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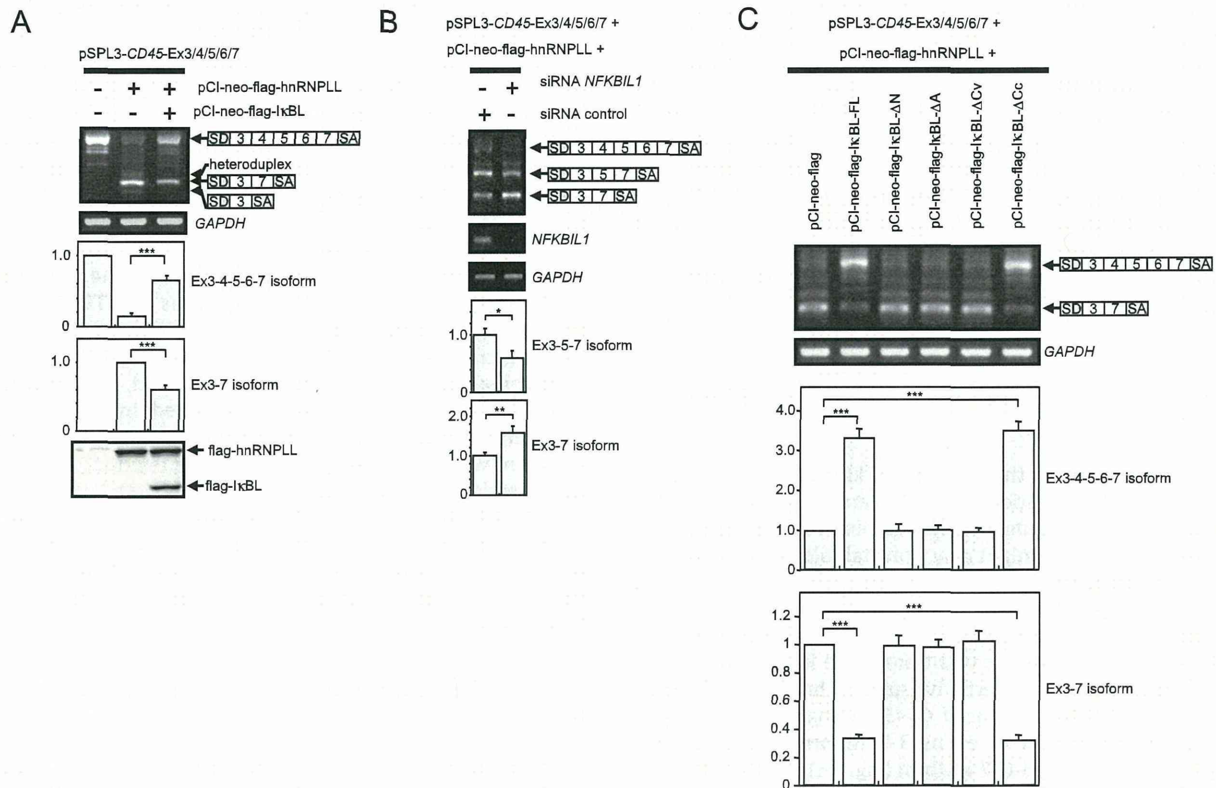

Fig. 2. Suppression of exon exclusion in the alternative splicing of *CD45* mini-gene by IκBL. (A) COS7 cells were transfected with *CD45* mini-gene and flag-hnRNPLL. RT-PCR analysis showed the effect of hnRNPLL on the alternative splicing of *CD45* transcripts derived from the mini-gene. COS7 cells 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

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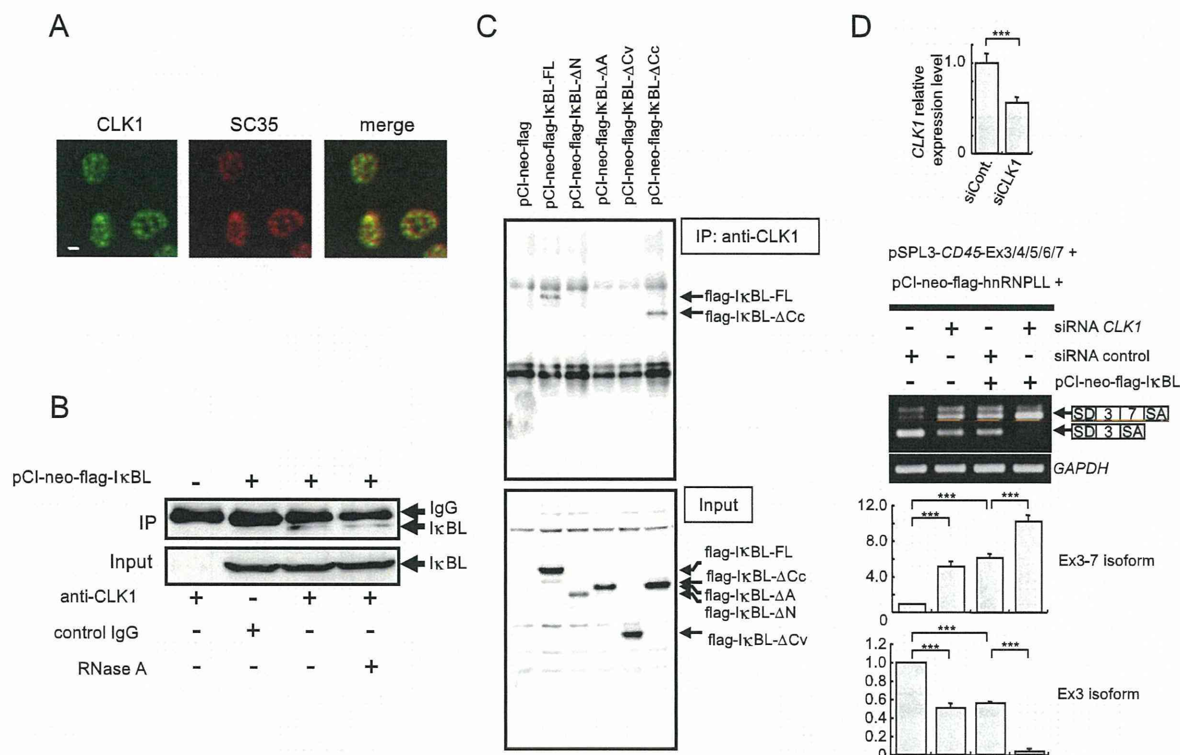

