO mRNA observed varied with the timing of blood

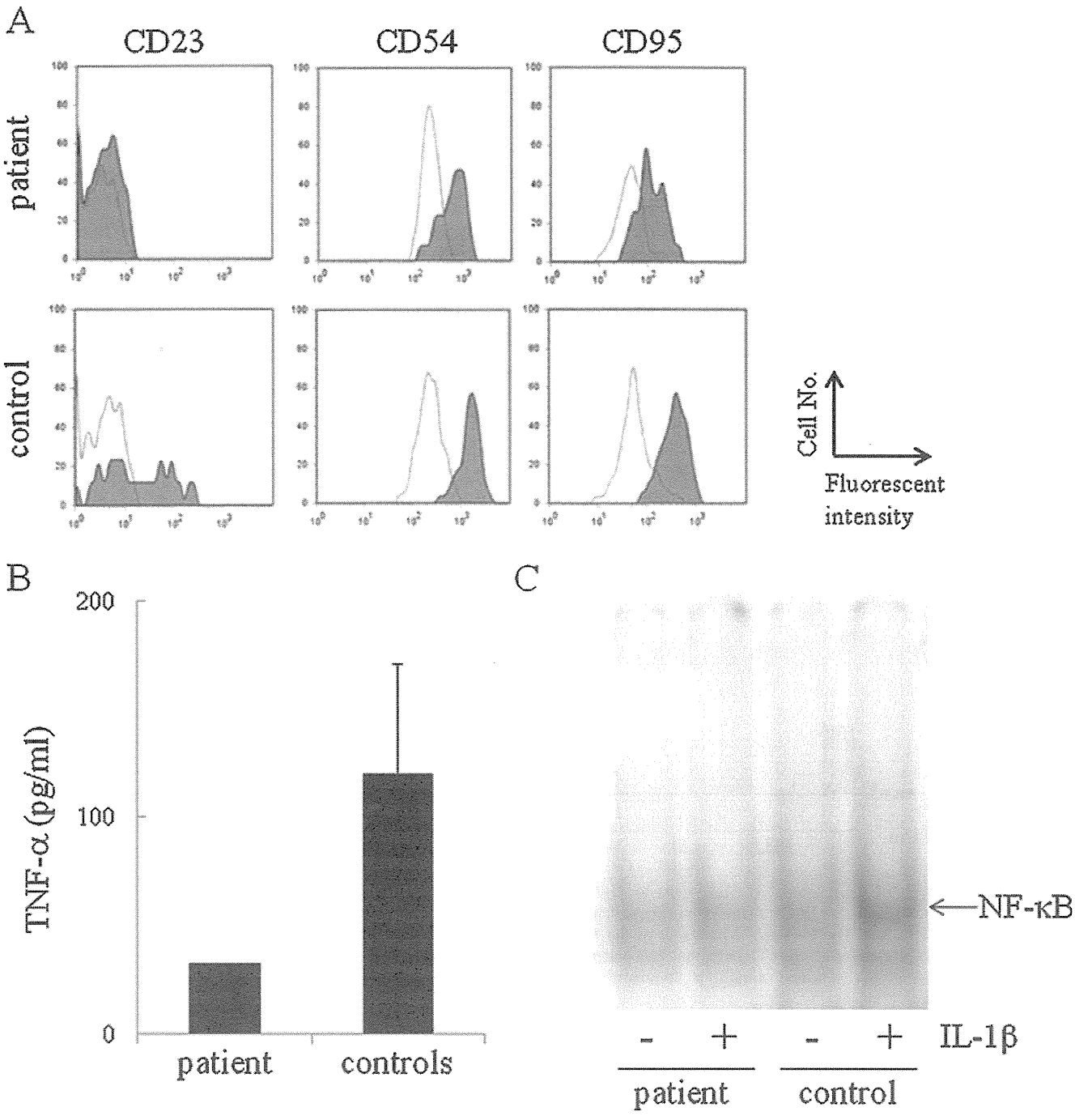

Fig. 4 Analysis of functional activity via NEMO. **a** Expression of CD23, CD54, and CD95, the surface markers of activated B cells, was measured using flow cytometry. PBMCs from the patient and healthy controls were treated with (*shaded histograms*) or without (*open histograms*) CD40L. **b** TNF-α production in response to LPS by $CD14^+$ cells was measured by Luminex. Data from the healthy subjects are represented as mean ± SD (n=4). **c** The NF-κB DNA-binding ability in response to IL-1β was measured by electrophoretic mobility shift assay. EBV-B cells from the patient showed a lower level of DNA-binding ability than healthy controls

collection in the patient with a 1056−1 G>A mutation. Curiously, however, there is a difference between the expression of WT-NEMO protein and the frequency of WT-NEMO mRNA in our patient. Although the frequency of WT-NEMO mRNA observed in the patient in our splicing assay was approximately 30% of all splice transcripts, expression of NEMO protein from the patient was only 12.5% that of WT levels. We suspect that the influence of nonsense-mediated RNA decay can explain this inconsistency between WT-NEMO expression at the mRNA and protein level. Some abnormally spliced forms of NEMO, such as 64-NEMO, result in premature stop codon. These

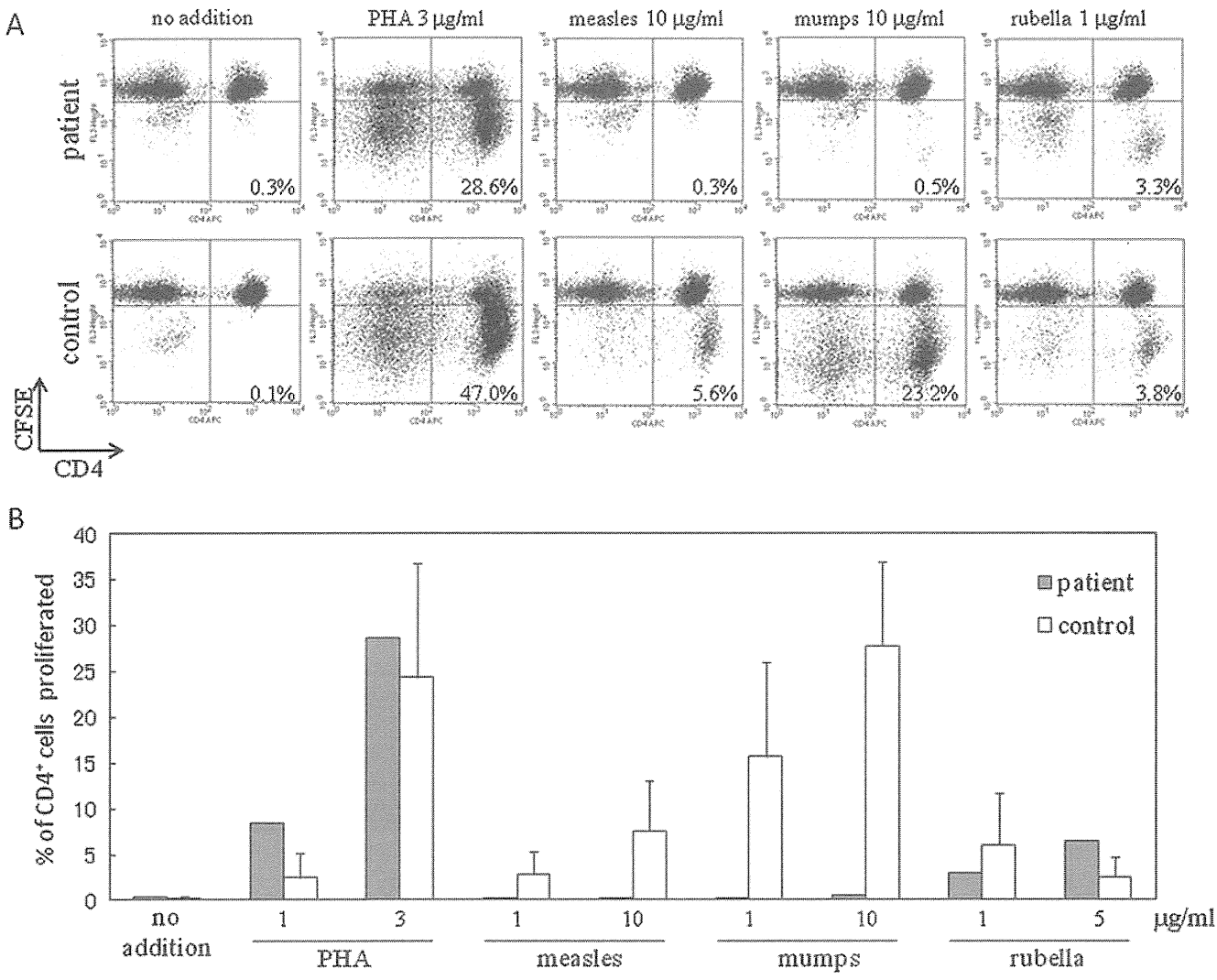

Fig. 5 CFSE analysis of the response of $CD4^+$ cells to PHA and various viruses. **a** Representative FACS figures from the patient and healthy subjects are shown. The *lower-right quadrant* of the FACS profile indicates the proportion of $CD4^+$ T cells that had undergone division in response to the indicated stimuli. **b** Summary of the percentage of proliferating $CD4^+$ T cells is shown. The data in the *white columns* represent the mean ± SD of five healthy subjects. Although $CD4^+$ T cells from the patient proliferated in response to the rubella virus, few divided cells were observed upon stimulation with the measles or mumps viruses

products are predicted to be susceptible to nonsense-mediated RNA decay. Therefore, although the splicing assay in this study is an effective way to detect variously spliced transcripts derived from the 769−1 G>C mutation, it may overestimate the proportion of in-frame transcripts which include WT-NEMO.

The other possibility to explain the existence of both WT and mutant mRNAs is germ-line or reversion mosaicism of WT and mutant NEMO-containing cells, as has previously been reported in patients with immunological disorders [28–31]. Furthermore, a reversion mosaicism has been identified in one patient with XL-ED-ID [12]. This patient exhibited NEMO protein expression that varied among cell lineages. Two types of NEMO-expressing cells, NEMO high and NEMO low, were observed by flow cytometric analysis. However, the pattern of NEMO expression did not differ among the lineages in our current study (Fig. 3a). In addition, we did not identify the WT-NEMO sequence from Sanger sequence using genomic DNA extracted from peripheral blood leukocytes or buccal mucosa from the patient (Fig. 1a). Taken together, although we could not completely exclude the possibility of low frequency mosaicism, we presume that normal NEMO mRNAs are derived from leakage through the splice-site mutation that may give rise to XL-ED-ID.

