a serine/threonine and tyrosine kinase, localizes in nuclear speckles and nucleoplasm, and CLK1 regulates alternative splicing through phosphorylation of serine/arginine rich (SR) proteins [14–16]. SR proteins play an important role in both constitutive splicing and alternative splicing of pre-mRNA [6,7]. Functional domain of SR proteins contains one or two RNA-recognition motifs (RRMs), where pre-mRNAs were bound to be processed.

We report here that IκBL physically interacts with CLK1 and SR protein, and functions as a novel regulator in the alternative splicing of both human and viral genes.

2. Materials and methods

2.1. Calculation of Ka/Ks value for amino acid substitution

Ka/Ks value was used to estimate the potential functional domain of IκBL. The Ka and the Ks values are calculated by DnaSP v3.0 [17], by comparing the human and murine *NFKBIL1* sequences. Ks is the number of synonymous substitutions per synonymous site, whereas Ka is the number of nonsynonymous substitutions per nonsynonymous site.

2.2. Plasmids

An exon trapping vector, pSPL3 (Invitrogen, Carlsbad, CA, USA), was used to construct mini-genes analyzed for the alternative splicing. A *CD45* mini-gene construct covered exons 3–7 and their intron–exon boundary segments from human *CD45*, while *CD72* and *CTLA4* mini-gene constructs encompassed exons 7–8, and exons 2–4, respectively, with their intron–exon boundary segments from human the genes. We cloned human cDNAs encoding IκBL, CLK1, hnRNPLL, hnRNPL, FOX1 and ASF/SF2 into mammalian expression vectors, pCI-neo (Promega, Madison, WI, USA) and pEGFP (Clontech, Mountain View, CA, USA). Deletion mutants of IκBL (IκBL-ΔN, -ΔA, -ΔCv and -ΔCc), CLK1 (CLK1-ΔN, -Δkinase) and ASF/SF2 (ASF/SF2-ΔRRM1β1, -ΔRRM2β1, -ΔRRM1&2β1 and -ΔRS) were generated by the standard PCR-based method. All constructs were sequenced to ensure that undesired mutations were not introduced during the cloning procedure. The constructs used in the plasmid-based rescue system for the influenza A virus, including pPOLI-M-RT, pcDNA-NP, pcDNA-PB1, pcDNA-PB2 and pcDNA-PA, were kindly provided by Dr. George G. Brownlee and Dr. Ervin Fodor. Plasmid DNAs for transfection were prepared using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany).

2.3. Cell culture and transfection

COS7, HeLa and HEK293T cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% de-complemented fetal calf serum (FCS) (Nichirei Biosciences, Tokyo, Japan) and Penicillin–Streptomycin–Glutamine (PSG) (Invitrogen). JSL1 cells were kindly provided by Dr. Kristen W. Lynch and maintained in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 5% FCS plus PSG. Transfection was done using COSFectin (Bio-Rad, Hercules, CA, USA) or Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. Hygromycin (Invitrogen) was used for selection of a stably transfected JSL1 line, JSL-IκBL.

2.4. RNA interference

Knockdown of endogenous *NFKBIL1* and *CLK1* was done by using pre-designed siRNAs (siRNA ID for *NFKBIL1*: s9517 and s194653; siRNA ID for *CLK1*: s3162 and s3163) (Ambion, Austin, TX, USA). A non-targeting siRNA was used as a negative control.

2.5. Immunofluorescence staining

Fixed and permeabilized HeLa cells were incubated with anti-SC35 (BD Biosciences Pharmingen, San Diego, CA, USA) and/or anti-CLK1 (Abcam, Cambridge, MA, USA) antibodies, followed by incubation with fluorescence-conjugated second antibodies. Images were analyzed with an LSM510 laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.6. RNA isolation, RT-PCR and real-time RT-PCR

Total cellular RNAs from human tissues were purchased from Agilent Technologies. Total RNAs from cells were purified by using RNeasy Mini kit (Qiagen) and cDNAs were synthesized by the reverse transcription (RT) reaction from 1 μg of RNA using PrimeScript RT reagent Kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. To evaluate the amount of splicing variants, cDNA was applied to PCR and the PCR products were separated by electrophoresis on agarose gels, visualized by ethidium bromide staining, and quantified by using ImageJ Version 1.36. The endogenous expression of mRNA was quantified by real-time RT-PCR using iCycler iQ Real-Time PCR Detection System (Bio-Rad).

2.7. Yeast-two-hybrid (Y2H) screening

All procedures for Y2H were performed according to the manufacturer's instructions for the Matchmaker GAL4 Two-Hybrid System 3 (Clontech).

2.8. Immunoprecipitation (IP) and immunoblotting

IP products were prepared by precipitation of antigen–antibody complex using Protein G Sepharose beads (GE Healthcare, Uppsala, Sweden). For immunoblotting, samples were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Invitrogen). After the incubation with antibodies, signals were visualized by Image Reader LAS-3000 (FUJIFILM, Tokyo, Japan).

2.9. Flow cytometry analysis

JSL1 and JSL1-IκBL cells with or without activation by 12-myristate 13-acetate (PMA) (Calbiochem, San Diego, CA, USA) were incubated with specific antibodies to CD45RA (eBioscience, San Diego, CA, USA) or CD45RO (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Flow cytometry analysis was performed on FACSCalibur (BD Biosciences, San Jose, CA, USA) according to the standard protocol.

2.10. Statistical analysis

Statistical comparisons were performed using Student's *t*-tests or one-way ANOVA followed by a post-hoc Bonferroni's or Dunnett's multiple comparison tests. The results were considered statistically significant when the p value was less than 0.05.

Additional information to *2.4.*, *2.5.*, *2.7.*, *2.8.* and antibodies used in this study can be found in Supplementary information.

3. Results

3.1. Domain structure of IκBL for localization to nuclear speckles

Based on the domain structure and Ka/Ks value, IκBL was divided into four segments; N-terminal segment (N) (amino acids 1–66) containing a putative NLS, ankyrin repeat domain segment (A) (amino acids 67–137), central variable segment (Cv)

(amino acids 138–297), and C-terminal conserved segment (Cc) containing a leucine zipper motif (amino acids 298–381) (Fig. 1A and B). Cellular localization of IκBL was investigated in HeLa cells transfected with EGFP-tagged IκBL. As shown in Fig. 1C, IκBL-linked EGFP signal was co-localized with SC35, a member of SR protein family, in the nuclear speckles. On the other hand, HeLa cells expressing EGFP-IκBL-ΔN showed diffuse cytoplasmic EGFP signals, demonstrating that the segment N was essential for the nuclear localization. Segments A and Cv were indispensable for the nuclear localization of IκBL, because their deletions impaired the localization to the nuclear speckles. In contrast, deletion of the segment Cc had no effect on the subnuclear localization of IκBL.

3.2. *IκBL inhibits exon exclusion in alternative splicing of immune-related genes*

Localization of IκBL in the nuclear speckles, along with the evidence that genetic variations of IκBL were associated with the susceptibility to inflammatory and/or autoimmune diseases, leads to a hypothesis that IκBL might play a pivotal role in the alternative splicing of immune-related genes. Because human *CD45* gene is known to undergo alternative splicing of exons from 3 to 7, we generated a mini-gene construct for human *CD45* covering exons 3–7. The mini-gene construct was transfected into a monkey cell line COS7 with inducers of alternative splicing, hnRNPLL or hnRNPL [18–21]. In the hnRNPLL-induced *CD45* alternative splicing, IκBL decreased the generation of exons 3-7 isoform and oppositely increased the exons 3-4-5-6-7 isoform (Fig. 2A). Similar effects of IκBL on the *CD45* alternative splicing were also observed in human cell lines, HeLa and HEK293T (data not shown). In addition, it was observed that IκBL also suppressed the hnRNPL-induced alternative splicing of *CD45* (Supplementary Fig. S1).

We next examined whether the silencing of endogenous *NFKBIL1* would affect the hnRNPLL-induced *CD45* alternative splicing. *CD45* mini-gene and hnRNPLL were transfected into HeLa, in which the endogenous *NFKBIL1* was interfered by human *NFKBIL1*-specific siRNA. It was found that the knock-down of *NFKBIL1* increased the exons 3-7 isoform and concomitantly decreased the exons 3-5-7 isoform (Fig. 2B), indicating that IκBL facilitated the exon inclusion in alternative splicing of *CD45*.

To examine the effect of IκBL on other human immune-related genes, we created mini-genes of *CD72* and *CTLA4*. A *CD72* mini-gene covered exons 7 and 8, whereas a *CTLA4* mini-gene encompassed exons 2–4. The hnRNPLL-induced *CD72* alternative splicing was counteracted by the expression of IκBL (Supplementary Fig. S2A). On the other hand, hnRNPLL-induced *CD72* alternative splicing was accelerated in cells where the endogenous *NFKBIL1* was silenced (Supplementary Fig. S2B). In addition, we found a suppression of FOX1-induced *CTLA4* alternative splicing by IκBL (Supplementary Fig. S3). Furthermore, we studied which domain of IκBL was involved in the regulation of alternative splicing. It was revealed that IκBL-ΔN, -ΔA and -ΔCv failed to suppress the hnRNPLL-induced *CD45* alternative splicing, whereas -ΔCc could suppress it similar to the intact (-FL) IκBL (Fig. 2C).

3.3. *Identification of molecules interacting with IκBL by Y2H screening*

Expression of *NFKBIL1* in human tissues was examined by real-time RT-PCR. It was found that *NFKBIL1* was ubiquitously expressed with the prominent expression in spleen (Supplementary Fig. S4A). Next, a Y2H screening of human spleen cDNA library was performed to identify interacting molecules

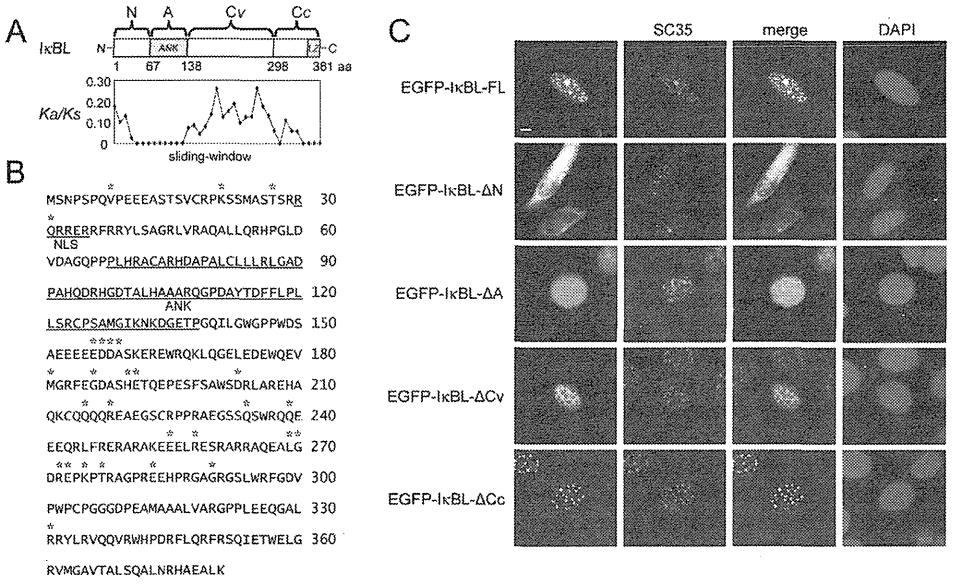

Fig. 1. Structure and cellular localization of IκBL. (A) Ka/Ks value based on the sliding window plot analysis for the *NFKBIL1* gene. Ks is the number of synonymous substitutions per synonymous site, whereas Ka is the number of nonsynonymous substitutions per nonsynonymous site, by comparing the human and murine *NFKBIL1* sequences. The Ka and the Ks values are calculated by DnaSP (v3.0). According to Ka/Ks value, IκBL was divided into four segments; N-terminal segment (N) (amino acids 1–66) containing a putative NLS, ankyrin repeats domain segment (A) (amino acids 67–137), central variable segment (Cv) (amino acids 138–297) and C-terminal conserved segment (Cc) with leucine zipper motif (amino acids 298–381). Amino acids are numbered starting from the first in-frame methionine codon. (B) Amino acid sequences of human IκBL. NLS and ankyrin repeats domain (ANK) are underlined. Asterisks indicate the positions of amino acids that are different from the amino acid sequences of murine IκBL. (C) HeLa cells were transfected with EGFP-IκBL-FL, -ΔN, -ΔA, -ΔCv or -ΔCc (EGFP signal, green) and immunofluorescence staining was performed by using anti-SC35 antibody (Alexa Fluor 568-labeled, red). IκBL-FL co-localized with SC35 in nuclear speckles. IκBL-ΔN localized in the cytosol. Both IκBL-ΔA and -ΔCv were found in the nuclei, but the localization to nuclear speckles was impaired. IκBL-ΔCc could localize to nuclear speckles, similar as IκBL-FL. A bar represents 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