The levels of NEMO protein expression decreased markedly, and the functional activity via NEMO in response to various stimuli were impaired in our patient. Recently, Mooster et al. reported a patient with immunodeficiency caused by a splice-site mutation in the 5′ UTR of the

IKBKG [20]. This patient also showed decreased expression of the NEMO protein, thus resulting in reduced NF-κB activity. In addition, the authors proposed that inadequate levels of normal NEMO protein played a role in the molecular pathogenesis of this patient. Similarly, decreased expression of NEMO protein was also suspected to have played an important role in the clinical manifestations of our patient. However, in contrast to our patient, neither the patients with 1056−1 G>C nor 5′ UTR mutation that demonstrated a residual expression of WT-NEMO presented with ectodermal dysplasia. Further studies will therefore be required to elucidate the factor that is associated with the development of the ectodermal phenotype.

$CD4^+$ T cells from the patient exhibit impaired proliferation in response to measles and mumps viruses. On the other hand, normal proliferation was observed upon stimulation with the rubella virus. To our knowledge, this is the first study to clarify an impairment of T cell proliferation in response to viral infections by CSFE analysis in a patient with NEMO mutation. These results were completely consistent with the laboratory finding of specific antibody production against rubella, but not measles and mumps viruses. Furthermore, the impairment of antibody production against measles, but not rubella, was also observed in another patient with ED-ID carrying a D311E hypomorphic mutation in *NEMO* (Imai et al., in revision in J Clin Immunol). It is interesting to speculate how the impairment of the NEMO protein disturbs the response against measles. Generally, the first line of host defense against viral infection is the innate immune system [32]. Viral infections induce inflammatory reactions via induction of IFNs and via the activation of NF-κB. The activation of interferon regulatory factor-3 (IRF-3) plays an important role in the induction of IFNs against viral infections. IRF-3 recognizes the measles virus nucleocapsid and triggers the induction of interferon production. However, IRF-3 activation and IRF-3-dependent gene induction are abrogated in NEMO-deficient cells [33]. Indeed, impairment of TLR3-induced NF-κB- and IRF-3-dependent IFN induction has also been documented in a patient with NEMO mutation (Audry et al. J Allergy Clin Immunol. in press, reference number: YMAI 8998). In addition, the activation of NF-κB also plays a pivotal role in the host defense against measles. The measles virus phosphoprotein upregulates the ubiquitin-modifying enzyme A20, a negative feedback regulator of NF-κB, resulting in viral escape from the host immune system [34, 35]. Therefore, the impairments of acquired immunity against viral infections observed in the patient may be derived from an impairment of innate immunity caused by NEMO mutation. Further studies will therefore be required to confirm the clinical and cellular phenotype against viral infections in other patients with NEMO mutation.

Conclusion

The 769−1 G>C mutation was shown to cause a decrease in NF-κB activation through the decreased expression level of NEMO protein, thus resulting in the development of XL-ED-ID.

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In conclusion, the associations among asthma, biofilm-forming bacteria, and revision ESS are strong and robust after adjusting for other factors in patients with CRS from a tertiary medical center. Despite its limitations, this study may improve our understanding of refractory CRS pathogenesis, possibly leading to more effective treatment strategies, such as incorporating the treatments of asthma and biofilm infection into conventional CRS therapies. Prospective cohort studies in diverse populations are needed to assess the causality of these associations.

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Zi Zhang, MD[a]
Darren R. Linkin, MD, MSCE[b]
Brian S. Finkelman, BS[a]
Bert W. O'Malley, Jr, MD[c]
Erica R. Thaler, MD[c]
Laurel Doghramji, RN, BSN[c]
David W. Kennedy, MD[c]
Noam A. Cohen, MD, PhD[c]
James N. Palmer, MD[c]

From [a]the Center for Clinical Epidemiology and Biostatistics, [b]the Department of Medicine, Division of Infectious Diseases, and [c]the Department of Otorhinolaryngology–Head and Neck Surgery, University of Pennsylvania School of Medicine, Philadelphia, Pa. E-mail: james.palmer@uphs.upenn.edu.

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Quantification of κ-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects

To the Editor:

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by severely decreased numbers of mature peripheral B lymphocytes as a result of a mutation in the *BTK* gene. Non-XLA is characterized by hypogammaglobulinemia with decreased B-cell counts (less than 2% of mature B cells) in the absence of the *BTK* gene mutation. Both XLA and non-XLA are caused by an early B-cell maturation defect.[1] In patients with XLA and non-XLA, recurrent infections appear between 3 and 18 months of age, whereas the mean age at diagnosis is 3 years.[2] This delayed diagnosis results in frequent hospitalization because of pneumonia, sepsis, meningitis, and other bacterial infections, which frequently require intravenous administration of antibiotics and can be fatal. Frequent pneumonia results in a high incidence of chronic lung diseases.[3] Thus, early diagnosis and early treatment, including periodical intravenous immunoglobulin replacement therapy, is essential to improve the prognosis and the quality of life of patients with XLA and non-XLA.

In the process of B-cell maturation, immunoglobulin κ-deleting recombination excision circles (KRECs) are produced during κ-deleting recombination allelic exclusion and isotypic exclusion of the λ chain.[4] Coding joint (cj) KRECs reside within the chromosome, whereas signal joint (sj) KRECs are excised from genomic DNA. cjKREC levels remain the same after B-cell division, whereas sjKREC levels decrease, because sjKRECs are not replicated during cell division.[5] Because the B-cell maturation defects in XLA and non-XLA occur before κ-deleting recombination, KRECs are not supposed to be produced. Therefore, measurements of KRECs have the potential to be applied to the identification of these types of B-cell deficiencies in patients, which consist of around 20% of all B-cell defects.[6] In addition, some types of combined immunodeficiencies show an arrest in B-cell maturation and can also be identified by this method. The success of newborn screening for T-cell deficiencies by measuring T-cell–receptor excision circles[7] prompted us to develop a newborn screening method for XLA and non-XLA by measuring KRECs derived from neonatal Guthrie cards.

The study protocol was approved by the National Defense Medical College institutional review board, and written informed consent was obtained from the parents of normal controls, the affected children, and adult patients, in accordance with the Declaration of Helsinki.

First, we determined the sensitivity of detection levels of cjKRECs and sjKRECs in Guthrie cards using real-time quantitative PCR.[5] Normal B cells from a healthy adult were isolated from peripheral blood (PB; mean purity, 88.5%). PB was also obtained from 1 patient with XLA (P20) whose B-cell number was 0.09 in 1 μL whole blood and who was negative for sjKRECs ($<1.0 \times 10^2$ copies/μg DNA). Various numbers of normal B cells were serially added to 1 mL whole PB obtained from this patient with XLA. The B-cell–added XLA whole blood was then applied to filter papers, and 3 punches (3 mm in diameter) of dried blood spots were used for DNA extraction. At least 3 DNA samples containing the same B-cell concentrations (0.09-400 B cells/μL) were used for the real-time quantitative PCR of cjKRECs and sjKRECs. The percentages of the positive samples ($>1.0 \times 10^2$ copies/μg DNA) of cjKRECs and sjKRECs increased constantly

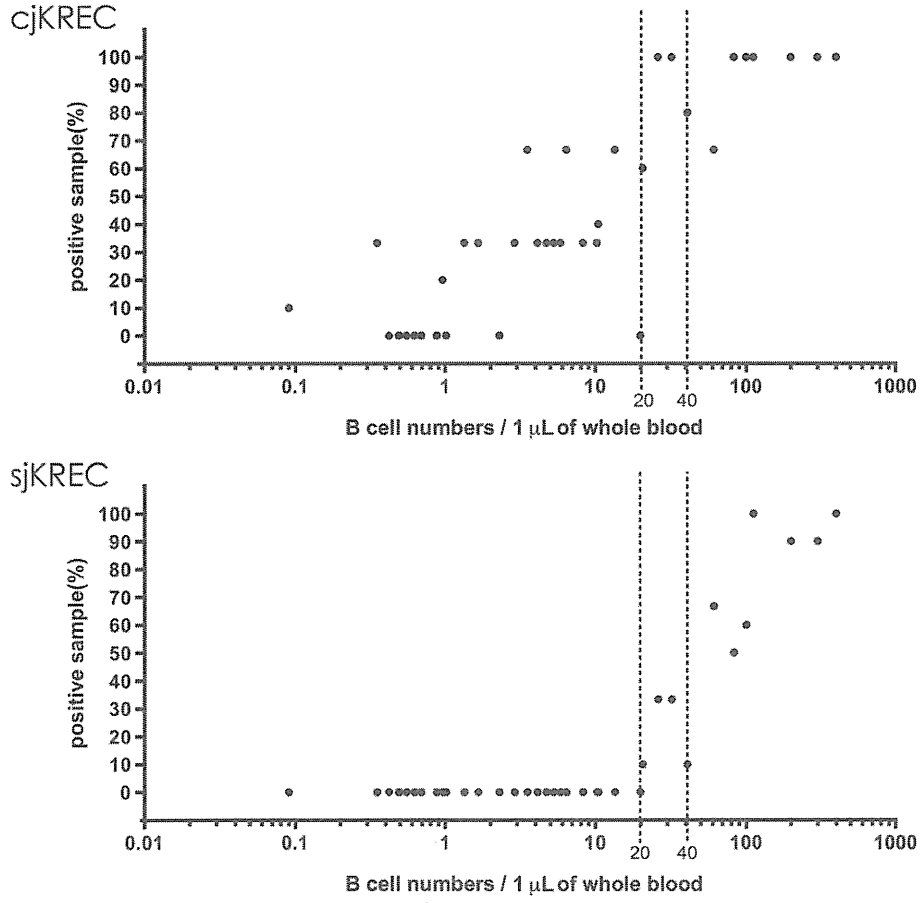

FIG 1. Sensitivity levels of cjKRECs and sjKRECs. Various numbers of purified normal B cells were serially added to whole PB from a patient with XLA (P20) to obtain B-cell–added XLA whole blood. cjKRECs and sjKRECs were measured in 3 to 10 samples of each concentration in triplicate. In all analyses, RNaseP (internal control) was positive ($2.3 \pm 0.2 \times 10^5$ copies/μg DNA). *X-axis,* B-cell numbers in 1 μL whole blood from a patient with XLA. *Y-axis,* Percentages of the KREC-positive results in the tests.

as the B-cell concentrations increased (Fig 1). None of the samples were positive for sjKRECs when the B-cell numbers were less than 20/μL, but cjKRECs were often positive. It has been reported that 90% of patients with XLA have less than 0.2% B cells in the PB at diagnosis.[1] Because peripheral lymphocyte numbers in neonates range from 1200 to 9800/μL,[8] the absolute B-cell numbers of 90% of patients with XLA are estimated to be 2.4 to 19.6/μL at the time of blood collection for Guthrie cards, although exact B-cell numbers of XLA in neonatal periods are not known at this moment. Because neonates are known to have fewer B cells than infants,[9] and we observed that B-cell numbers are constantly low in patients with XLA throughout infancy (Nakagawa, unpublished data, June 2010), which is consistent with the fact that BTK plays an essential role in B-cell maturation. It is likely that neonates with XLA also have severely decreased B cells. On the other hand, all samples obtained from 400 B cells/μL were positive for both cjKRECs and sjKRECs. We also observed that all healthy infants (1-11 months old; n = 15) were sjKREC-positive (Nakagawa, unpublished data, June 2010) and might have at least 600 B cells/μL whole blood.[9] From these data, it is assumed that at least 90% of patients with XLA are sjKREC-negative, and healthy neonates are positive for sjKRECs on neonatal Guthrie cards.

Next, we measured cjKRECs and sjKRECs in dried blood spots in filter papers or Guthrie cards from 30 patients with XLA and 5 patients with non-XLA and from 133 neonates born at the National Defense Medical College Hospital during this study period (August 2008 to October 2009) and 138 healthy subjects of various ages (1 month to 35 years old) to investigate the validity of this method. The levels of B cells of the patients ranged from 0.0% to 1.1% of total lymphocytes and 0.0 to 35.78/μL. IgG levels were 10 to 462 mg/dL (see this article's Tables E1 and E2 in the Online Repository at www.jacionline.org). Patients with leaky phenotypes[1,10] were included; 1 patient (P30) had more than 1% B cells and 34.22/μL total B cells, and 4 patients had more than 300 mg/dL serum IgG (P12, P30, P31, P33). All of the normal neonatal Guthrie cards were positive for both cjKRECs and sjKRECs ($7.2 \pm 0.7 \times 10^3$ and $4.8 \pm 0.6 \times 10^3$ copies/μg DNA, respectively). All healthy subjects of various ages were also positive for both cjKRECs and sjKRECs (Nakagawa, unpublished data, June 2010). In contrast, specimens from all 35 B-cell–deficient patients were sjKREC-negative ($<1.0 \times 10^2$ copies/μg DNA; Fig 2). All 5 patients with leaky phenotypes were also sjKREC-negative, which might be explained by the hypothesis that leaky B cells of patients with XLA are long-lived B cells that divided several times and have fewer sjKRECs than naive B cells.

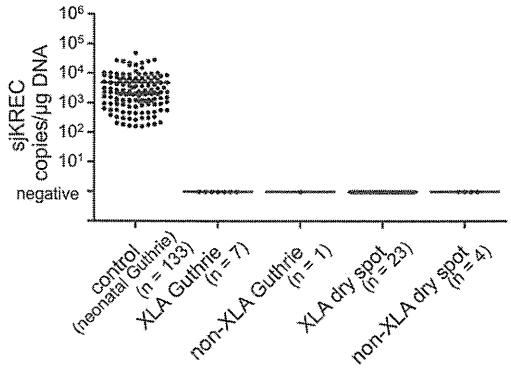

FIG 2. Copy numbers of sjKRECs measured in neonatal Guthrie cards or dried blood spots obtained from B-cell–deficient patients. On all samples from control, neonatal Guthrie cards (n = 133) were sjKREC-positive (4.8 ± 0.6 × 10^3 copies/μg DNA). B-cell–deficient patients were negative for sjKRECs in neonatal Guthrie cards (XLA, n = 7; non-XLA, n = 1) and dried blood spots (XLA, n = 23; non-XLA, n = 4).

One patient (P27) was positive for cjKRECs, but other patients were negative for it. *RPPH1* (internal control) was detectable at the same level as in normal controls in all samples.

These results indicate that sjKRECs are undetectable in XLA and non-XLA and suggest that measurement of sjKRECs in neonatal Guthrie cards has the potential for the use of newborn mass screening to identify neonates with early B-cell maturation defects. Greater numbers of neonatal Guthrie cards should be examined to confirm this potential, and the data obtained from dried blood spots on filter papers must be examined to prove that they truly reflect the data obtained from neonatal Guthrie cards. We should also examine whether screening can reduce the cost of treatment of the bacterial infections and chronic lung diseases in patients with XLA and non-XLA and increase the benefits for these patients. An anticipated pilot study using a large cohort of newborns must address these problems. We also found that T-cell–receptor excision circles and sjKRECs can be measured simultaneously on the same plate. Thus, a pilot study of neonatal screening for both T-cell and B-cell deficiencies could be performed simultaneously.

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Noriko Nakagawa, MD[a]
Kohsuke Imai, MD, PhD[a,b]
Hirokazu Kanegane, MD, PhD[c]
Hiroki Sato, MS[b]
Masafumi Yamada, MD, PhD[d]
Kensuke Kondoh, MD, PhD[e]
Satoshi Okada, MD, PhD[f]
Masao Kobayashi, MD, PhD[f]
Kazunaga Agematsu, MD, PhD[g]
Hidetoshi Takada, MD, PhD[h]
Noriko Mitsuiki, MD[i,j]
Koichi Oshima, MD[j,k]
Osamu Ohara, PhD[j]
Deepti Suri, MD[l]
Amit Rawat, MD[l]
Surjit Singh, MD[l]
Qiang Pan-Hammarström, MD, PhD[m]
Lennart Hammarström, MD, PhD[m]
Janine Reichenbach, MD[n]
Reinhard Seger, MD[n]
Tadashi Ariga, MD, PhD[d]
Toshiro Hara, MD, PhD[h]
Toshio Miyawaki, MD, PhD[c]
Shigeaki Nonoyama, MD, PhD[a]

From [a]the Department of Pediatrics, National Defense Medical College, and [b]the Department of Medical Informatics, National Defense Medical College Hospital, Saitama, Japan; [c]the Department of Pediatrics, University of Toyama, Toyama, Japan; [d]the Department of Pediatrics, Hokkaido University, Hokkaido, Japan; [e]the Department of Pediatrics, St Marianna University School of Medicine, Kanagawa, Japan; [f]the Department of Pediatrics, Hiroshima University, Hiroshima, Japan; [g]the Department of Pediatrics, Shinshu University, Nagano, Japan; [h]the Department of Pediatrics, Kyushu University, Fukuoka, Japan; [i]the Department of Pediatrics, Tokyo Medical and Dental University, Tokyo, Japan; [j]the Department of Human Genome Technology, Kazusa DNA Research Institute, Chiba, Japan; [k]the Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan; [l]the Advanced Pediatric Centre Post Graduate Institute of Medical Education and Research, Chandigarh, India; [m]the Division of Clinical Immunology, Department of Laboratory Medicine, Huddinge Hospital, Karolinska Institute, Stockholm, Sweden; and [n]the Department of Immunology/Hematology/BMT, University Children's Hospital Zurich, Zurich, Switzerland. E-mail: kimai@ndmc.ac.jp.

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TABLE E1. Characteristics of patients with XLA

Patient no.	Unique patient no.	Age (y)	Sex	Serum Ig (mg/dL)			$CD19^+$		*BTK* mutation			Source	
				IgG	IgA	IgM	% Lymph	/μL	Genomic DNA	cDNA	Amino acid	Guthrie	Dry spot
P1	670	0	M	87	<6	10	0.21	12.99	29269G>T	1178-1G>T	Splice acceptor defect	x	
P2	718	0	M	215	<10	<10	0.07	7.04	11593_11594 insA	144_145insA	Arg49 frameshift	x	
P3	722	0	M	80	<1	1	<1.00	NA	25644C>T	763C>T	Arg255X	x	
P4	727	8	M	295	59	57	0.11	3.52	29269G>T	1178-1G>T	Splice acceptor defect		x
P5	732	34	M	1140*	<6	8	0.02	0.24	11631T>A	182T>A	Ile61Asn		x
P6	811	24	M	458*	0	13	0.50	5.32	23570T>G	426T>G	Tyr142X		x
P7	813	18	M	628*	109	6	0.60	6.87	23570T>G	426T>G	Tyr142X		x
P8	814	19	M	260	0	NA	0.20	3.01	16180C>T	344C>T	Ser115Phe		x
P9	815	13	M	600*	<10	<5	0.08	1.72	11590G>T	142-1G>T	Splice acceptor defect		x
P10	816	11	M	12	0	5	0.00	0.00	150kb deletion of *BTK, TIMM8A, TAF7L, DRP2*				x
P11	817	10	M	10	2	24	0.80	35.78	36288C>T	1928C>T	Thr643Ile		x
P12	824	13	M	462	6	27	0.41	14.49	27518C>A	895-11C>A	Splice acceptor defect		x
P13	834	5	M	<237	<37	43	0.00	0.00	25715_26210del	776+57_839+73del	Exon 9 deletion		x
P14	838	21	M	<50	<5	7	0.00	0.00	31596G>C	1631+1G>C	Splice donor defect		x
P15	839	16	M	604*	<1	<2	0.04	0.66	31596G>C	1631+1G>C	Splice donor defect		x
P16	847	11	M	698*	26	11	0.08	1.86	25536delG	655delG	Val219 frameshift		x
P17	877	14	M	20	19	8	0.21	NA	32357T>C	1750+2T>C	Splice donor defect		x
P18	880	5	M	233	39	41	0.06	NA	10941-?_14592+?del	1-?_240+?del	Exon 1-3 deletion		x
P19	888	8	M	<212	<37	150	0.15	6.60	11023G>A	83G>A	Arg28His		x
P20	891	21	M	195	<6	37	0.02	0.09	32243C>G	1638C>G	Cys502Trp		x
P21	958	0	M	<50	<10	9	0.80	27.14	31544_31547 delGTTT	1580_1583del GTTT	Cys527 frameshift		x
P22	701	2	M	115	<2	4	0.09	1.99	16172C>A	336C>A	Tyr112X		x
P23	911	0	M	<10	<6	<4	0.00	0.00	29955A>C	1350-2A>C	Splice acceptor defect	x	
P24	937	0	M	60	<2	58	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P25	938	0	M	<20	<4	<6	0.00	0.00	36269-?_36778+?del	1909-?_2418+?del	Exon 19 deletion	x	
P26	939	0	M	60	<2	22	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P27	890	12	M	<237	<37	<20	0.03	NA	36261G>A	1909-8G>A	Splice acceptor defect		x
P28	944	6	M	12	<1	1	0.02	NA	36281C>T	1921C>T	Arg641Cys		x
P29	948	5	M	<237	<37	<20	0.01	0.70	36261G>A	1909-8G>A	Splice acceptor defect		x
P30	1053	5	M	386	5	113	1.10	34.22	32259A>C	1654A>C	Thr552Pro		x

Age, Age at analysis of KRECs; *$CD19^+$ % Lymph,* CD19-positive cell percentage in lymphocytes; *$CD19^+$ /μL,* CD19-positive cell number in 1 μL whole peripheral blood; *M,* male; *NA,* not available; *Serum Ig,* serum levels of immunoglobulins at diagnosis.