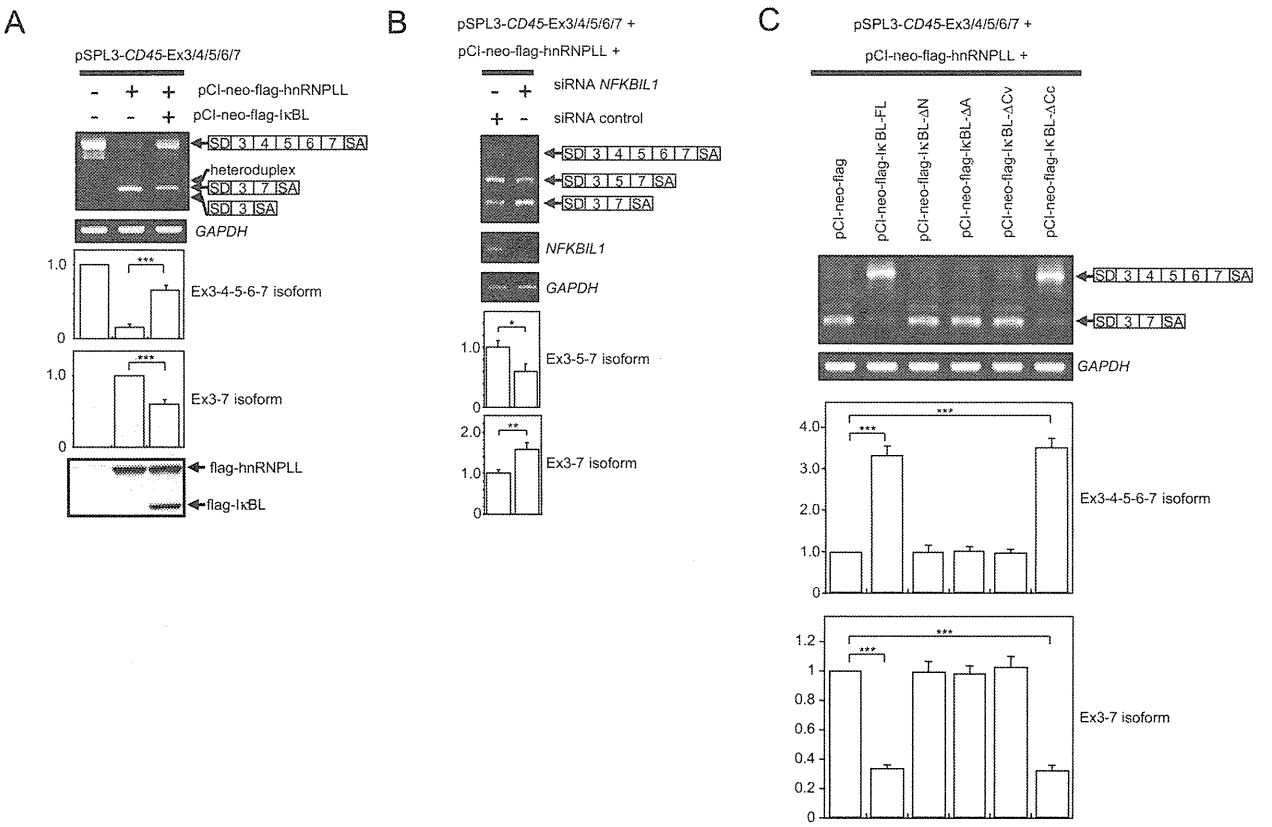

Fig. 2. Suppression of exon exclusion in the alternative splicing of *CD45* mini-gene by IκBL. (A) COS7 cells were transfected with *CD45* mini-gene and flag-hnRNPLL. RT-PCR analysis showed the effect of hnRNPLL on the alternative splicing of *CD45* transcripts derived from the mini-gene. COS7 cells were additionally transfected with flag-IκBL. The suppressive effect of IκBL on the hnRNPLL-induced alternative splicing of *CD45* is shown. Relative amounts of exons 3-4-5-6-7 isoform and exons 3-7 isoform were quantified and normalized to *GAPDH* transcripts. (B) HeLa cells were treated with siRNA specific to *NFKBIL1*, and then subjected to transfection with *CD45* mini-gene and flag-hnRNPLL. The hnRNPLL-induced alternative splicing of *CD45* was shown. Relative amounts of exons 3-5-7 isoform and exons 3-7 isoform were quantified and normalized to *GAPDH* transcripts. (C) COS7 cells were transfected with *CD45* mini-gene, flag-hnRNPLL plus one of flag-IκBL-FL, -ΔN, -ΔA, -ΔCv or -ΔCc constructs. RT-PCR analysis showed the effects of IκBL-FL, -ΔN, -ΔA, -ΔCv and -ΔCc on the hnRNPLL-induced alternative splicing of *CD45*. Relative amounts of exons 3-4-5-6-7 isoform and exons 3-7 isoform were quantified and normalized to *GAPDH* transcripts. Bar graphs in (A)–(C) represent the quantification of indicated transcripts. Data are shown as means ± SD of three replicates. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$.

with IκBL, which would provide us with useful information for the molecular mechanism of IκBL-dependent splicing regulation. A total of 11 different interacting proteins, including CLK1, were picked-up in the Y2H screening (Supplementary Fig. S4B). Interestingly, it was found that IκBL bound itself, suggesting that IκBL could form a multimer.

3.4. IκBL interacts with CLK1

The interaction of IκBL with CLK1 was further investigated, because CLK1 was known to play an important role in the alternative splicing [14–16] and the endogenous CLK1 localized in nuclear speckles (Fig. 3A). To confirm the interaction between IκBL and CLK1, COS7 were transfected with a flag-IκBL construct, followed by immunoprecipitation (IP) with an anti-CLK1 antibody and subsequent immunoblotting of IκBL using an anti-flag antibody. As shown in Fig. 3B, IκBL was found in the IP products of endogenous CLK1 and treatment with RNase A had little effect on the interaction. In addition, deletion mutants of IκBL, IκBL-ΔN, -ΔA and -ΔCv, failed to associate with CLK1 (Fig. 3C). To examine the role of CLK1 in the alternative splicing, HEK293T cells were pre-treated with siRNA to knockdown the endogenous expression level of *CLK1* (Fig. 4D). Knockdown of *CLK1* impeded hnRNPLL-induced alternative splicing of both *CD45* and *CD72*, as similar to the inhibition by IκBL (Fig. 4D and Supplementary Fig. S2C, respectively).

3.5. Regulation of alternative splicing by IκBL was independent from kinase activity of CLK1

We next asked how IκBL suppressed the exon skipping in alternative splicing. Given that IκBL interacted with CLK1, and knockdown of *CLK1* impeded alternative splicing, IκBL might inhibit the function of CLK1. CLK1 is composed of N-terminal regulatory domain and C-terminal kinase domain (Supplementary Fig. S5A) and is known to phosphorylate SR proteins, which are involved in the splicing. COS7 were transfected with a myc-tagged construct for an SR protein, ASF/SF2, with or without CLK1 full-length (CLK1-FL) or kinase domain deleted (CLK1-Δkinase) constructs. It was confirmed that the kinase domain was indispensable for CLK1 to phosphorylate ASF/SF2, whereas IκBL failed to affect the CLK1-induced phosphorylation of ASF/SF2 (Fig. 4A). On the other hand, the functional domain of CLK1 indispensable for the regulation of alternative splicing in *CD45* was, to our surprise, the N-terminal regulatory domain, but not the kinase domain (Supplementary Fig. S5B). These data indicated that both IκBL and CLK1 regulated the alternative splicing of *CD45*, in which the kinase activity of CLK1 was not involved.

N-terminal regulatory domain of CLK1 was reported to interact with ASF/SF2 [14], and we confirmed that N-terminal domain of CLK1 bound ASF/SF2 (Supplementary Fig. S6A). On the other hand, when we transfected COS7 with constructs of flag-ASF/SF2-FL, -ΔRRM1β1, -ΔRRM2β1, -ΔRRM1&2β1 or -ΔRS, followed by

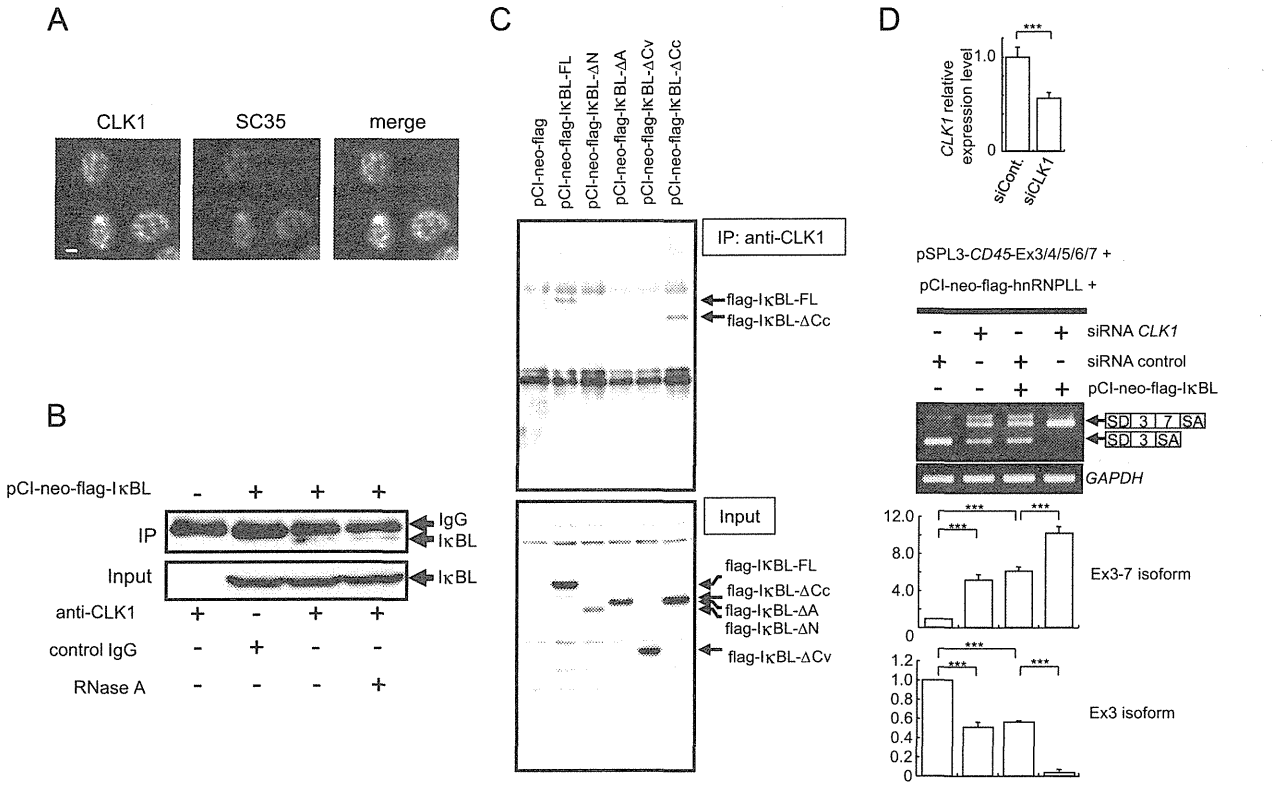

Fig. 3. Interaction of IκBL with CLK1. (A) Subcellular localization of endogenous CLK1 (FITC-labeled, green) and endogenous SC35 (Alexa Fluor 568-labeled, red) were visualized by immunofluorescent staining in HeLa cells. Bar indicates 5 μm. (B) COS7 cells were transfected with or without flag-tagged IκBL, followed by IP using an antibody against CLK1 in the presence or absence of RNase A. The IP products and input lysates were immunoblotted with anti-flag antibody. (C) Co-IP of endogenous CLK1 with flag-IκBL-FL, -ΔN, -ΔA, -ΔCv or -ΔCc. The IP products and input lysates were immunoblotted with anti-flag antibody. The results in (B) and (C) were representatives of three independent experiments. (D) HEK293T cells were treated with siRNA specific to *CLK1*, and then subjected to transfection with *CD45* mini-gene and flag-hnRNPLL with or without flag-IκBL. The transcripts derived from alternative splicing of *CD45* mini-gene were shown. Relative amounts of exons 3-7 isoform and exon 3 isoform were quantified and normalized to *GAPDH* transcripts. Bar graphs represent the quantification of indicated transcripts. Data are shown as means ± SD of four replicates. $^{***}p < 0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immunoprecipitation with anti-CLK1 antibody, it was found that ASF/SF2 lacking both RRMs failed to associate with CLK1 (Supplementary Fig. S6B and S6C). Because ASF/SF2 was suggested to be involved in the regulation of *CD45* alternative splicing [22–24], we analyzed the hnRNPLL-induced splicing of *CD45* mini-gene in the presence of ASF/SF2-FL or deletion mutant constructs. It was observed that ASF/SF2 inhibited the hnRNPLL-induced exon exclusion of *CD45*. In addition, ASF/SF2 without RRMs lost the inhibitory function, indicating that RRMs of ASF/SF2 were crucial for the regulation of *CD45* alternative splicing (Supplementary Fig. S7).

To investigate the interaction of IκBL with CLK1 and ASF/SF2, we transfected COS7 with EGFP-tagged IκBL in combination with flag-tagged CLK1-FL or deletion constructs. CLK1-FL and CLK1-Δkinase, but not CLK1-ΔN, were co-immunoprecipitated with EGFP-IκBL, indicating that IκBL bound the N-terminal regulatory domain of CLK1 (Fig. 4B). In addition, it was demonstrated that IκBL bound the ASF/SF2 at the RRMs (Fig. 4C). These observations implied that CLK1 and IκBL were competitively associated with RRMs of ASF/SF2.

3.6. Overexpression of IκBL impaired endogenous CD45 alternative splicing in JSL1 T cells

It was reported that a human T cell line, JSL1, expressed a variety of *CD45* isoforms and stimulation with PMA induced the expression of *CD45* transcripts with alternative splicing [25]. We found that steady-state level of mRNA for *NFKBIL1* was significantly reduced in JSL1 treated with PMA (Fig. 5A). To study the effects of IκBL on the endogenous *CD45* alternative splicing, we transfected JSL1 with flag-tagged IκBL followed by a hygromycin selection to obtain a stable cell line expressing IκBL, JSL1-IκBL (Fig. 5B). In comparison with PMA-induced alternative splicing of *CD45* in JSL1, PMA-treated JSL1-IκBL showed a decreased amount of the exons 3-7 isoform and reciprocally increased amount of longer isoforms including the exons 3-5-6-7 isoform (Fig. 5B). Flow cytometry analysis showed that PMA-treated JSL1-IκBL expressed higher amount of CD45RA isoform, that encompassed exon 4, and a slightly lower amount of CD45RO isoform corresponding to the exons 3-7 isoform (Fig. 5C), indicating that IκBL impeded PMA-induced exon exclusions in the alternative splicing of endogenous *CD45*. It also was found that IκBL associated with CLK1 in JSL1-IκBL (Supplementary Fig. S4C).

3.7. IκBL regulates the alternative splicing of influenza A virus M gene

It was reported that a knockdown of *CLK1* reduced the replication of influenza A virus, which was mediated by the impaired alternative splicing of viral *M2* mRNA [26]. To study a possible effect of IκBL on the alternative splicing of influenza *M* gene, we employed a plasmid-based rescue system [27,28]. The plasmid encoding influenza *M* gene was co-transfected with viral RNA polymerase complex constructs into COS7. It was found that IκBL

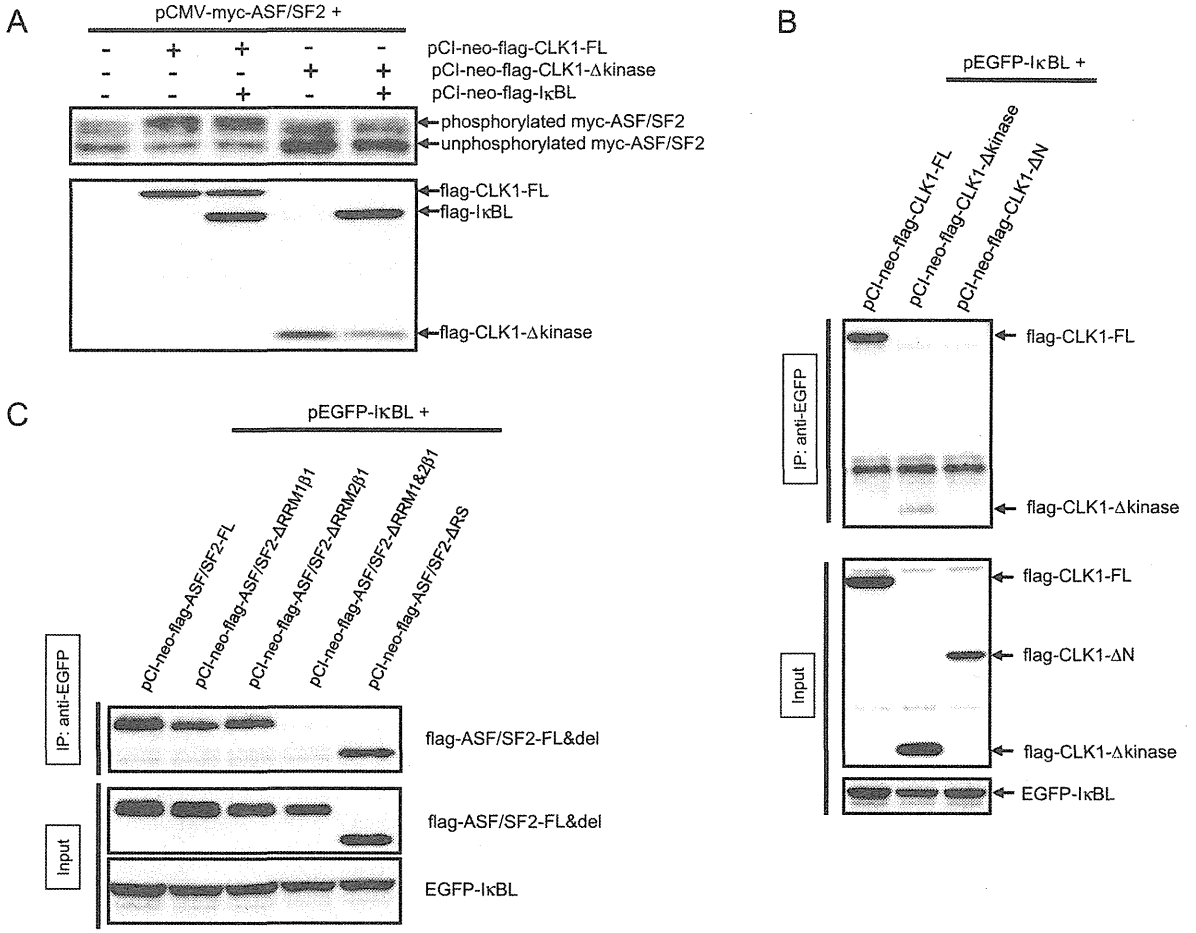

Fig. 4. IκBL did not interfere with CLK1-dependent phosphorylation. (A) COS7 cells were transfected with the constructs of myc-ASF/SF2 along with flag-CLK1-FL or -Δkinase. Immunoblotting showed unphosphorylated and phosphorylated ASF/SF2. In addition, COS7 cells were transfected with flag-IκBL. There was no effect of flag-IκBL on the phosphorylation level of ASF/SF2. (B) Co-IP of EGFP-IκBL with flag-CLK1-FL, Δkinase or -ΔN. (C) Co-IP of EGFP-IκBL with flag-ASF/SF2-FL, -ΔRRM1β1, -ΔRRM2β1, -ΔRRM1&2β1 or -ΔRS. The IP products and input lysates were immunoblotted with anti-flag and anti-EGFP antibodies. The results in (A)–(C) were representatives of three independent experiments.

reduced the amount of *M2* splice variant derived from the *M* gene in a dose-dependent manner (Fig. 6A). On the other hand, the synthesis of *M2* was promoted when *NFKBIL1* expression was interfered by siRNA (Fig. 6B), indicating the role of IκBL in the splicing regulation of viral *M* gene. It was also observed that exogenous expression of ASF/SF2 suppressed the alternative splicing of *M* gene, and this suppression was abolished in the absence RRMs (Supplementary Fig. S8).

4. Discussion

It has been accepted that abnormalities in the regulation of mRNA splicing are tightly linked to the pathogenesis of human disorders in that approximately 15% of the mutations that cause genetic diseases affect pre-mRNA splicing, and splicing mutations might be the most frequent causes of hereditary disease [6,29,30]. In addition, a number of association studies revealed the link of *NFKBIL1* to autoimmune or inflammatory diseases and it has been demonstrated that a sequence variation in the *NFKBIL1* promoter, which results in the reduced expression of IκBL, may confer the susceptibility to RA [4]. Given that IκBL localized in the nuclear speckles and associated with RNA, there is a possible link between the altered expression of IκBL and immune-related diseases via altered RNA splicing.

In this study, it was demonstrated that the exon skipping in alternative splicing of *CD45* was suppressed by IκBL. On the other hand, when the expression of *NFKBIL1* was silenced, the exon skipping of *CD45* was promoted. We also observed that IκBL affected alternative splicing of *CD72* and *CTLA4*. These results strongly indicated that IκBL enhanced exon inclusion in the alternative splicing of immune-related genes. Because *CD45* is expressed in nearly all hematopoietic cells and *CD72* is mainly expressed in B cells, whereas *CTLA4* is known as a surface receptor of T cells, the observations in this study suggested that the regulatory function of IκBL on the alternative splicing was not limited to specific gene or cell type. In addition, the suppressive effects of IκBL were observed in the alternative splicing events induced by hnRNPLL, hnRNPL and FOX1, implying a role of IκBL in a broad context of splicing regulation.