BTK mutation's reference sequences are NCBI NC_000023.9, NM_000061.2, and NP_000052.1.

*Trough level during intravenous immunoglobulin therapy.

TABLE E2. Characteristics of patients with non-XLA

				Serum Ig (mg/dL)			$CD19^+$			Source	
Patient no.	Unique patient no.	Age (y)	Sex	IgG	IgA	IgM	% Lymph	/μL	*BTK* mutation	Guthrie	Dry spot
P31	596	4	F	386	<6	6	0.42	21.27	Normal		x
P32	719	0	F	<50	<5	<5	0.00	0.00	Normal	x	
P33	835	8	M	311	323	20	0.09	1.88	Normal		x
P34	915	0	M	<212	<37	<20	0.00	0.00	Normal		x
P35	947	0	M	<21	<37	<39	0.00	0.00	Normal		x

Age, Age at analysis of KRECs; *$CD19^+$ % Lymph,* CD19-positive cell percentage in lymphocytes; *$CD19^+$ /μL,* CD19-positive cell number in 1 μL whole peripheral blood; *F,* female; *M,* male; *Serum Ig,* serum levels of immunoglobulins at diagnosis.

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Clinical and Host Genetic Characteristics of Mendelian Susceptibility to Mycobacterial Diseases in Japan

Takayuki Hoshina · Hidetoshi Takada · Yuka Sasaki-Mihara · Koichi Kusuhara · Koichi Ohshima · Satoshi Okada · Masao Kobayashi · Osamu Ohara · Toshiro Hara

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Abstract
Purpose The aim of this study is to investigate clinical characteristics and genetic backgrounds of Mendelian susceptibility to mycobacterial diseases (MSMD) in Japan.
Methods Forty-six patients diagnosed as having MSMD were enrolled in this study. All patients were analyzed for the *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* gene mutations known to be associated with MSMD.
Results Six patients and one patient were diagnosed as having partial interferon-γ receptor 1 deficiency and nuclear factor-κB-essential modulator deficiency, respectively. Six of the seven patients had recurrent disseminated mycobacterial infections, while 93% of the patients without these mutations had only one episode of infection.
Conclusions The patients with a genetic mutation were more susceptible to developing recurrent disseminated mycobacterial infections. Recurrent disseminated mycobacterial infections occurred in a small number of patients even without these mutations, suggesting the presence of as yet undetermined genetic factors underlying the development and progression of this disease.

Keywords Disseminated mycobacterial infection · IFN-γR1 deficiency · NEMO deficiency · flow cytometric analysis

T. Hoshina · H. Takada (✉) · Y. Sasaki-Mihara · K. Kusuhara · T. Hara
Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University,
3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan
e-mail: takadah@pediatr.med.kyushu-u.ac.jp

K. Ohshima · O. Ohara
Laboratory for Immunogenomics,
RIKEN Research Center for Allergy and Immunology,
Yokohama, Japan

K. Ohshima · O. Ohara
Department of Human Genome Technology,
Kazusa DNA Research Institute,
Chiba, Japan

K. Kusuhara
Department of Pediatrics,
University of Occupational and Environmental Health,
Fukouka, Japan

S. Okada · M. Kobayashi
Department of Pediatrics, Hiroshima University
Graduate School of Biomedical Sciences,
Hiroshima, Japan

Introduction

Although the outcome of mycobacterial infection is influenced by many factors, including the virulence of the pathogen and the environment of the host, it has been demonstrated that host genetic factors play important roles in the defense against mycobacteria [1]. Mendelian susceptibility to mycobacterial diseases (MSMD, MIM 209950) is a rare primary immunodeficiency syndrome characterized by a predisposition to develop infections caused by weakly virulent mycobacteria, such as *Mycobacterium bovis* bacille Calmette-Guerin (BCG) and environmental non-tuberculous mycobacteria (NTM) [2–4]. These patients are vulnerable to systemic salmonellosis and infections with *Mycobacterium tuberculosis*, the virulent mycobacterial species, to a lesser extent [5, 6]. Diseases caused by other intracellular pathogens, such as *Nocardia*, *Listeria*, *Paracoccidioides*, *Histoplasma*, and *Leishmania*, and some viruses, such as human herpes virus-8, have only rarely been reported, mostly in single patients [7–12].

To date, interferon (IFN)-γ receptor 1 (*IFNGR1*) [13–15], IFN-γ receptor 2 (*IFNGR2*) [16], interleukin (IL)-12 p40 subunit (*IL12B*) [17], IL-12 receptor β subunit (*IL12RB1*) [18–20], signal transducer and activator of transcription-1 (*STAT1*) [21], and nuclear factor-κB-essential modulator (*NEMO*) [22] mutations were identified as the causes of this primary immunodeficiency. On the other hand, no genetic etiology has yet been reported to be identified for about half of all patients with MSMD [3]. In addition, there have been no precise reports on the clinical characteristics and genetic backgrounds of MSMD in Asian countries, including Japan, which has a high prevalence of tuberculosis.

In this study, we analyzed patients who had a recurrent or disseminated infection with intracellular pathogens to clarify the clinical manifestations and host genetic backgrounds of MSMD in Japan.

Materials and Methods

Subjects

We studied 46 patients (30 males and 16 females) diagnosed as having MSMD because of recurrent infections, or blood-borne infections such as osteomyelitis/arthritis, and multiple infections at different anatomic sites by intracellular bacteria including BCG, NTM, *Salmonella* species, *Listeria monocytogenes*, or *M. tuberculosis* in 34 hospitals in Japan from 1999 to 2009. There was no consanguinity in these families. The clinical information on each patient was collected using a standardized case report form. Informed consent was obtained from the parents of the subjects before the study. This study was approved by the Ethics Committee of Kyushu University.

Flow Cytometric Analysis

Two-color flow cytometric analysis was performed to investigate IFN-γ receptor 1 (IFN-γR1) expression levels on the patients' monocytes by using an EPICS XL instrument (Beckman Coulter, Miami, FL, USA). Peripheral blood mononuclear cells (PBMCs) were stained with mouse anti-IFN-γR1 monoclonal antibody (MAb) (Genzyme, Cambridge, MA, USA), followed by rat phycoerythrin anti-mouse immunoglobulin antibody (BD Bioscience Pharmingen, San Diego, CA, USA). Cells were washed twice and stained with a phycoerythrin 5.1 (PC5)-anti-CD14 MAb (Beckman Coulter). IFN-γR1 expression was analyzed on monocytes determined by their side scatter and CD14 positivity.

Genomic DNA and cDNA Sequence Analysis

The *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes were analyzed for coding exons and flanking intronic sequences. These genes were amplified by polymerase chain reaction (PCR) after whole genome amplification with a GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, UK). The PCR products were treated with an Exo-SAP-IT kit (GE Healthcare, Amersham, UK) and then were analyzed by direct sequencing with an ABI 3130 DNA sequencer (Perkin-Elmer, Foster City, CA, USA). Detected mutations were confirmed by sequencing the PCR product using cDNA as a template.

Statistical Analysis

Comparisons of the proportions were analyzed by the χ^2 test. The Mann–Whitney *U* test was used to compare differences between quantitative variables. A *P* value less than 0.05 was considered to be statistically significant.

Results

The median age of the patients was 8 years (range, 6 months–41 years), and the median age at the onset of infection was 1 year and 4 months (range, 4 months–6 years). The male to female ratio was 1.9:1. Only one patient had not received a BCG vaccination. There were 59 episodes of disseminated mycobacterial infections in the 46 patients. Nine (19%) of 46 patients had two or more episodes of these infections. Two of the patients had three episodes, and one had four episodes of these infections. In all episodes, BCG was the most common pathogen (82.6%, Table I). The *Mycobacterium avium* complex (MAC) was isolated during eight episodes of these infections. *M. tuberculosis* was also confirmed in two episodes of infection. No severe *Salmonella* species, *L. monocytogenes*, or viral infections were observed.

The common clinical manifestations were osteomyelitis/arthritis, lymphadenitis, and subcutaneous abscess/dermatitis (Table I and Fig. 1a). Only one patient was diagnosed as having arthritis, and the lesion spread to the adjacent bone. Two patients showed hepatosplenomegaly during the BCG infection, and two patients with the MAC infection developed pulmonary abscess. Among the BCG infections, the median intervals of time between BCG vaccination and the development of primary BCG infection were 3 (1–10 months), 4 (2–36 months), and 11 months (5–46 months) for the subcutaneous abscess/dermatitis, lymphadenitis, and osteomyelitis/arthritis, respectively (Fig. 1b).

We performed the genetic analysis on these patients for the *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes. Six patients (five families) and one patient had mutations in the *IFNGR1* and *NEMO* genes, respectively (Table II). Five of the seven patients who had a mutation in the *IFNGR1* gene were the patients that we

Table I The clinical manifestations of the patients with MSMD

	Patients with genetic mutation, *n* (%)	Patients without a genetic mutation, *n* (%)	Total *n* (%)
Causative pathogen[a]			
BCG	3 (42.9)	35 (89.7)	38 (82.6)
M. avium complex	1 (14.3)	3 (10.2)	4 (8.7)
BCG+*M. avium* complex	2 (28.5)	0 (0)	2 (4.3)
M. avium complex+*M. tuberculosis*	1 (14.3)	1 (2.6)	2 (4.3)
Sites of infection[b]			
Osteomyelitis/arthritis	7 (43.8)	24 (55.8)	31 (52.5)
Lymphadenitis	8 (50.0)	8 (18.6)	16 (27.1)
Dermatitis/subcutaneous	3 (18.8)	11 (25.6)	14 (23.7)
Pulmonary abscess	0 (0)	2 (4.7)	2 (3.4)

The total number exceeds 59 because some patients had multiple lesions at the same time

[a] $n=7$ for patients with a genetic mutation and $n=39$ for patients without a genetic mutation

[b] $n=16$ for patients with a genetic mutation and $n=43$ for patients without a genetic mutation

reported previously [14, 15], and the other two patients were newly identified. All of the IFN-γR1-deficient patients were heterozygotes, and the mutation was in the transmembrane domain in one patient (774del4: patient 5) and in the intracellular domain in five patients (811del4: patient 1, 818del4: patients 2–4, and 832 G>T, E278X: patient 6), which led to the expression of a truncated protein with a dominant negative effect on the IFN-γR1 signaling (Table II and Fig. 2a). The IFN-γR1 expression levels were significantly increased in all six patients with IFN-γR1 deficiency (Fig. 2b). Patient 7 had a missense mutation in *NEMO* (943 G>C, E315Q). The CD14-positive cells from this patient produced a lower level of TNF in response to LPS stimulation (data not shown), which was consistent with the defect in NF-κB signaling.

The proportions of the patients with recurrent mycobacterial infection or multiple osteomyelitis/arthritis were

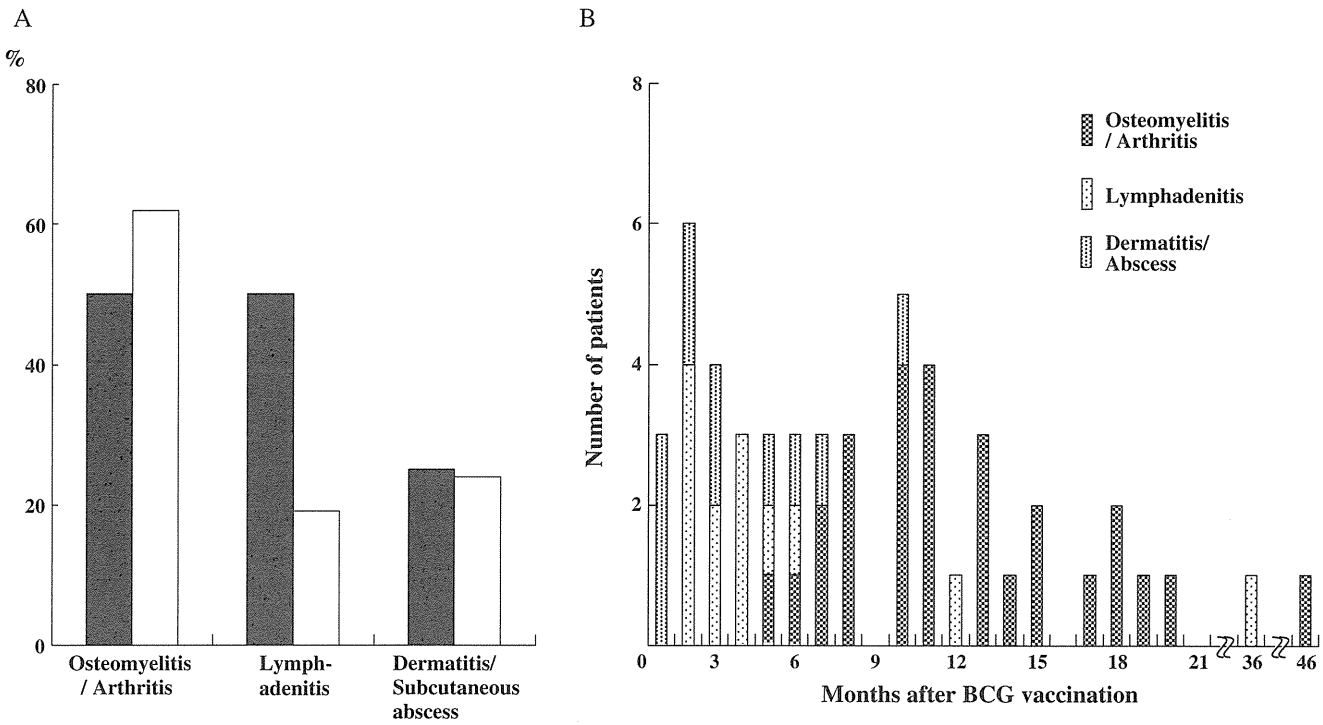

Fig. 1 The clinical features of the patients with BCG infection. The distribution of the sites of infections (**a**) and the intervals between BCG vaccination and the first onset of BCG infection (**b**) are shown. The *black bar* and the *white bar* represent the proportion of the patients with and without genetic mutations, respectively

Table II Characteristics of the patients with a genetic mutation

Patient no.	Sex	Age	Age of onset	Episodes of infections prior to detection of the genetic mutation	Genetic mutation
1[a] [14]	F	1 year 7 months	10 months	BCG lymphadenitis and dermatitis Multiple BCG osteomyelitis	*IFNGR1* 811del4
2[a] [14]	M	1 year 9 months	8 months	BCG lymphadenitis, hepatomegaly Multiple BCG osteomyelitis	*IFNGR1* 818del4
3[a] [14]	M	2 years	2 years	Multiple BCG osteomyelitis	*IFNGR1* 818del4
4[a] [14]	M	41 years	3 years	*M. tuberculosis* lymphadenitis (twice) Multiple MAC octeomyelitis	*IFNGR1* 818del4
5[a] [15]	F	12 years	6 months	BCG lymphadenitis Multiple MAN osteomyelitis	*IFNGR1* 774del4
6	M	19 years	4 months	BCG lymphadenitis and dermatitis Multiple BCG osteomyelitis MAC subcutaneous abscess Multiple MAC osteomyelitis	*IFNGR1* E278X
7	M	10 years	10 months	*M. tuberculosis* lymphadenitis Multiple MAC lymphadenitis Sepsis, bacterial pneumonia (four times)	*NEMO* E315Q

Patient 4 is the father of patient 2

MAC Mycobacterium avium complex

[a] These patients were reported previously

significantly higher in those with the genetic mutations (Table III). There were no significant differences in the age at the onset of mycobacterial infection, or in the interval of time between BCG vaccination and the first onset of BCG infection between the patients with and without genetic mutations. One patient diagnosed with BCG dermatitis died of persistent diarrhea of unknown etiology, while the others are still alive.

Discussion

In the present study, we investigated the clinical characteristics and the genetic backgrounds of the patients diagnosed as having MSMD in Japan. We observed that the patients with the genetic mutation were susceptible to developing recurrent mycobacterial infections and multiple osteomyelitis/arthritis, and IFN-γR1 deficiency was the most

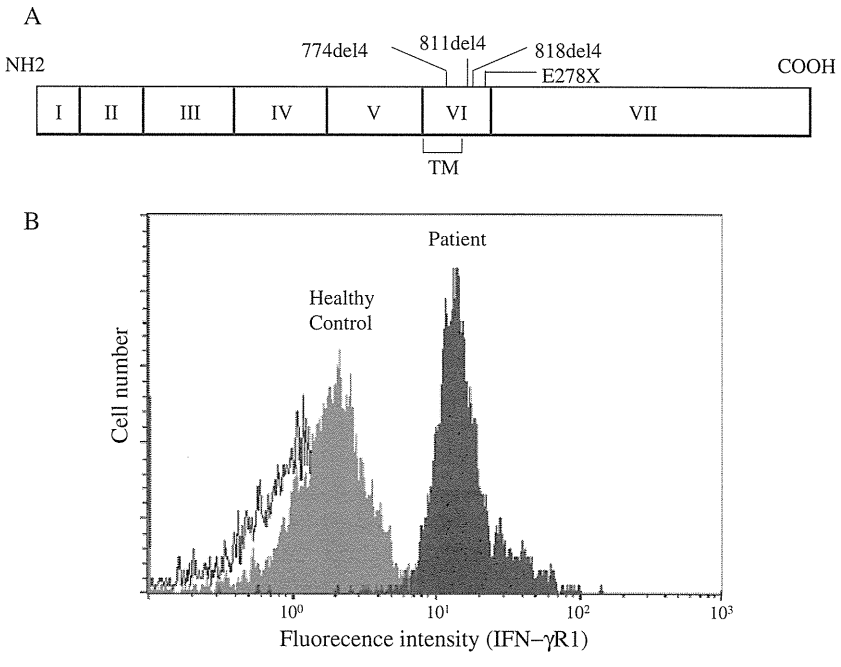

Fig. 2 *IFNGR1* gene mutations and the analysis of IFN-γR1 expression on monocytes. The sites of *IFNGR1* gene mutations in the six IFN-γR1-deficient patients (**a**) and the increased IFN-γR1 expression level on monocytes in patient 2 are shown (**b**)

Table III Comparison of the patients with and without a genetic mutation

	Patients with a genetic mutation (n=7)	Patients without a genetic mutation (n=39)
Age of onset (months)	10 (4–36)	14 (4–75)
Male to female ratio	2.5:1	1.8:1
Familial history (n)	2	0
Median interval between BCG vaccination and the first onset of BCG infection (months)	9.5 (7–15, n=4)	10 (1–46, n=35)
Recurrent cases (%)	85.7*	7.7
Patients with multiple osteomyelitis/arthritis (%)	100* (n=6)	4.2 (n=24)

*p<0.0001

frequent genetic defect identified in these patients. The prevalence of MSMD is estimated to be at least 0.59 cases per million births, and the disease does not seem to be confined to any ethnic group or geographic region, according to a national retrospective study of idiopathic disseminated BCG infection in France [23, 24]. This is the first epidemiological study associated with MSMD in Japan which showed the difference in the clinical manifestation and the genetic background between Japan and Western countries.

The *IFNGR1* mutations identified in this study were in exon IV, within the transmembrane domain, or the intracellular domain of the *IFNGR1* gene (Fig. 2a), which led to a truncated protein lacking signaling motifs [25]. The truncated protein also lacks the recycling motif, which leads to the overexpression of the mutant protein (Fig. 2b) [25]. These mutations are located in important hot spots in the patients diagnosed with dominant partial IFN-γR1 deficiency [13], and the flow cytometric analysis of IFN-γR1 expression levels may be a useful method for the screening for this disease [15]. The *NEMO* mutation found in patient 7 was in exon VIII within the leucine zipper domain of the *NEMO* gene. A previous study reported that a mutation in this region disrupted a common salt bridge in the leucine zipper domain and impaired T-cell-dependent IL-12 production [22].

The patients with the genetic mutations were susceptible to recurrent mycobacterial infections and multiple osteomyelitis/arthritis as described previously [3], but no fatal mycobacterial infection was observed in this study. Unlike complete IFN-γR1 and IFN-γR2 deficiencies, which often cause fatal mycobacterial infections [13, 16], the patients with dominant partial IFN-γR1 and NEMO deficiencies have been reported to have a relatively mild disease and a better prognosis [13, 22]. These factors might have contributed to the good outcome of the patients in this study. In addition, the low virulence of BCG might contribute to the characteristics of BCG infection in Japan, because the BCG Tokyo 172 strain that is used in Japan for vaccination is the least virulent BCG substrain.