To elucidate the molecular mechanisms of IκBL in the regulation of alternative splicing, we searched for IκBL-interacting proteins using Y2H screening and identified CLK1 to be a binding partner. The association with CLK1 was mediated by N-terminal segment, ankyrin repeat domain segment, and central variable segment of IκBL, all of which were indispensable for IκBL to correctly localize in the nuclear speckles, and to regulate the alternative splicing. These results suggested the involvement of association between IκBL and CLK1 in the alternative splicing.

Although it is not clarified how CLK1 functions on its substrate, it has been reported that CLK1 phosphorylates the SR proteins and plays a role in alternative splicing of target genes including *CLK1* itself [14–16]. In this study, a knockdown of *CLK1* impeded exon

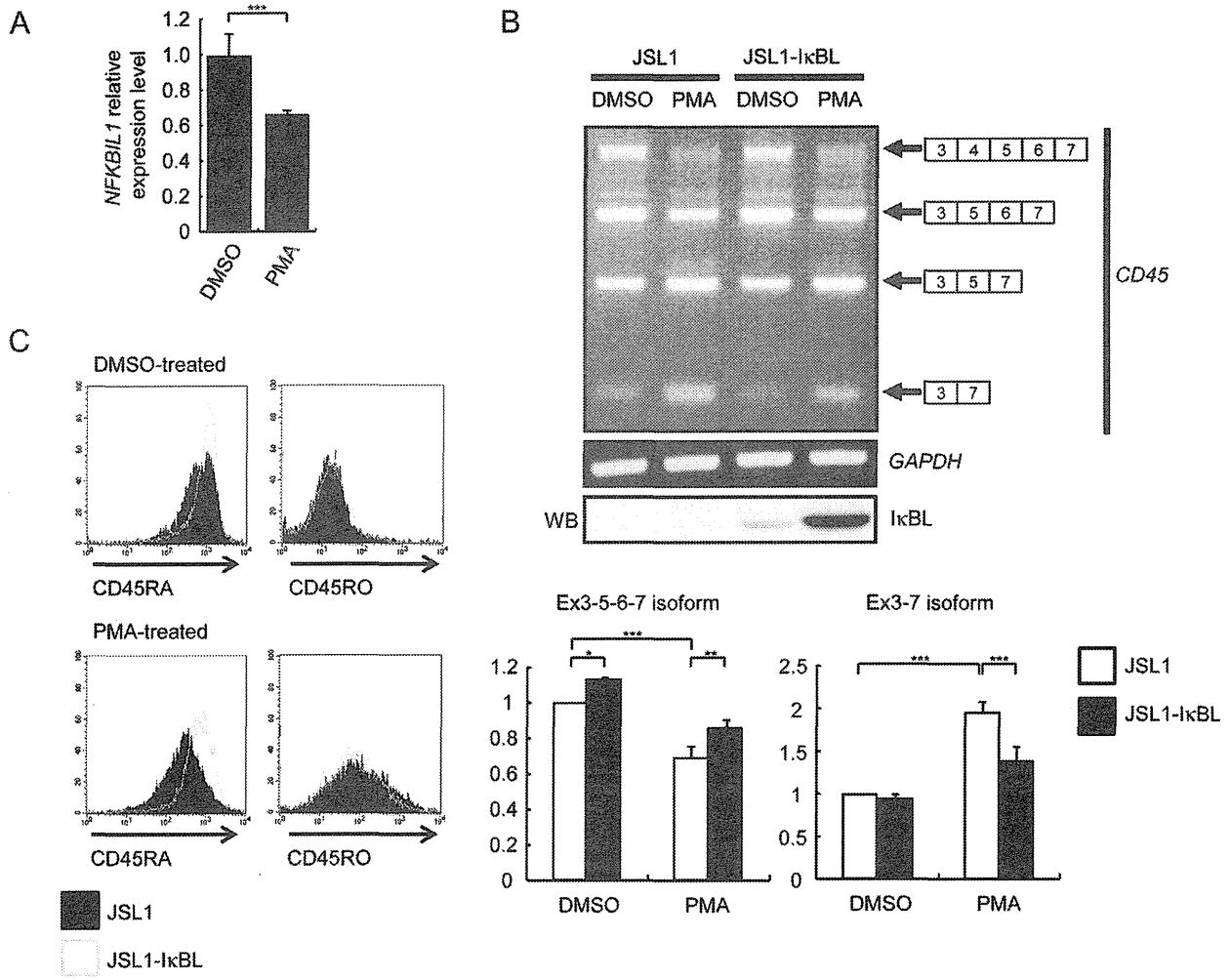

Fig. 5. Role of IκBL in the alternative splicing of endogenous *CD45* in JSL1. (A) Expression of endogenous *NFKBIL1* in JSL1 treated with DMSO (control) or PMA (10 ng/ml, 2 days) was analyzed by using real-time RT-PCR. *NFKBIL1* mRNA level was normalized to 18S rRNA. Data are shown as means ± SD of three replicates. ***$p < 0.005$. (B) RT-PCR analysis showed the alternative splicing of endogenous *CD45* in JSL1 and JSL1-IκBL with DMSO or PMA (10 ng/ml, 2 days) treatment. Relative amounts of exons 3-5-6-7 isoform and exons 3-7 isoform were quantified and normalized to *GAPDH*. Data are shown as means ± SD of three replicates. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$. (C) Flow cytometry analysis of JSL1 and JSL1-IκBL treated with DMSO or PMA (10 ng/ml, 4 days). PE-labeled CD45RA antibody was used to detect the longer CD45 protein isoforms encompassing exon 4, whereas APC-labeled CD45RO antibody was used to detect the short CD45 protein isoform of exons 3-7.

exclusion, implying that the mechanism by which IκBL regulates alternative splicing might be mediated by functional suppression of CLK1. Although IκBL might interfere with CLK1-induced phosphorylation of SR proteins, we demonstrated in this study that IκBL failed to alter the CLK1-induced phosphorylation of ASF/SF2. To our surprise, it was clearly showed that the functional domain of CLK1 to regulate the alternative splicing of *CD45* was the N-terminal regulatory domain, but not the C-terminal kinase domain. In this context, it is noteworthy that we deciphered a novel mechanism of alternative splicing, where IκBL was involved in, which is independent from the kinase activity of CLK1.

We further investigated the kinase-independent mechanism in the alternative splicing of *CD45*. Our results demonstrated that IκBL bound the N-terminal regulatory domain of CLK1. Albeit that function of the N-terminal domain of CLK1 remained to be clarified, a previous study using Y2H system showed that the N-terminal domain mediated the association with several splicing factors including ASF/SF2, that is one of the most intensively investigated SR proteins [14]. Domain structure of ASF/SF2 is that there are two RRMs followed by a C-terminal RS domain. Each RRM consists of four antiparallel β-strands and two α-helices, which determines the RNA-binding specificity. Importantly, deletion of β1-strand, where RNP submotif locates, is supposed to disrupt the tertiary structure of RRMs. The RS domain includes multiple consecutive phosphorylatable RS/SR dipeptide repeats of which phosphorylation status affects protein–RNA and protein–protein interactions [31,32]. ASF/SF2 plays a pivotal role in the *CD45* alternative splicing [22–24]. In this study, we found that ASF/SF2 counteracted the hnRNPLL-induced *CD45* alternative splicing. Furthermore, it was observed that ASF/SF2 lacking the RRMs, but not RS domain, failed to regulate the *CD45* splicing, which was consistent with that RRMs of ASF/SF2 was indispensable for the alternative splicing [31,32]. We assessed the interaction of IκBL with ASF/SF2, in which IκBL bound the RRMs of ASF/SF2. Therefore, it was supposed that IκBL and CLK1, presumably in a competitive way, interacted with the RRMs of ASF/SF2 to modulate the splicing of *CD45*.

To examine the role of IκBL in alternative splicing of endogenous immune-related genes, JSL1-IκBL, a JSL1 cell line over-expressing flag-IκBL, was established. JSL1-IκBL showed an impeded exon skipping in the PMA-induced alternative splicing of endogenous *CD45*. On the other hand, PMA-induced alternative splicing was accompanied by the reduced expression of endogenous *NFKBIL1*,

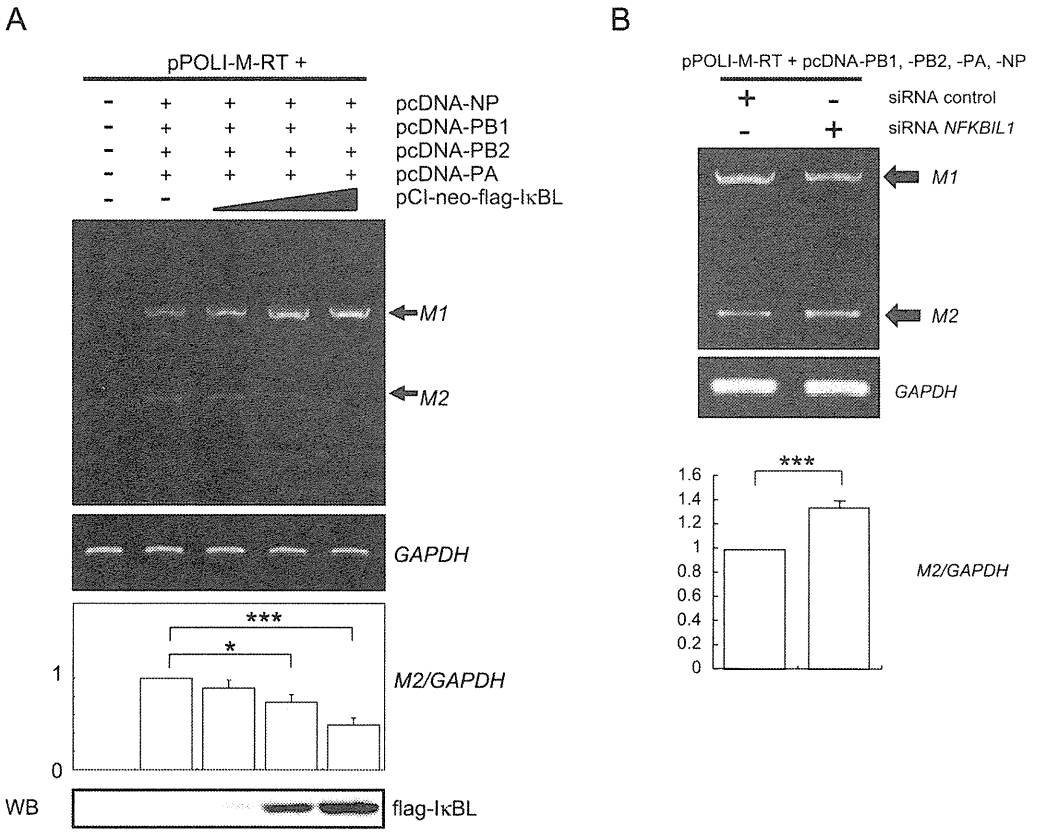

Fig. 6. Regulation of alternative splicing in influenza A virus *M* gene by IκBL. (A) Influenza A virus *M* gene plasmid was co-transfected with plasmids encoding viral RNA polymerase complex factors, i.e. PB1, PB2, PA and NP along with different dose of flag-IκBL into COS7 cells. Expression of flag-IκBL inhibited the generation of spliced *M2* viral RNA in a dose-dependent manner. (B) The effect of *NFKBIL1* knockdown on the alternative splicing of *M* gene was shown. Bar graph indicates the quantification of *M2* viral transcripts normalized to *GAPDH*. Data are shown as means ± SD of three replicates. $^{*}p < 0.05$; $^{***}p < 0.005$.

suggesting that the altered splicing of *CD45* in PMA-stimulated JSL1 was mediated in part by the reduced expression of IκBL. It is well known that abnormally high amount of CD45RO+ T cells predominated in synovial fluid of RA patients [33,34], and our study suggested that the lower expression of IκBL might modulate the activation of T cells and hence would be associated with the susceptibility to RA.