The *IL12RB1* mutation has been reported to be the most common cause of MSMD [4]. However, none of the patients in this study was diagnosed as having an IL-12 receptor β1 deficiency. In Japan, this disease was reported in only one patient with disseminated lymphadenitis caused by *M. avium* complex [18]. It has been suggested that most complete IL-12 receptor β1-deficient individuals may be asymptomatic, and only those that also have a second mutation in another gene may be more prone to infections [26, 27]. These symptomatic IL-12 receptor β1-deficient patients are mainly found in families with consanguineous parents [19, 27]. Consanguineous marriages are uncommon in Japan, and there were no consanguineous families in this study. This might be the reason why no IL-12 receptor β1-deficient patients were observed. Alternatively, it is possible that the causative gene mutations associated with MSMD are different among races, because the number of patients with IL-12 receptor β1 deficiency was also lower than those with IFN-γR1 deficiency in Taiwan [28].

Although another patient had multiple osteomyelitis, and three patients had recurrent disseminated mycobacterial infections in these studies, they did not have mutations in any of the six genes. It was previously reported that no genetic etiology has yet been identified in about half of patients with disseminated and recurrent mycobacterial infections [3, 4]. This suggests the presence of as yet undetermined genetic factors in the development of this disease.

In the present study, the number of patients with genetic mutations might be too small to conclusively indicate the differences in the clinical manifestations and the host genetic backgrounds of MSMD between Japan and Western countries. However, in terms of the genetic etiology and the prognosis, it remains possible that the features of the patients diagnosed as having MSMD in the present study are different from those in previous reports [3]. Further investigations of a large number of patients are therefore warranted to more precisely evaluate the clinical manifestations and the host genetic background of MSMD in Japan.

Conclusions

We found that the patients diagnosed as having MSMD in Japan seem to have different genetic features, as well as

different clinical manifestations, compared with those in Western countries. A few patients with recurrent mycobacterial infections without mutations in the six known genes might suggest a contribution of other genetic, as well as environmental, factors in the susceptibility to recurrent infections.

Acknowledgments We thank the physicians for providing detailed information and allowing us to analyze blood samples of the MSMD patients, and we appreciate the assistance of Dr. Brain Quinn for editing the English usage. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan.

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Hoxb4 transduction down-regulates Geminin protein, providing hematopoietic stem and progenitor cells with proliferation potential

Yoshinori Ohno[a,1], Shin'ichiro Yasunaga[a,1], Motoaki Ohtsubo[a], Sayaka Mori[a], Miyuki Tsumura[a,b], Satoshi Okada[a,b], Tomohiko Ohta[c], Kiyoshi Ohtani[d], Masao Kobayashi[b], and Yoshihiro Takihara[a,2]

[a]Department of Stem Cell Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8553, Japan; [b]Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8551, Japan; [c]Division of Breast and Endocrine Surgery, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan; and [d]Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, Sanda 669-1337, Japan

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Retrovirus-mediated transduction of Hoxb4 enhances hematopoietic stem cell (HSC) activity and enforced expression of Hoxb4 induces in vitro development of HSCs from differentiating mouse embryonic stem cells, but the underlying molecular mechanism remains unclear. We previously showed that the HSC activity was abrogated by accumulated Geminin, an inhibitor for the DNA replication licensing factor Cdt1 in mice deficient in *Rae28* (also known as *Phc1*), which encodes a member of Polycomb-group complex 1. In this study we found that Hoxb4 transduction reduced accumulated Geminin in *Rae28*-deficient mice, despite increasing the mRNA, and restored the impaired HSC activity. Supertransduction of Geminin suppressed the HSC activity induced by Hoxb4 transduction, whereas knockdown of Geminin promoted the clonogenic and replating activities, indicating the importance of Geminin regulation in the molecular mechanism underlying Hoxb4 transduction-mediated enhancement of the HSC activity. This facilitated our investigation of how transduced Hoxb4 reduced Geminin. We showed in vitro and in vivo that Hoxb4 and the Roc1 (also known as Rbx1)-Ddb1-Cul4a ubiquitin ligase core component formed a complex designated as RDCOXB4, which acted as an E3 ubiquitin ligase for Geminin and down-regulated Geminin through the ubiquitin-proteasome system. Down-regulated Geminin and the resultant E2F activation may provide cells with proliferation potential by increasing a DNA prereplicative complex loaded onto chromatin. Here we suggest that transduced Hoxb4 down-regulates Geminin protein probably by constituting the E3 ubiquitin ligase for Geminin to provide hematopoietic stem and progenitor cells with proliferation potential.

Retrovirus-mediated transduction of Hoxb4 has been shown to enhance activities of hematopoietic stem cells (HSCs), including self-renewal capacity in vivo and ex vivo in mice and humans (1–3). Moreover, enforced expression of Hoxb4 induces in vitro development of HSCs from differentiating mouse embryonic stem cells on OP9 stroma, suggesting that Hoxb4 also promotes developmental maturation of HSCs (4). It has therefore been anticipated that Hoxb4 can aid the development of a technological procedure for preparing a sufficient number of HSCs ex vivo (5, 6) as well as elucidate the molecular mechanism supporting HSC activity. Hox genes are widely conserved and share a homeobox encoding the homeodomain. Because the homeobox was ascertained to provide a sequence-specific DNA-binding activity, Hox genes have long been believed to specify antero-posterior positional identity through their transcriptional regulatory activity (7). Hoxb4 with an N-to-A substitution at amino acid 212 within helix 3 of the homeodomain (Hoxb4N > A) lacks DNA-binding capacity and it cannot enhance HSC activity (8). This has supported the hypothesis that Hoxb4 enhances HSC activity through its transcriptional regulatory activity. It has been further reported that Hoxb4 transcriptionally activates *c-Myc* (also known as *Myc*) (9) and down-regulates genes involved in TNF-α and FGF signaling in bone marrow cells (BMCs) (10). The molecular mechanism underlying Hoxb4-mediated activation of HSCs, however, currently remains insufficiently understood.

Rae28 and Bmi1, members of Polycomb-group (PcG) complex 1, have been shown to be essential for sustaining HSC activity (11, 12). PcG complex 1 maintains the transcriptionally repressed state of Hox genes through ubiquitination of histone H2A at lysine 119 (13), and Hoxb4 is one of the downstream targets for PcG complex 1 during early development (14). It is, however, presumed that Hoxb4 does not act as a downstream mediator for PcG complex 1 in sustaining HSC activity because Hoxb4 expression was not affected in hematopoietic cells deficient in *Rae28* and *Bmi1* (11, 12). *Bmi1* was shown to maintain HSC activity through direct repression of the INK4a locus encoding the p16 cyclin-dependent kinase inhibitor and p19ARF (12, 15) as well as through direct interaction with E4F1 (16). p19ARF and E4F1 are known to regulate p53 through ubiquitination (17, 18). On the other hand, we recently demonstrated that PcG complex 1, consisting of Ring1B, Bmi1, Rae28, and Scmh1, functions as an E3 ubiquitin ligase for Geminin, an inhibitor of DNA replication licensing factor Cdt1 (19), and that abnormal accumulation of Geminin impairs HSC activity in *Rae28*-deficient ($Rae^{-/-}$) mice (20). In this study, we find that the impaired HSC activity in $Rae^{-/-}$ fetal liver cells (FLCs) was genetically complemented by Hoxb4 transduction and provide evidence suggesting that Hoxb4 acts as an E3 ubiquitin ligase for Geminin through the direct interaction with the Roc1-Ddb1-Cul4a ubiquitin ligase core component to regulate the protein's stability. Subsequently, down-regulated Geminin, in conjunction with its E2F activation, may facilitate DNA replication licensing to provide cells with proliferation potential (19). Geminin is further known to regulate chromatin remodeling (21) and transcription (22, 23). Here we indicate that Geminin regulation is crucial for Hoxb4 transduction-mediated enhancement of the hematopoietic stem and progenitor cell activity.

Results

Expression of Roc1, Ddb1, and Cul4a and Their Complex Formation with Hoxb4. Murine BMCs were sorted for purification of a $CD34^-$ $c\text{-}kit^+$ $Sca1^+$ lineage marker-negative (lin^-) subpopulation ($CD34^-KSL$)

Author contributions: Y.T. designed research; Y.O., S.Y., M.O., S.M., M.T., and S.O. performed research; T.O. and K.O. contributed new reagents/analytic tools; M.K. and Y.T. analyzed data; and Y.T. wrote the paper.

The authors declare no conflict of interest.

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[1]Y.O. and S.Y. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: takihara@hiroshima-u.ac.jp.

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[long-term repopulating (LTR)-HSCs], CD34$^+$KSL (multipotential progenitor cells), c-kit$^+$ Sca1$^-$ lin$^-$ cells (progenitors), and their progeny subpopulations. Expression of *Roc1*, *Ddb1*, and *Cul4a* was detected in each of the hematopoietic subpopulations by RT-PCR analysis (Fig. 1*A*). Although *Cul4a* expression was predominant in lymphoid cells, that in HSC and progenitor subpopulations is presumed to be functionally significant because the HSC activity was reportedly defective in the heterozygous *Cul4a*-deficient mice (24). Because the yeast two-hybrid analysis with Hoxb4 as bait and Cul4a as prey clearly suggested that Hoxb4 directly interacts with Cul4a (Fig. S1*A*) similarly to Hoxa9 (25), we examined whether Hoxb4 forms a complex (designated as RDCOXB4) with Roc1-Ddb1-Cul4a in a cell line derived from the human kidney cells, HEK-293 cells (HEK-293), transfected with Flag-tagged Hoxb4. Roc1, Cul4a, and Ddb1 were detected in the immunoprecipitates prepared with an anti-Flag antibody (Fig. 1*B*), indicating that exogeneous Hoxb4 formed the RDCOXB4 complex in HEK-293. The similar complex formation was observed in a Hoxb4-transduced myeloid cell line, 32D cells (32D) (Fig. S1*B*). The RDCOXB4 complex may directly interact with Geminin because the yeast two-hybrid and immunoprecipitation analyses showed that Hoxb4 interacted with Geminin through the homeodomain (Fig. S1 *G* and *H*) as the other Hox proteins did (22).