NFKBIL1 is mapped within *HLA*, which comprises a number of genes involved in the protection of host from microorganisms. It has been reported that the knockdown of *CLK1* reduces the replication of influenza A virus, which is associated with the impaired splicing of viral M2 isoform [26]. In this study, it was revealed that IκBL could regulate the level of *M2* RNA transcript, implying that IκBL was capable to inhibit the influenza viral replication. This is a so far unraveled mechanism for fighting against invading microorganisms; by regulating alternative splicing of target viral genes by the *HLA*-linked gene, *NFKBIL1*.

5. Conclusions

IκBL, which interacts with CLK1 and SR proteins in the nuclear speckles, is one of the factors playing crucial roles in the alternative splicing in both human and viral genes. We revealed that IκBL was involved in a novel mechanism for alternative splicing in which CLK1 played a kinase-independent role. The study also provided us with a novel insight into the association of *NFKBIL1* with the susceptibility to inflammatory and/or autoimmune disorders, which is a novel link of *HLA* locus to both immunity and infection in humans, via regulation of alternative splicing.

Author contributions

JA conducted most of the experiments, contributed to data analysis, and wrote the paper. TN participated in the experiments of alternative splicing using mini-genes. TA conducted immunofluorescence staining. HS and MY participated in the Y2H experiment, biochemical study and data analysis. AK designed the study, supervised the experiments, and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2013.07.010.

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Review

IκBL mapped within the HLA region is a novel regulator of alternative splicing involved in the pathogenesis of immune-related diseases

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HLA region contains a set of genes that play crucial roles in the immune system. In addition to the central function of antigen-presentation, which is conducted by HLA class I and II genes, function of the other HLA-linked genes may also contribute to the immune regulation. *IKBL*, alternatively named as *NFKBIL1*, mapped within the HLA class III region is a newly emerged gene, of which sequence variations are associated with the susceptibility or resistance to autoimmune and/or inflammatory diseases. We recently have revealed that the *IKBL*-coded protein, IκBL, is involved in the regulation of alternative splicing in human immune-related genes and a viral gene, which unravel an unexpected function of the HLA-linked gene and provided a novel understanding of *HLA* in the regulation of immunity and infection. In this review, we summarize the latest trends in the study of *IKBL*.

Key Words: NFKBIL1, CLK1, alternative splicing, susceptibility, autoimmune disease, influenza virus

HLA region in immune regulation

Human leukocyte antigen (HLA) system located on chromosome 6p21.31 is the major histocompatibility complex in human. HLA genes have initially been recognized as the major determinants in the allo-recognition in blood transfusion and tissue transplantation. HLA region contains a large number of genes, of which products are essential in the immune regulation and coordinate the innate and adaptive immune responses.

HLA region is usually classified into three subregions, named HLA class I, II and III. HLA class I region contains genes encoding for HLA class I molecules, *HLA-A*, *-B* and *-C*, which are expressed by nearly all nucleated cells. Cytoplasmic proteins including pathogens like virus are degraded into short peptides by proteasome, which are subsequently presented in the context with HLA class I molecules to be recognized by $CD8^+$ killer T cells. $CD8^+$ T cells recognize a complex of HLA molecules with the "non-self" peptides to eliminate the virus-infected cells by exhibiting cytotoxicity. HLA class I molecules also play a role in the interaction with NK cells. Cells expressing HLA class I molecules bound by self- or non-self-peptides are the prerequisite determinants whether attacked by NK cells or not.

HLA class II region contains genes encoding for HLA class II molecules, *HLA-DR*, *-DQ* and *-DP*, mainly expressed by antigen-presenting cells (APCs) such as macrophages, dendritic cells and B cells. Exogenous proteins including outer microorganisms are digested into peptides in endosomes of APCs, which are bound and presented by HLA class II molecules on the cell surface. $CD4^+$ T cells are mainly sensing HLA class II molecules, of which activation may result in induction of inflammation and immune response, via for example macrophages to secret inflammatory cytokines and B cells to produce specific antibodies, respectively.

HLA class I and II molecules as described above are

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provided with the function in antigen presentation to $CD4^+$ or $CD8^+$ T cells, respectively. On the other hand, HLA class III region contains a number of genes not involved in the antigen-presentation. It has been well known that these non-antigen-presentation genes are also important in the immune regulation. For examples, genes in the HLA class III region encode components of complement system, Bf, C2, and C4, which are involved in the clearance of pathogens. In addition, lymphotoxins including TNF-α are encoded by the genes in the HLA class III region and play roles as central mediators in the inflammatory response as well as in the programmed cell death.

A potential role of IκBL in the immune regulation

Inhibitor of κB-like (*IKBL*), also named as NF-κB inhibitor-like 1 (*NFKBIL1*), is mapped within the HLA class III region about 25 kb telomeric to *TNFA*. A considerable number of studies reported the association between genetic variations of *IKBL* with the susceptibility or resistance to autoimmune and/or inflammatory diseases, suggesting that *IKBL* might mediate underlying mechanisms in the immune regulation.

As far as we know, the genetic variations of *IKBL*, which are reported to link with immune-related diseases, include five different single nucleotide polymorphisms (SNPs); –421 8T/9T (rs3219186), –324 C/G (rs3219185), –262 A/G (rs3219184), –62 A/T (rs2071592) and +738 T/C (rs3130062), as well as haplotypes composed of promoter SNPs, from IkBLp*01 to IkBLp*05[1]. The first study carried out by Okamoto et al identified *IKBL* as a candidate risk locus for rheumatoid arthritis (RA), in which the –62T allele conferred the susceptibility[2]. Subsequent study conducted by different group using independent samples supported that the –62T allele was associated with RA[3], but the other SNPs in close linkage disequilibrium (LD) with the –62T may also shape the susceptibility to RA[1]. Another autoimmune disease, systemic lupus erythematosus (SLE), was also reported to be associated with SNPs of *IKBL*. The –62A and +738C alleles showed decreased and increased odds risk for SLE, respectively, while the –62A+738T haplotype was found to decrease the risk[4]. Furthermore, +738C allele in an ancestral haplotype 7.1 was reported to confer a resistance to multiple sclerosis (MS)[5]. The associations with *IKBL* were also reported for other autoimmune diseases; Graves disease (susceptibility with –62A)[6] and type I diabetes (T1D) (resistance with IkBLp*03 haplotype)[7].

Genetic variations of *IKBL* are also associated with series of chronic inflammatory diseases. A meta-analysis in Japanese populations revealed that –262G and –62T were the candidate loci for susceptibility to ulcerative colitis[8], although another European group additionally reported an association with +738C[9]. In addition, the associations were found for other inflammatory diseases such as chronic Chagas cardiomyopathy (susceptibility with –262A and –62A alleles, and –262A–62A haplotype)[10], Takayasu arteritis (TA) (susceptibility with IkBLp*03 haplotype)[11], and chronic thromboembolic pulmonary hypertension (susceptibility with IkBLp*03 haplotype)[11]. These lines of evidence strongly suggested the involvement of *IKBL* in autoimmune and/or inflammatory diseases. However, the molecular function of IκBL, as well as the molecular basis underlying the pathogenesis of these immune-related diseases, remained largely unknown.

Molecular function of IκBL

Evidence has mounted that SNPs in the promoter region of *IKBL* influence the expression of *IKBL*. Shibata *et al.* have reported that the promoter SNPs consist of five different haplotypes, IkBLp*01 to IkBLp*05, which conferred different transcriptional activities of *IKBL*[1]. Interestingly, IkBLp*01 and p*03, which showed the lowest and highest promoter activities, were associated with the susceptibility to RA and TA, respectively[1]. Furthermore, the –62 position was predicted to be a binding site for δEF1, USF1 and E47 transcription factors, and the –62 SNP was indeed demonstrated to affect the binding of these transcription factors, which was supposed to have an impact on the expression of *IKBL*[2,12]. Taken these observations into account, it could be speculated that the association between *IKBL* with immune-related diseases may attribute to the altered expression of IκBL.

Overexpression and/or knockdown of *IKBL* were reported for investigating the functional role of IκBL in the context of immune regulation. First, the role of IκBL in IKK-IκB-NF-κB signaling pathway was examined. Inflammatory signal-induced phosphorylation of IκB leads to its degradation, releasing NF-κB dimer to translocate into nucleus and to initiate transcription. As compared with the members of IκB family, such as IκBα and IκBβ, which are central molecules in the inflammatory signaling, the amino acids sequences of IκBL showed only a limited homology.

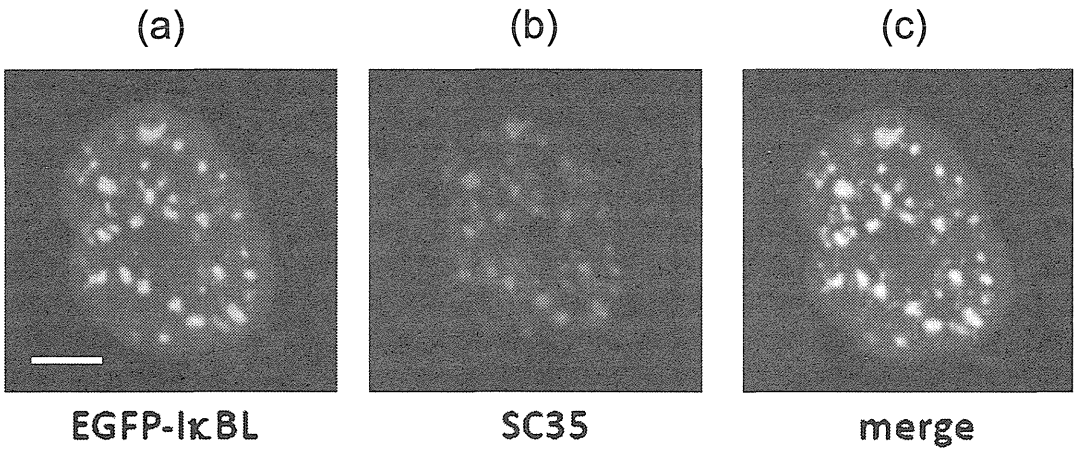

Figure 1 Subcellular localization of IκBL

EGFP-tagged *IKBL* construct was transfected into HeLa cells. The transfected cells were immunostained by anti-SC35 antibody followed by Alexa-Fluor 568-conjugated secondary antibody. (a) EGFP signals (green) representing the localization of IkBL, (b) localization of SC35 (red), and (c) merged image of left and middle images. Scale bar; 5 μm.

In addition, IκBL did not show any transactivation activity[13] (our unpublished observation).

We and others investigated the intracellular localization of IκBL[13–15]. It was found that EGFP-tagged IκBL localized within nuclear speckles, the punctuate staining pattern under microscope, which are known as typical localization pattern of RNA splicing factors, such as serine/arginine rich (SR) proteins[16], as evidenced by the co-localization of IκBL and a SR protein, SC35 (Figure 1). In addition, immunoprecipitation assay revealed that IκBL bound RNA[13]. These lines of evidence implied that IκBL might participate in the processing of RNA. Transcribed pre-mRNA undergoes post-transcriptional splicing, categorized into constitutive and alternative splicing. Depending on the *cis*-regulatory elements and splicing-related factors, splicing events discriminate introns from pre-mRNAs and combine exons to form mature RNA transcripts in the constitutive splicing. On the other hand, the alternative splicing is an important mechanism in the post-transcriptional control of gene function in eukaryotes, in which target exons in pre-mRNAs could be either excluded or included depending on specific cellular contexts.

To clarify the role of IκBL, we made an effort to investigate its function in the alternative splicing. Because abnormal alternative splicing in several immune-related genes was reported to link with autoimmune diseases including MS, SLE and T1D[17–19], mini-gene of *CD45*, *CD72* and *CTLA4* were designed and constructed to be tested for the alternative splicing in the context of IκBL function. It was found that knockdown of *IKBL* promoted the exon exclusion, whereas overexpression of *IKBL* counteracted the exon skipping[15]. On the other hand, IκBL affected the alternative splicing of Influenza A virus *M* gene[15]. These results for the first time demonstrated that IκBL played role as a regulator of alternative splicing in the immunity and infection (Figure 2).

Molecular mechanism of IκBL in the alternative splicing

We further asked the molecular mechanism of IκBL-mediated regulation of alternative splicing. By yeast two hybrid screening, IκBL was found to interact with CDC-like kinase 1 (CLK1), a well-known factor to regulate the alternative splicing by phosphorylating SR proteins[20–22]. The effects of CLK1 in the alternative splicing of immune-related genes were found to counteract IκBL, leading to a hypothesis that IκBL may interfere with the kinase activity of CLK1. However, IκBL did not affect the CLK1-induced phosphorylation of SR protein[15]. Furthermore, kinase activity of CLK1 was dispensable for the alternative splicing[15]. These results have suggested that IκBL and CLK1 regulate the alternative splicing by a novel mechanism distinct from the CLK1-dependent phosphorylation (Figure 2).

Our works contribute to understanding the function of IκBL. However, there are several topics to be discussed. First, CLK1, as the interacting partner of IκBL, may serve as a clue to investigate the mechanism of IκBL-mediated

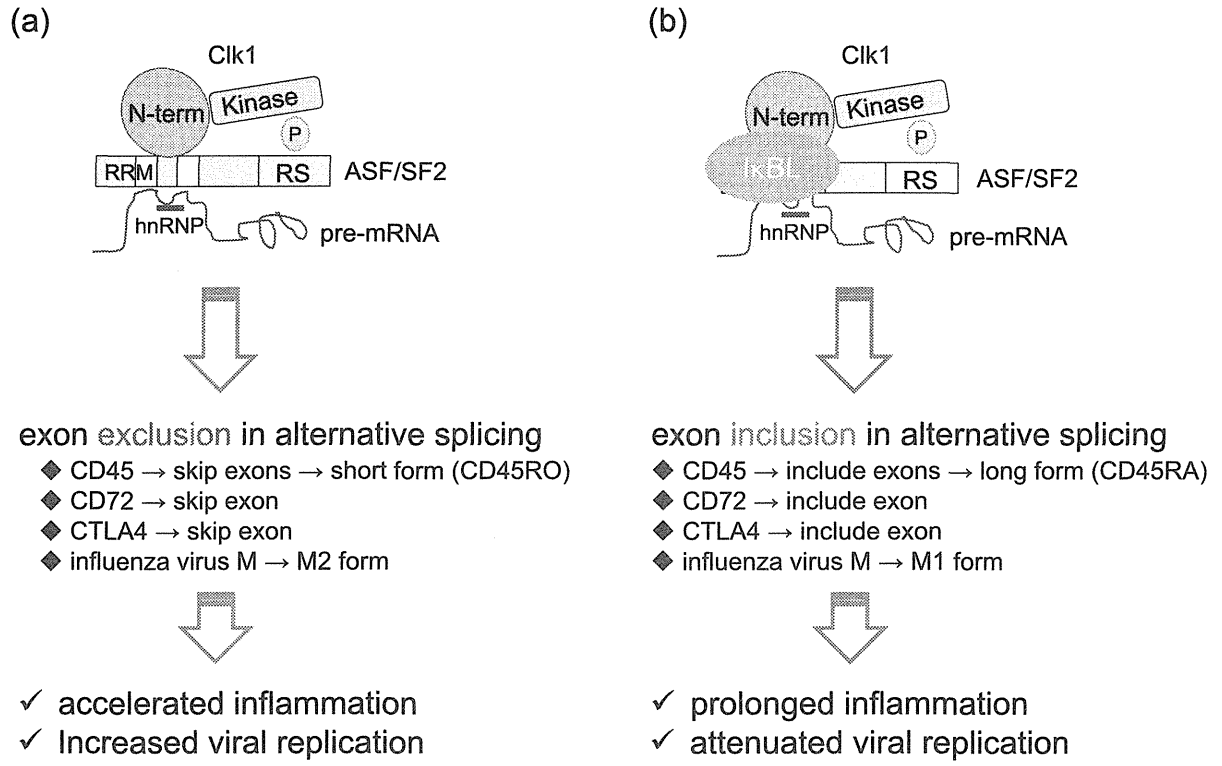

Figure 2 Involvement of IκBL in the Clk1-mediated alternative splicing

Clk1-mediated alternative splicing process is schematically represented. (a) In the absence of IκBL, pre-mRNA binds RRM domain of ASF/SF2 and undergoes splicing mediated by heterogeneous nuclear ribonucleoprotein (hnRNP) such as hnRNPL, hnRNPLL and FOX1. Clk1 usually enhances the splicing process by phosphorylating RS domain of ASF/SF2. In the Clk1-mediated phosphorylation process by its kinase domain, N-terminal domain of Clk1 binds RRM domain of ASF/SF2. The process may result in skipping exons of human immune-related genes including CD45, CD72 and CTLA4 as well as an influenza virus M gene, which might lead to accelerated inflammation and increased viral replication. It should be noted that the alternative splicing process of these genes could be found in the absence of kinase function. (b) In the presence of IκBL, Clk1-mediated alternative splicing is attenuated. IκBL binds both RRM domain of ASF/SF2 and N-terminal domain of Clk1. Clk1-mediated phosphorylation of RS domain of ASF/SF2 is not inhibited by IκBL. The attenuated splicing process may result in the inclusion of exons, leading to prolonged inflammation and attenuated viral replication.

alternative splicing. We found that the N-terminal regulatory domain of CLK1 played an important role in the alternative splicing[15], but no definite function was deciphered for the N-terminal domain of CLK1. Second, it is well known that phosphorylation of SR proteins has significant impacts on the RNA splicing[23]. Albeit that IκBL did not affect the phosphorylation of ASF/SF2, it should be considered that IκBL might affect the phosphorylation status of other splicing factors. In addition, SR proteins interacting with IκBL may not limit to ASF/SF2. Third, given that the regulation of alternative splicing by IκBL is independent from the kinase activity of CLK1, the exact mechanism for the involvement of IκBL in the alternative splicing remains elusive. IκBL was found to associate with the RNA recognition motifs (RRMs) of ASF/SF2 (Figure 2), implying that IκBL would interfere with the RNA binding of SR proteins. On the other hand, it was reported that RRM2 of ASF/SF2 mediated autoregulation in their expression[24]. The fact that IκBL associates with RRMs of ASF/SF2 l suggests that IκBL might control the expression of ASF/SF2 or other SR proteins. Fourth, a fundamental issue still remains to be uncovered; that is, how IκBL is induced and where it is expressed in the context of immune-related diseases. It was found that the expression of *IKBL* was relatively low in human tissues and organs, although the overexpression and knockdown assays demonstrated that altered expression of *IKBL* could affect the alternative splicing events. Indeed, the expression of *IKBL* was inhibited by activation stimuli with PMA to affect the alternative splicing in an established human T cell line[15]. It is worth to assess whether stimulations of primary immune cells would change the *IKBL* expression.

IκBL and diseases

We have demonstrated that IκBL might regulate the immune system via modulating alternative splicing of immune-related genes, which coincides with the notion that the disturbance of alternative splicing in immune-related genes would link with autoimmune diseases[17–19]. However, functional evidence for that the pathogenesis of immune-related diseases is attributable to the deregulation of alternative splicing is still lacking. Even though splice variants of *CD45*, *CD72* and *CTLA4* have been suggested to regulate the function of B and T cells[25–27], further studies illustrating the causal relationship between the alternative splicing and diseases are required. Furthermore, IκBL appears to control a large variety of alternative splicing, but the mechanisms controlling the gene-specificity are waiting to be identified, and a comprehensive analysis of target genes is particularly essential. For this purpose, next generation sequencing could be applied for exploring the RNAs regulated by IκBL in cells involved in the immune regulation, appended with the information of exact interacting sites or motifs. These results will not only propose the characteristics of IκBL-interacting RNAs, but also provide an overview to which extent IκBL is involved in the alternative splicing of immune-related genes.

In order to investigate the role of IκBL in the autoimmune and inflammatory diseases, *IKBL*-knockout (KO) mice will undoubtedly be required. On the other hand, it was reported that *IKBL*-transgenic (Tg) mice show resistance to collagen-induced arthritis, an experimental model for RA[28]. It is worth trying to apply *IKBL*-KO or -Tg mice into other models of immune-related diseases such as myelin-induced experimental autoimmune encephalomyelitis, a model of MS. Besides, examining the alternative splicing of target genes in *IKBL*-KO or -Tg mice will be valuable for establishing the link between the alternative splicing and immune-related diseases.

IKBL also regulates the alternative splicing of influenza A virus *M* gene[15]. Given that inhibition of the synthesis of M2 variant accounts for decreased virus titer[29], *IKBL* provides us with an insight into the host-dependent control of viral replication. It also suggests that IκBL, as well as splicing factors, would be useful to prevent viral infection by modulating alternative splicing of viral genes. Beside of influenza A virus *M* gene, genes of other virus are known to undergo alternative splicing in infected cells, such as *tat*, *rev* genes of human immunodeficiency virus (HIV)[30]. Whether IκBL affects expressivity of HIV genes and lead to an impact on virus replication will be an attractive issue for investigation.

Conclusion remark

Acknowledging to genetic association studies, *IKBL* was identified to be a candidate gene involved in the immune regulation. Albeit several issues remain to be clarified, recent studies have suggested that IκBL modulates the alternative splicing in both human and viral genes. These observations led to further understanding about the function of HLA region in the immune system and in the pathogenesis of immune-related diseases. In the future, as an excellent achievement of biomedical research, we expect IκBL as a potential target of therapeutic strategy in clinical treatments.