Hoxb4 Restores Impaired HSC Activity and Geminin Protein Level in Rae$^{-/-}$FLC. We then determined whether Hoxb4 compensated for impaired HSC activity in Rae$^{-/-}$ mice, which resulted from accumulated Geminin (20). Hoxb4 was transduced into wild-type FLC (Rae$^{+/+}$FLC) and Rae$^{-/-}$FLC by using a murine stem cell virus vector with the enhanced yellow fluorescence protein (EYFP) gene (MEP). Hoxb4 transduction increased cell population in the S phase, stopped apoptosis, and recovered the impaired clonogenic, long-term culture-initiating cell (LTC-IC) and LTR activities in Rae$^{-/-}$FLC, whereas Hoxb4N>A exerted little effect (Fig. S2 *A–D* and Fig. 2*A*). Because we previously showed that accumulated Geminin gave rise to HSC deficiency in Rae$^{-/-}$FLC, we next examined the effect of Hoxb4 transduction on Geminin. Although Geminin mRNA was increased by Hoxb4 transduction as similar to mRNAs for *Cdt1* and *Cyclin A2*, target genes for E2F (Fig. S2*E*), cell sorting analysis showed that Geminin protein was significantly reduced by Hoxb4 transduction in each phase of the cell cycle (Fig. 2*B*). Down-regulation of Geminin protein was also detected in Lin$^+$, KSL, and CD34$^-$KSL subpopulations of Hoxb4-transduced BMCs (Fig. S3). Down-regulation of Geminin protein was further confirmed by immunoblot analysis in Hoxb4-transduced BMCs and 32D where the mRNA and S-phase cells were increased (Fig. 2*C* and Fig. S1 *C–F*).

Effect of Geminin on Hoxb4-Mediated Hematopoietic Induction. To examine whether down-regulation of Geminin protein is involved in the molecular mechanism underlying the Hoxb4-mediated hematopoietic induction, we examined the effect of Geminin on Hoxb4-transduced BMCs. BMCs were first transduced with Hoxb4 by using the murine stem-cell virus vector with the resistance gene for puromycin (MPI) and then were supertransduced by using the MEP vector with either Geminin or destruction box-deleted Geminin (Geminin-DBD), which is resistant to ubiquitination by the anaphase-promoting complex/cyclosome (APC/C) (26). Geminin protein was reduced by Hoxb4 transduction throughout the cell cycle, and Geminin supertransduction reverted the reduced Geminin protein level to that in control cells (Fig. 3*A*). Transduction of Geminin-DBD further up-regulated Geminin

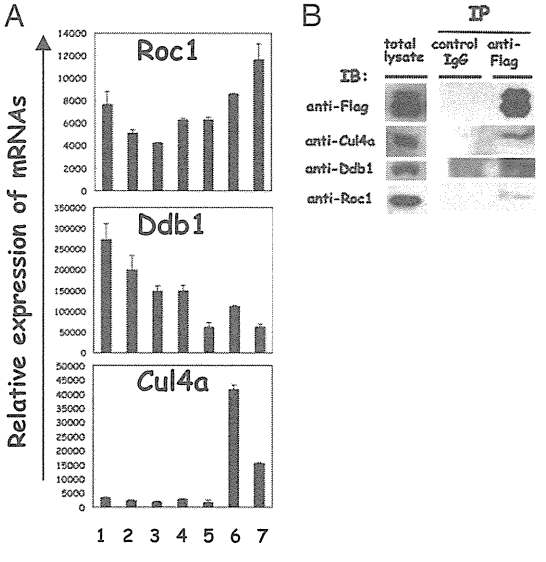

Fig. 1. Expression of Roc1, Ddb1, and Cul4a and the complex formation with Hoxb4. (*A*) mRNA expression examined by quantitative RT-PCR. The mRNA expression levels are shown as ratios to the level in GAPDH. 1, CD34$^-$KSL; 2, CD34$^+$KSL; 3, progenitors; 4, Ter119$^+$ cells (erythroid cells); 5, Gr1$^+$ cells (granulocytes); 6, CD3$^+$ cells (T cells); 7, B220$^+$ cells (B cells). (*B*) Immunoprecipitation analysis of the RDCOXB4 complex in HEK-293 transfected with Flag-Hoxb4. An anti-HA polyclonal antibody was used as a control antibody in the immunoprecipitation. IP, immunoprecipitation; IB, immunoblotting.

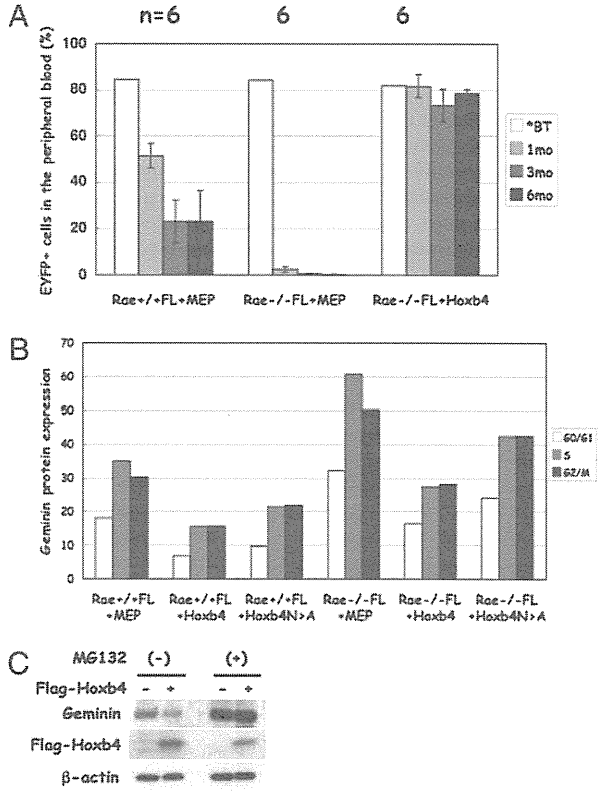

Fig. 2. Effect of Hoxb4 transduction on FLCs and BMCs. (*A*) LTR activity. Percentages of EYFP$^+$ cells in the peripheral blood cells of recipient mice were examined 1, 3, and 6 mo after transplantation. BT: before transplantation. Number of recipient mice is shown above the graph. (*B*) Geminin protein in FLCs, which were examined in each phase of the cell cycle by flow cytometry. (*C*) Geminin protein in BMCs, which were examined by immunoblot analysis. Hoxb4 transduction-mediated down-regulation of Geminin was suppressed by MG132 treatment.

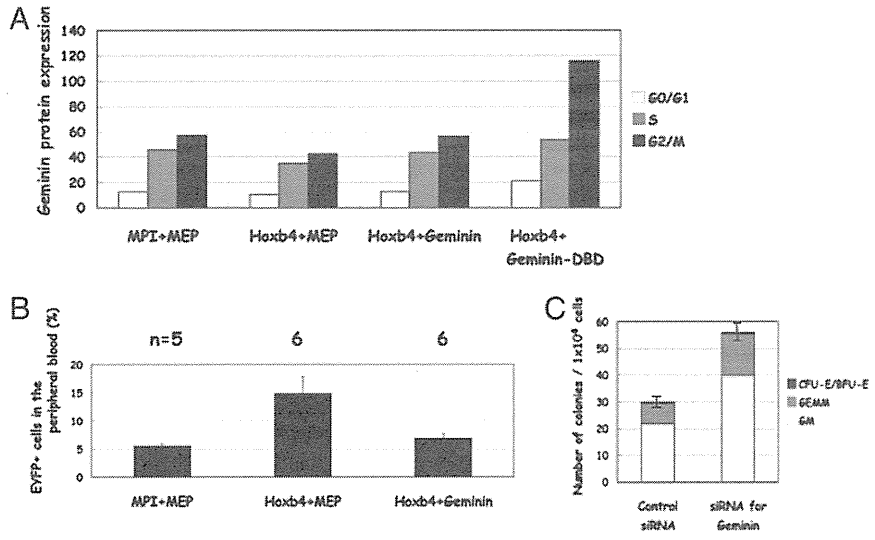

Fig. 3. Effect of Geminin transduction or knockdown in BMCs. (*A*) Effect of Geminin transduction on Geminin protein. BMCs were transduced with Hoxb4 and either Geminin or Geminin-DBD, and Geminin protein in each phase of the cell cycle was determined by flow cytometry. (*B*) Effect of Geminin transduction on LTR activity. (*C*) Effect of siRNA-induced Geminin knockdown on clonogenic activity.

protein (Fig. 3*A*). Geminin and Geminin-DBD transduction efficiently abrogated the clonogenic activity enhanced by Hoxb4 transduction (Fig. S4*A*). Geminin transduction also remarkably affected the replating, LTC-IC, and LTR activities enhanced by Hoxb4 transduction (Fig. 3*B* and Fig. S4 *B* and *C*). On the other hand, siRNA-mediated Geminin knockdown did not affect cell cycling (Fig. S5 *A* and *B*) but clearly promoted clonogenic and replating activities (Fig. 3*C* and Fig. S5 *C* and *D*). We further observed that the enhanced clonogenic activity was suppressed by restoration of Geminin (Fig. S5 *E* and *F*), confirming that the effect of the siRNA was mediated by specific down-regulation of Geminin. These findings indicated that Geminin down-regulation is crucial for Hoxb4-mediated induction of the HSC activity.

Effect of Hoxb4 on Geminin in HEK-293. We next examined the molecular mechanism of how Hoxb4 transduction down-regulated Geminin protein. Transient transfection of Hoxb4 reduced endogeneous Geminin protein in HEK-293 (Fig. 4*A*) despite increasing the mRNA (Fig. S6*A*), whereas the reduction was completely suppressed by treatment of MG132, an inhibitor of proteasome (Fig. 4*A*). Geminin down-regulation in Hoxb4-transduced BMCs and 32D was also suppressed by MG132 treatment (Fig. 2*C* and Fig. S1*F*). Pulse-chase–labeled Geminin with [^{35}S]methionine was shown to be destabilized in Hoxb4-transduced HEK-293 (Fig. S6*B*). Cul4a overexpression induced down-regulation of Geminin protein synergistically with Hoxb4 (Fig. S6*C*). siRNA-mediated knockdown of Cul4a eliminated the downregulating effect of Hoxb4 on Geminin protein (Fig. 4*B*), which facilitated our examination of the involvement of Cul4a in Hoxb4-mediated Geminin regulation. Mobility-shifted Geminin bands were detected in extracts from HEK-293 cotransfected with Geminin, hemagglutinin (HA)-tagged ubiquitin (HA-Ub), and Hoxb4 or Cul4a in the presence of MG132 (Fig. S7*A*). Mobility-shifted Geminin bands were confirmed to be ubiquitin-conjugated Geminin by means of immunoprecipitation analysis (Fig. S7*B*). Ubiquitination of Geminin-DBD through Hoxb4 was similar to that of Geminin (Fig. S7*A*), suggesting that the Hoxb4-mediated ubiquitination was independent of APC/C. The above-mentioned findings support a hypothesis that transduced Hoxb4 down-regulates Geminin protein through the ubiquitin-proteasome system (UPS) with the RDCOXB4 complex as the E3 ubiquitin ligase.