Acknowledgments

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IκBL mapped within the HLA region is a novel regulator of alternative splicing involved in the pathogenesis of immune-related diseases

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HLA 領域には免疫にかかわる多数の遺伝子が存在するが，抗原提示において重要な役割を果たす HLA クラス I およびクラス II 遺伝子群以外の遺伝子も免疫制御に関わると考えられる。なかでも，HLA クラス III 領域内にマップされる IKBL（NFKBIL1）遺伝子は，その多型自己免疫疾患や慢性炎症疾患などの疾患感受性と関連することが知られている遺伝子であるが，その機能は不明であった。最近我々は，IKBL がコードする IκBL タンパクがヒト免疫関連遺伝子やインフルエンザウイルス遺伝子の選択的スプライシングを制御することを明らかにしたが，この知見は HLA 領域による免疫と感染の制御する機構として新たな視点をもたらすものである。本総説では，IKBL 研究に関する最近の動向を紹介する。

キーワード：NFKBIL1，CLK1，選択的スプライシング，疾患感受性，自己免疫疾患，インフルエンザウイルス

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ORIGINAL PAPER

Divergence and diversity of *ULBP2* genes in rhesus and cynomolgus macaques

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Abstract Non-human primates such as rhesus macaque and cynomolgus macaque are important animals for medical research fields and they are classified as Old World monkey, in which genome structure is characterized by gene duplications. In the present study, we investigated polymorphisms in two genes for ULBP2 molecules that are ligands for NKG2D. A total of 15 and 11 *ULBP2.1* alleles and 11 and 10 *ULBP2.2* alleles were identified in rhesus macaques and cynomolgus macaques, respectively. Nucleotide sequences of exons for extra cellular domain were highly polymorphic and more than 70 % were non-synonymous variations in both *ULBP2.1* and *ULBP2.2*. In addition, phylogenetic analyses revealed that the *ULBP2.2* was diverged from a branch of *ULBP2.1* along with *ULBP2*s of higher primates. Moreover, when 3D structural models were constructed for the rhesus ULBP2 molecules, residues at presumed contact sites with NKG2D were polymorphic in *ULBP2.1* and *ULBP2.2* in the rhesus macaque and cynomolgus macaque, respectively. These observations suggest that amino acid replacements at the interaction sites with NKG2D might shape a specific nature of ULBP2 molecules in the Old World monkeys.

Keywords Rhesus macaque · Cynomolgus macaque · *ULBP2/RAET1H* · NKG2D · Polymorphisms

Introduction

Natural-killer group 2 member D (NKG2D), a C-type lectin molecule, is an activating receptor expressing on the surface of NK, $\gamma\delta^+$ and $CD8^+$ T cells, which plays an important role in the immune system (Wu et al., 1999; Raulet 2003). In humans, several MHC class I-like molecules are known as ligands for NKG2D, including MHC class I chain-related (MIC) and UL-16 binding protein (ULBP)/retinoic acid early transcript 1 (RAET1) (Bauer et al. 1999; Cosman et al. 2001; Chalupny et al. 2003; Bacon et al. 2004). These ligands are usually stress-inducible, and their recognition by NKG2D leads to the activation of NK cells, resulting in the killing of virus-infected cells and tumor cells (Pende et al. 2002; Eagle et al. 2006, Pappworth et al. 2007; Ward et al. 2007).

The human ULBP/RAET1 molecules are encoded by the *ULBP/RAET1* gene family located on the 6q24.2, which is composed of 10 members including six functional genes, *ULBP1*, *2*, *3*, *4*, *5*, and *6*, corresponding to *RAET1I*, *H*, *N*, *E*, *G*, and *L*, respectively (Radosavljevic et al. 2001; Chalupny et al. 2003; Eagle et al. 2009a, b; Eagle et al. 2009b). In addition, several sequence variations in each *ULBP* have been identified (Romphruk et al. 2009; Antoun et al. 2010). Although it is evident that the cell surface expression of the ligand molecules on target cells is differentially regulated (Eagle et al. 2006), genetic variations or polymorphisms in the coding region might also have a functional impact.

In the medical field, non-human primates including rhesus and cynomolgus macaques are used as animal models in the

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immunological studies for infectious diseases, autoimmune diseases, organ transplantation, and development of vaccines. These macaques are members of the Old World monkey and it has been reported that the genetic diversity in the rhesus macaque is quite unique, i.e., more than 60 % of the rhesus macaque-specific expansions are found in the protein coding sequences (Gibbs et al. 2007). To fully evaluate the results of immunological experiments using macaque models, it is essential to characterize the genetic diversity of immune-related molecules, which may shape the basis of individual differences in the immune response against foreign antigens and/or pathogens. It has been reported that the copy numbers of genes in the major histocompatibility complex (MHC) loci in the Old World monkey are higher than those in humans (Kulski et al. 2004; Gibbs et al. 2007; Otting et al. 2007). In addition, the extent of genetic diversity in MHC differed, in part, depending on the geographic area, and we have reported that the diversity of MHC class I genes in the rhesus and cynomolgus macaques is considerably different depending on habitat (Naruse et al. 2010, Saito et al. 2012). In our previous study, we have demonstrated that *ULBP4* is more polymorphic in the Old World monkey than in humans (Naruse et al. 2011). It also was revealed that each member of the *ULBP/RAET1* gene family, except for *ULBP6*, had been duplicated in the rhesus genome (Naruse et al. 2011).

Recent reports have indicated that the expression of *ULBP2* is upregulated in HIV infection (Richard et al. 2013, Matusali et al. 2013). Because the innate immune system may be involved in the response to environmental pathogens, it is important to investigate the polymorphisms in the ligands of NK receptors in the experimental animal models for developing HIV vaccine. Here, we report the *ULBP2* polymorphisms focusing on the divergence and diversity in the Old World monkey.

Materials and methods

Animals

A total of 37 rhesus macaques and 24 cynomolgus macaques, previously analyzed for the polymorphisms in MHC class I genes (Naruse et al. 2010, Saito et al. 2012) were the subjects. They were maintained in the breeding colonies in Japan. The founders of the rhesus macaque colonies were captured in Myanmar and Laos, whereas the founders of cynomolgus macaque colonies were captured in Indonesia, Malaysia, and the Philippines. All care including blood sampling of animals were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised 1985) and the study protocol was subjected to prior approval by the local animal protection authority.

DNA extraction and sequencing analysis

Genomic DNAs of B lymphoblastoid cell lines from rhesus macaques and whole blood samples of cynomolgus macaques were prepared, as previously reported (Naruse et al. 2010, Saito et al. 2012). Amplification of *ULBP2* from macaques was done by polymerase chain reaction (PCR) with specific primer pairs designed for the region spanning from intron 1 to intron 3 of rhesus *ULBP2*, LOC694466 (designated as *ULBP2.1*) and LOC694600 (designated as *ULBP2.2*), using FastStart Taq DNA polymerase (Roche, Mannheim, Germany). Primer sequences are as follows: UL2.1NF (5′-AGGGGCTAACTAGGGGTCTTTC) and UL2.1NR (5′ ACCGTTTCTGATCTCATTCCA) for *ULBP2.1*, and UL2.2NF (5′-GAGGGCTAACTAGGGGTCTCT) and UL2.2NR (5′-ACCATTTCTGATCTCATTCCAGA) for *ULBP2.2*. The PCR program was composed of following steps: denaturation at 95 °C for 4 min; 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s; and additional extension at 72 °C for 7 min. The PCR products, about 1,400 bp for *ULBP2.1* and about 1,080 bp for *ULBP2.2*, were cloned into pSTBlue-1 AccepTer vector (Novagen, WI, USA) according to the manufacturer's instructions and transformed into Nova Blue Single™ competent cells (Merck Biosciences Japan, Tokyo, Japan). Ten to 20 independent transformed colonies were picked up for each sample and subjected to sequencing on both strands by using a BigDye Terminator cycling system and an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analysis

Nucleotide sequences from cloned DNAs were aligned using the Genetyx software package (version 8.0, Genetyx Corp., Japan). When at least three clones from independent PCR or from different subjects showed identical sequences, the sequences were submitted to the DNA Data Bank of Japan (DDBJ). A neighbor-joining tree was constructed by Kimura's two-parameter method for a phylogenetic analysis of *ULBP2* sequences from exon 2 to exon 3, excluding intron 2 sequences, by using the Genetyx software. Bootstrap values were based on 5,000 replications. The *ULBP2* and *ULBP6* sequences from human (GenBank accession numbers AL583835 and AL355497, respectively), and *ULBP2* sequences from chimpanzee (NC006473), western gorilla (NC018430), rhesus (NC007861), and another member of Old World monkey, olive baboon (NC018155) were included in the phylogenetic analysis. The *ULBP1* (LOC694341), *ULBP3* (LOC694525), *ULBP4* (LOC695031), and *ULBP5* (LOC694265) sequences from rhesus macaque, and *ULBP1* (NM025218), *ULBP3* (AL355497), *ULBP4* (AL355312), and *ULBP5* (AL583835) sequences from human were also included in the analysis.

Table 1 Alleles of *ULBP2.1* and *ULBP2.2* in rhesus and cynomolgus macaques

Gene	Species	Allele name	Accession no	ID of reference animal	Clone name
ULBP2.1	*Macaca mulatta*	*Mamu-ULBP2.1*1*	NC007861[a]	Not found in the subjects of this study	
		*Mamu-ULBP2.1*2*	AB826205	R491	UL2.1NR491F-9
		*Mamu-ULBP2.1*3*	AB826206	R312, R314, R496	UL2.1NR314F-2
		*Mamu-ULBP2.1*4*	AB826207	R277, R316, R350, R396, R429, R434, R437, R455, R465, R473, R492, R495	UL2-1R227-13 F
		*Mamu-ULBP2.1*5*	AB826208	R325, R333, R337, R384, R434, R491	UL2-1R434-2 F
		*Mamu-ULBP2.1*6*	AB826209	R350	UL2.1NR350F-13
		*Mamu-ULBP2.1*7*	AB826210	R227, R234, R283, R314, R320, R321, R328, R337, R346, R384,R396, R446, R455, R465, R490, R496	UL2-1R227-7 F
		*Mamu-ULBP2.1*8*	AB826211	R495	UL2.1NR495F-8
		*Mamu-ULBP2.1*9*	AB826212	R321, R333, R360	UL2.1NR321F-8
		*Mamu-ULBP2.1*10*	AB826213	R316, R342, R408	UL2-1R408-12 F
		*Mamu-ULBP2.1*11*	AB826214	R346	UL2.1NR346F-20
		*Mamu-ULBP2.1*12*	AB826215	R342	UL2.1NR342F-14
		*Mamu-ULBP2.1*13*	AB826216	R325, R346, R360, R361, R379, R408, R429, R430, R437, R439, R446, R473, R490	UL2.1NR439F-11
		*Mamu-ULBP2.1*14*	AB826217	R453	UL2-1R453-1 F
		*Mamu-ULBP2.1*15*	AB826204	R234, R312, R361	UL2.1NR234F-7
	Macaca fascicularis	*Mafa-ULBP2.1*1*	NC007861[a]	M04, C09	2.1-2UL2-1 M04-5 F
		*Mafa-ULBP2.1*2*	AB826219	M05, C10, C11	UL2.1NFM05-12
		*Mafa-ULBP2.1*3*	AB826220	M03, C07	UL2.1NFM03-8
		*Mafa-ULBP2.1*4*	AB826221	P01, P02, P03, M01, C01, C03, C04, C05, C07, C08	2.1-1UL2-1 M01-10 F
		*Mafa-ULBP2.1*5*	AB826222	P02, C06	UL2-1P02-2 F
		*Mafa-ULBP2.1*6*	AB826223	M02, C05	2.1-6UL2-1 M02-17 F
		*Mafa-ULBP2.1*7*	AB826224	M03, M04, C06, C08, C09	UL2-1 M03-1 F
		*Mafa-ULBP2.1*8*	AB826225	P04, P05, M01, M05, M06, C02, C12, C13	2.1-3UL2-1 M01-12 F
		*Mafa-ULBP2.1*9*	AB826226	P04, M06, C10, C11, C12, C13	2.1-4UL2-1 M06-10 F
		*Mafa-ULBP2.1*10*	AB826228	M02, C04	UL2-1 M02-20 F
		*Mafa-ULBP2.1*11*	AB826218	P01, C02	UL2NP01-F-2
ULBP2.2	*Macaca mulatta*	*Mamu-ULBP2.2*1*	NC007861[b]	R283, R316, R320, R321, R325, R328, R333, R337, R342, R346, R360, R379, R384, R396, R408, R429, R430, R437, R439, R446, R453, R473, R490, R495	UL2-2R396-3 F
		*Mamu-ULBP2.2*2*	AB827340	R491	UL2.2NR491F-5
		*Mamu-ULBP2.2*3*	AB827341	R314, R321	UL2-2R314-7 F
		*Mamu-ULBP2.2*4*	AB827342	R350	UL2.2NR350F-3
		*Mamu-ULBP2.2*5*	AB827343	R234, R320	UL2-2R361-8 F
		*Mamu-ULBP2.2*6*	AB827344	R325, R333, R337, R384, R491, R492	UL2-2R325-12 F
		*Mamu-ULBP2.2*7*	AB827345	R237, R312, R453	UL2-2R237-5 F
		*Mamu-ULBP2.2*8*	AB827346	R228, R314, R396, R492, R495	UL2-2R383-3 F
		*Mamu-ULBP2.2*9*	AB827347	R496	UL2-2R496-12 F
		*Mamu-ULBP2.2*10*	AB827339	R234, R312, R328, R439,R446, R490, R496	R234UL2.2NF-16
		*Mamu-ULBP2.2*11*	AB827348	R367, R430	UL2-2R367-12 F