Reconstitution of E3 Ubiquitin Ligase Activity of RDCOXB4 for Geminin. To determine the E3 ubiquitin ligase activity of the RDCOXB4 complex for Geminin, we reconstituted the recombinant protein complex in *Spodoptera frugiperda* insect cells, named Sf9. Sf9 were coinfected with baculoviruses including His6-Roc1, Ddb1, Cul4a (27), and Flag-Hoxb4. Cell extracts were then prepared from Sf9-expressing (His6-Roc1)-Ddb1-Cul4a-(Flag-Hoxb4)[RDCOXB4], which was purified with metal affinity column chromatography. Gel filtration fractionation analysis showed that one of the peak fractions of Flag-Hoxb4 corresponded with the complex with a molecular weight similar to that of the recombinant complex consisting of stoichiometrically determined amounts of the components (260 kDa) (Fig. S8*A*). We also prepared and purified (GST-Roc1)-Ddb1-Cul4a-(Flag-Hoxb4) [RDCOXB4] with glutathione affinity column chromatography (Fig. 5*A*). The affinity-purified recombinant

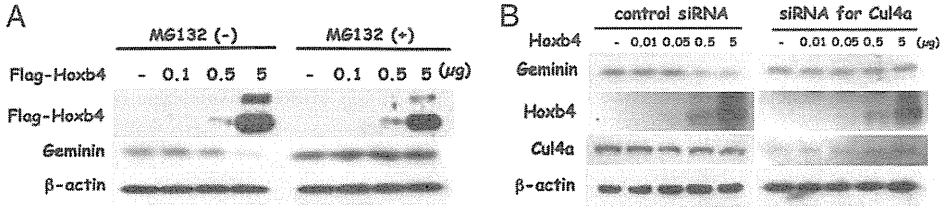

Fig. 4. Effect of Hoxb4 or Cul4a on Geminin protein in HEK-293. (*A*) Effect of Hoxb4 transfection on Geminin protein, which was examined by immunoblot analysis. The effect was suppressed by MG132 treatment. (*B*) Effect of Cul4a knockdown on Hoxb4-mediated down-regulation of Geminin protein.

RDCOXB4 was then subjected to an in vitro ubiquitination assay with purified bacterially produced recombinant His6- and myc-tagged Geminin (myc-Geminin). Mobility-shifted Geminin bands were detected in the reaction products (Fig. S8*B*). Intensity of the bands increased and mobility also shifted according to dosage of the RDCOXB4 complex and the reaction time. Next, an in vitro ubiquitination assay with biotin-tagged ubiquitin (biotin-ubiquitin) was performed to determine whether the shifted bands corresponded with ubiquitinated Geminin (Fig. 5*B*). myc-Geminin was then immunoprecipitated with an anti-myc polyclonal antibody after the reaction, and similar mobility-shifted bands were detected in the immunoprecipitate through biotin–avidin interaction, confirming that the mobility-shifted bands represented ubiquitinated Geminin. The lower two mobility-shifted bands (Fig. S8*C*) were detectable in the reaction products obtained with methyl-ubiquitin, whereas more mobility-shifted bands were not, indicating that the former corresponded to mono-ubiquitinated Geminin and the latter to Geminin with more elongated ubiquitin chains.

Hoxb4N>A tended to form the RDCOXB4 complex more efficiently and/or stably than did wild-type Hoxb4 (Fig. 5*A*). The poly-ubiquitination activity was, however, abrogated by a single amino acid substitution (Fig. 5*C*), suggesting that the E3 ubiquitin ligase activity for Geminin of RDCOXB4 was mediated by a homeodomain in Hoxb4, which provides an interaction domain with Geminin. These findings clearly showed in vitro that Hoxb4 formed the RDCOXB4 complex and acted as the E3 ubiquitin ligase for Geminin. We also compared E3 ubiquitin ligase activities in GST-Roc1, RDOXB4(-Cul4a), RDC(-Hoxb4), RCOXB4 (-Ddb1), and RDCOXB4 (Fig. 5 *A* and *C*). The mobility-shifted bands for poly-ubiquitinated Geminin were undetectable in GST-Roc1, RDOXB4(-Cul4a), RDC(-Hoxb4), and RCOXB4(-Ddb1) although those for mono-ubiquitinated Geminin were detectable (Fig. 5*C*). Even in the absence of either Cul4a or Ddb1, GST-Roc1 interacted with Hoxb4 but displayed mono-ubiquitination activity only for Geminin (Fig. 5*C*). Each of the RDCOXB4 members may thus be required for an effective induction of poly-ubiquitination. To eliminate the possibility that Geminin was ubiquitinated by contaminated APC/C, we confirmed that a similar activity occurred in Geminin-DBD (Fig. S8*D*). We also examined ubiquitination of each of the RDCOXB4 members in the reaction products. The RDCOXB4 complex itself may thus also be subjected to self-ubiquitination (Fig. S8*E*).

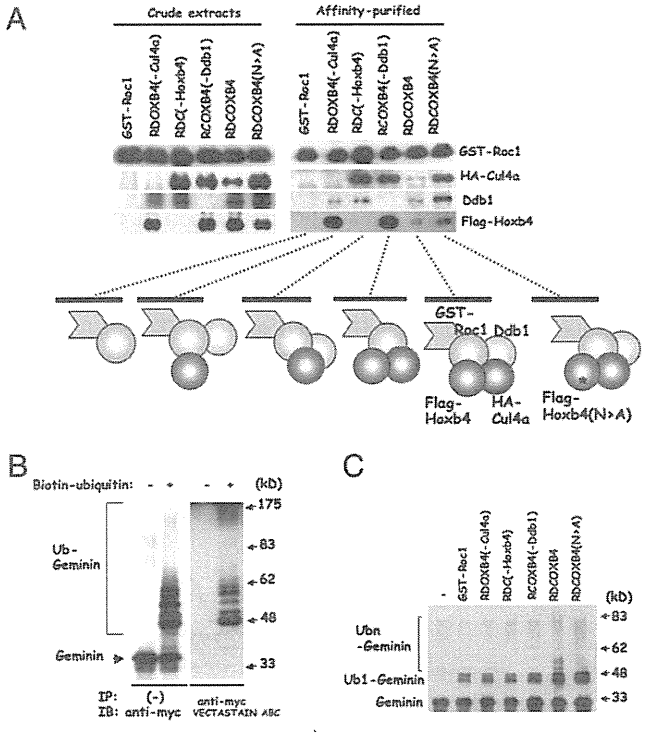

Fig. 5. Reconstitution and purification of the RDCOXB4 complex in Sf9 and E3 ubiquitin ligase activity for Geminin. (*A*) Crude extracts: expression of each member of the complex was detected in crude extracts by immunoblot analysis. Affinity-purified: pull-down assay of the complex with GST-Roc1. (*Lower*) Schematic representation of the complex. *, N>A mutation in the homeodomain of Hoxb4. (*B* and *C*) E3 ubiquitin ligase activity for Geminin. The affinity-purified recombinant complex was subjected to in vitro ubiquitination reaction (myc-Geminin + E1 + E2 + ubiquitin). (*B*) Reaction with biotin-tagged ubiquitin. Ubiquitinated Geminin was detected through biotin–avidin interaction in immunoprecipitated myc-Geminin. (*C*) The E3 ubiquitin ligase activity for Geminin in GST-Roc1, RDOXB4(-Cul4a), RDC(-Hoxb4), RCOXB4(-Ddb1), RDCOXB4, and RDCOXB4 (N>A). The amount of GST-Roc1 in the complex was adjusted to that of RDCOXB4 (1 μg).

Effect of Hoxb4 Transduction on E2F Activity and Its Target Gene Expression. Hoxb4 transduction increased mRNA for *Geminin*, *Cdt1*, and *Cyclin A2* in either Rae$^{+/+}$FLC or Rae$^{-/-}$FLC, whereas Hoxb4N>A did so less efficiently (Fig. S2*E*). Because these genes are under the regulation of E2F (28, 29), the induction was presumed to be mediated by E2F activation. We next examined the effect of Hoxb4 on E2F activity by means of a transient transfection experiment with an E2F-firefly luciferase reporter plasmid, pE2WTx4-Luc, in HEK-293 (Fig. 6*A*) (30). Hoxb4 overexpression induced luciferase activity in a dosage-dependent manner, but that of Hoxb4N>A did so less efficiently (Fig. 6*A*). Because Hoxb4 transfection reduced Geminin protein through UPS as mentioned above, we examined the effect of Geminin on E2F activity (Fig. 6*A*). siRNA-mediated knockdown of Geminin-induced E2F activity and restoration of reduced Geminin by 6myc-tagged Geminin transfection significantly reversed the effect, suggesting that Hoxb4 induced E2F activity at least in part through the direct regulation of Geminin.

Effect of Hoxb4 Transduction on Cdt1 and Mcm2. Finally, we examined by immunoblot analysis the effect of Hoxb4 on Cdt1 in the whole extract (Fig. S2*F*) as well as in the chromatin fraction (Fig. 6*B*). Transduction of Hoxb4 increased Cdt1 in the whole extract (Fig. S2*F*), probably through the aforementioned E2F activation, whereas that of Hoxb4N>A increased less efficiently. Similar induction was observed in Cyclin A2 (Fig. S2*F*) and Mcm2 (Fig. 6*B*). Hoxb4 transduction more prominently increased Cdt1 and Mcm2 in the chromatin fraction of Rae$^{-/-}$FLC (Fig. 6*B*), in which chromatin-loaded Cdt1 and Mcm2 were markedly reduced by accumulated Geminin as described previously (20). The down-regulation of Geminin protein was thus presumed to increase chromatin-loaded Cdt1 and Mcm2 either by E2F activation or by relieving the Geminin-mediated direct inhibition of Cdt1, promoting the prereplicative complex formation on chromatin to provide cells with higher proliferation potential.

Discussion

We show here that Hoxb4 directly interacts with Geminin through the homeodomain. Hoxb4 transduction induced formation of the RDCOXB4 complex, which may act as the E3 ubiquitin ligase for Geminin, whereas Hoxb4N>A constituted a similar complex that displayed little of the E3 ubiquitin ligase activity. Although the homeodomain of Hox proteins has long been believed to function as a DNA-binding domain (7), these findings indicate that the homeodomain may provide the RDCOXB4 complex with a recognition domain for Geminin. The involvement of the Roc1-Ddb1-Cul4a ubiquitin ligase core component in sustaining HSC activity is further supported by recently reported