Table 1 (continued)

Gene	Species	Allele name	Accession no	ID of reference animal	Clone name
	Macaca fascicularis	*Mafa-ULBP2.2*1*	NC007861[b]	M05, C10, C11	UL2-2FM05-2
		*Mafa-ULBP2.2*2*	AB827350	M03, M04, C06, C08	UL2-2FM03-1
		*Mafa-ULBP2.2*3*	AB827351	P03, C08	P03UL2-2-6 F
		*Mafa-ULBP2.2*4*	AB827352	P01, C03, C04, C05	UL2-2P01-1 F
		*Mafa-ULBP2.2*5*	AB827353	P01,P03, P04, M01, M04, M06, C01, C02, C09, C11, C13	UL2-2P01-7 F
		*Mafa-ULBP2.2*6*	AB827354	P04, P05, M05, M06, C12, C13	UL2-2P04-19 F
		*Mafa-ULBP2.2*7*	AB827355	M02, C05,	UL2-2FM02-2
		*Mafa-ULBP2.2*8*	AB827356	M03, C07	UL2-2FM03-11
		*Mafa-ULBP2.2*9*	AB828102	P02, M01, C01, C03, C06, C07	UL2-2FM01-1
		*Mafa-ULBP2.2*10*	AB827349	M02, C04	UL2-2FM02-11

[a] Identical to LOC694466

[b] Identical to LOC694600

Structure model analysis

Three-dimensional (3D) structure models of ULBP2 molecules were created for amino acid positions from 1 to191, by using a molecular visualization software RasTop2.2 (http://sourceforge.net/projects/rastop/), by referring the human ULPB3 molecule in complex with NKG2D (Radaev et al. 2001) from the Molecular Modeling Database (MMCB No.18231). Polymorphic sites were mapped on the 3D structure models by using the Cn3D 4.1 program (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

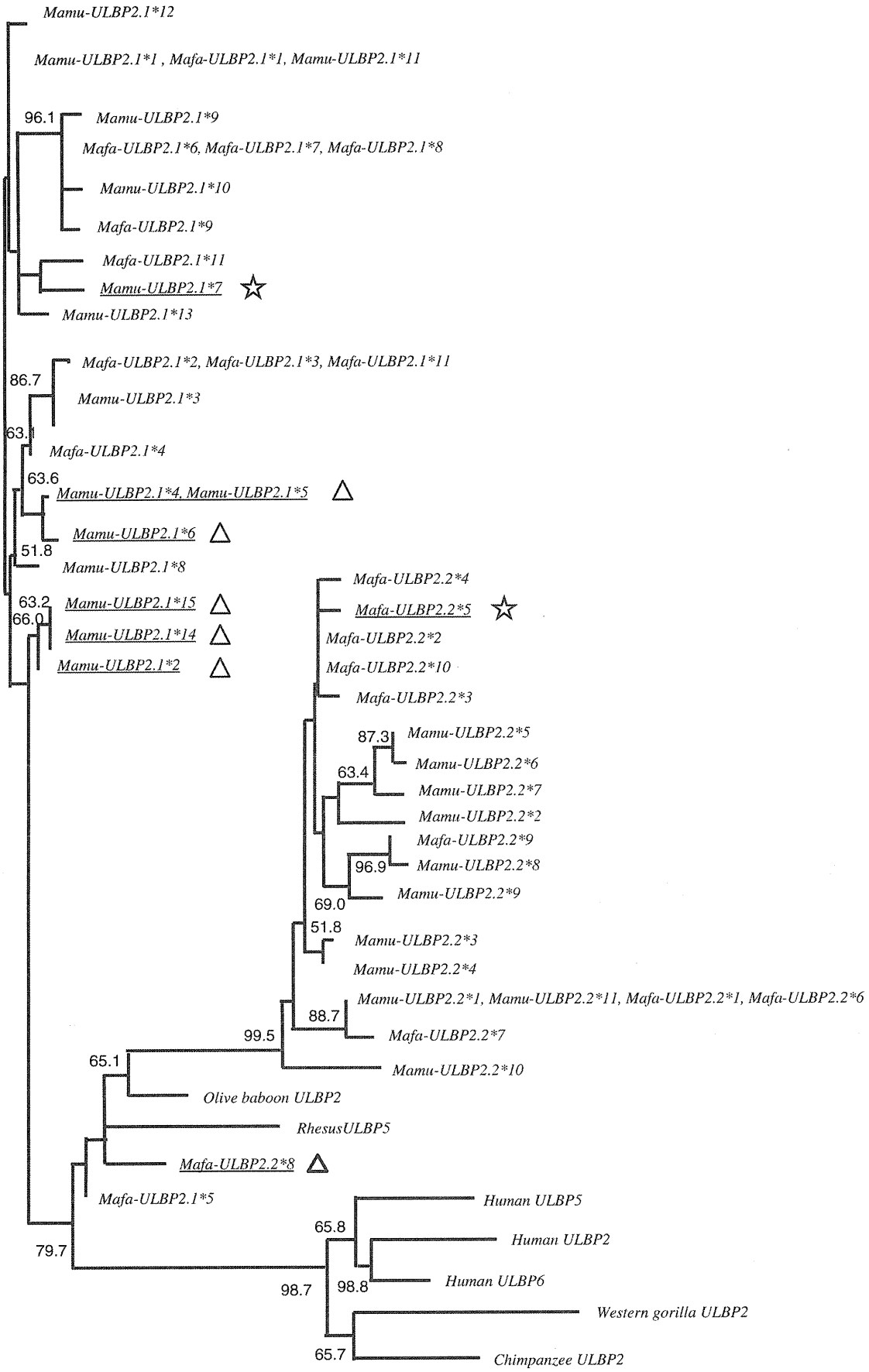
Results

Identification of alleles for a ULBP2 gene, *ULBP2.1*

There are two orthologous genes for *ULBP2*, LOC694466 and LOC694600, in the rhesus macaque genome. In the present study, we designated LOC694466 and LOC694600 as *ULBP2.1* and *ULBP 2.2*, respectively, and we designed primer pairs to separately amplify the *ULBP2.1* and *ULBP 2.2*. As expected, PCR products from each gene could be obtained and distinguished by their lengths, although minor length differences due to single nucleotide repeat number polymorphisms in an A stretch were found in the intron 2 sequences.

We obtained nucleotide sequences for the region from exon 2 to exon 3 of *ULBP2.1* from 37 rhesus macaques and 24 cynomolgus macaques by sequencing the cloned PCR products of 1,370–1,395 bp. The *ULBP2.1* sequences from the rhesus macaques were classified into 15 different alleles (Table 1), designated as *Mamu-ULBP2.1*1* to *-ULBP2.1*15*. The LOC4964466 sequences were given with the allele name of *Mamu-ULBP2.1*1*, although it was not found in the analyzed subjects of current study. In the cynomolgus macaques, 11 different alleles, *Mafa-ULBP2.1*01* to *-ULBP2.1*11*, were identified (Table 1). The nucleotide sequences of *Mafa-ULBP2.1*1* were identical to those of *Mamu-ULBP2.1*1* reported for rhesus macaque LOC694466.

Fig. 1 Phylogenetic tree of *ULBP2.1* and *ULBP2.2* alleles and related *ULBP2*. The tree was constructed using neighbor-joining method with bootstrap values of 5,000 replications. The values are indicated as percentages and those values less than 50 % are not shown. The sequences of human *ULBP2* (AY026825), human *ULBP5* (AL583835), human *ULBP6* (AL355497), rhesus *ULBP5* (LOC694265), chimpanzee *ULBP2* (NC006473), western gorilla *ULBP2* (NC018430), and olive baboon *ULBP2* (NC018155) were included in the analysis. The *underlined alleles* indicated with *triangles* and *stars* carried polymorphisms on the α helix structure and contact sites with NKG2D, respectively


Mamu-ULBP2.1*12
Mamu-ULBP2.1*1 , Mafa-ULBP2.1*1, Mamu-ULBP2.1*11
96.1
Mamu-ULBP2.1*9
Mafa-ULBP2.1*6, Mafa-ULBP2.1*7, Mafa-ULBP2.1*8
Mamu-ULBP2.1*10
Mafa-ULBP2.1*9
Mafa-ULBP2.1*11
Mamu-ULBP2.1*7
Mamu-ULBP2.1*13
Mafa-ULBP2.1*2, Mafa-ULBP2.1*3, Mafa-ULBP2.1*11
86.7
Mamu-ULBP2.1*3
63.1
Mafa-ULBP2.1*4
63.6
Mamu-ULBP2.1*4, Mamu-ULBP2.1*5
Mamu-ULBP2.1*6
51.8
Mamu-ULBP2.1*8
63.2
Mamu-ULBP2.1*15
66.0
Mamu-ULBP2.1*14
Mamu-ULBP2.1*2
Mafa-ULBP2.2*4
Mafa-ULBP2.2*5
Mafa-ULBP2.2*2
Mafa-ULBP2.2*10
Mafa-ULBP2.2*3
87.3
Mamu-ULBP2.2*5
Mamu-ULBP2.2*6
63.4
Mamu-ULBP2.2*7
Mamu-ULBP2.2*2
Mafa-ULBP2.2*9
96.9
Mamu-ULBP2.2*8
Mamu-ULBP2.2*9
69.0
51.8
Mamu-ULBP2.2*3
Mamu-ULBP2.2*4
Mamu-ULBP2.2*1, Mamu-ULBP2.2*11, Mafa-ULBP2.2*1, Mafa-ULBP2.2*6
88.7
Mafa-ULBP2.2*7
99.5
65.1
Mamu-ULBP2.2*10
Olive baboon ULBP2
RhesusULBP5
Mafa-ULBP2.2*8
Mafa-ULBP2.1*5
Human ULBP5
65.8
Human ULBP2
79.7
Human ULBP6
98.7
98.8
Western gorilla ULBP2
65.7
Chimpanzee ULBP2