Fig. 3. Interaction of IκBL with CLK1. (A) Subcellular localization of endogenous CLK1 (FITC-labeled, green) and endogenous SC35 (Alexa Fluor 568-labeled, red) were visualized by immunofluorescent staining in HeLa cells. Bar indicates 5 μm. (B) COS7 cells were transfected with or without flag-tagged 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

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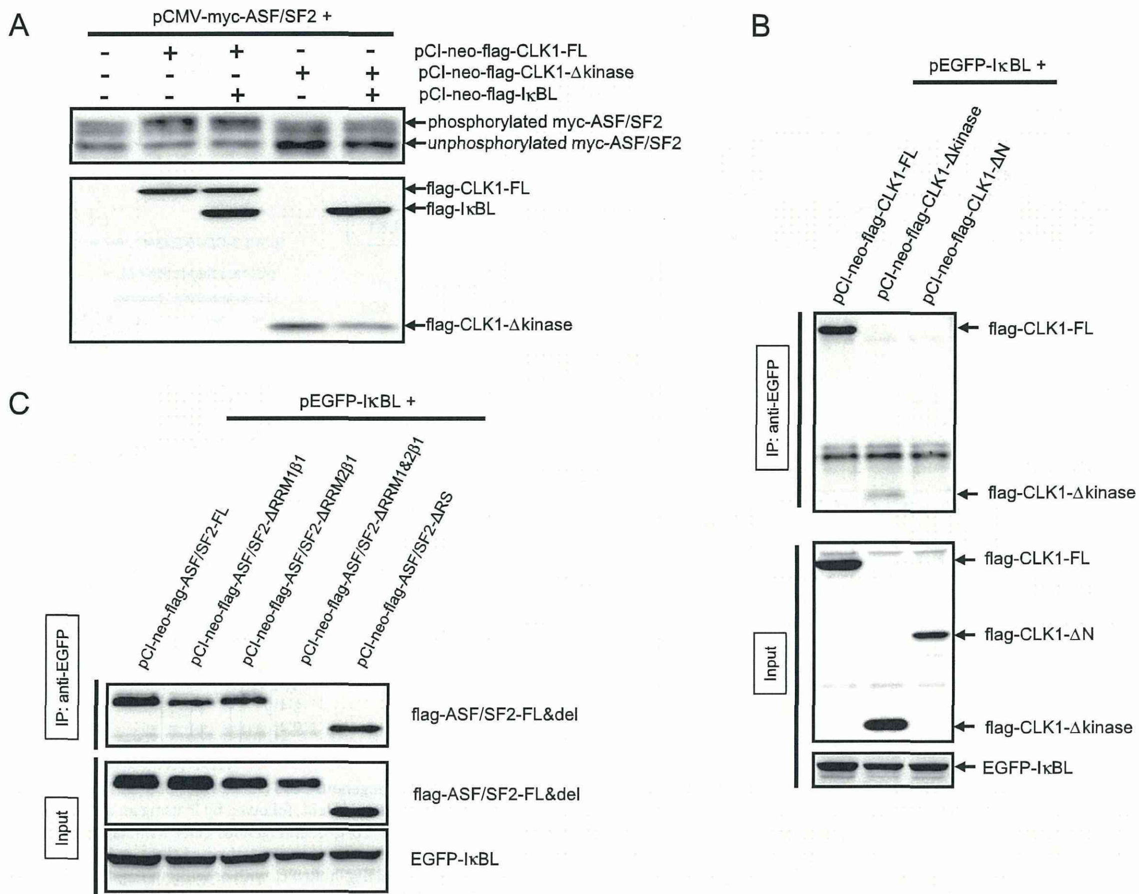

Fig. 4. IκBL did not interfere with CLK1-dependent phosphorylation. (A) COS7 cells were transfected with the constructs of myc-ASF/SF2 along with flag-CLK1-FL or -Δkinase. Immunoblotting showed unphosphorylated and phosphorylated ASF/SF2. In addition, COS7 cells were transfected with flag-IκBL. There 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

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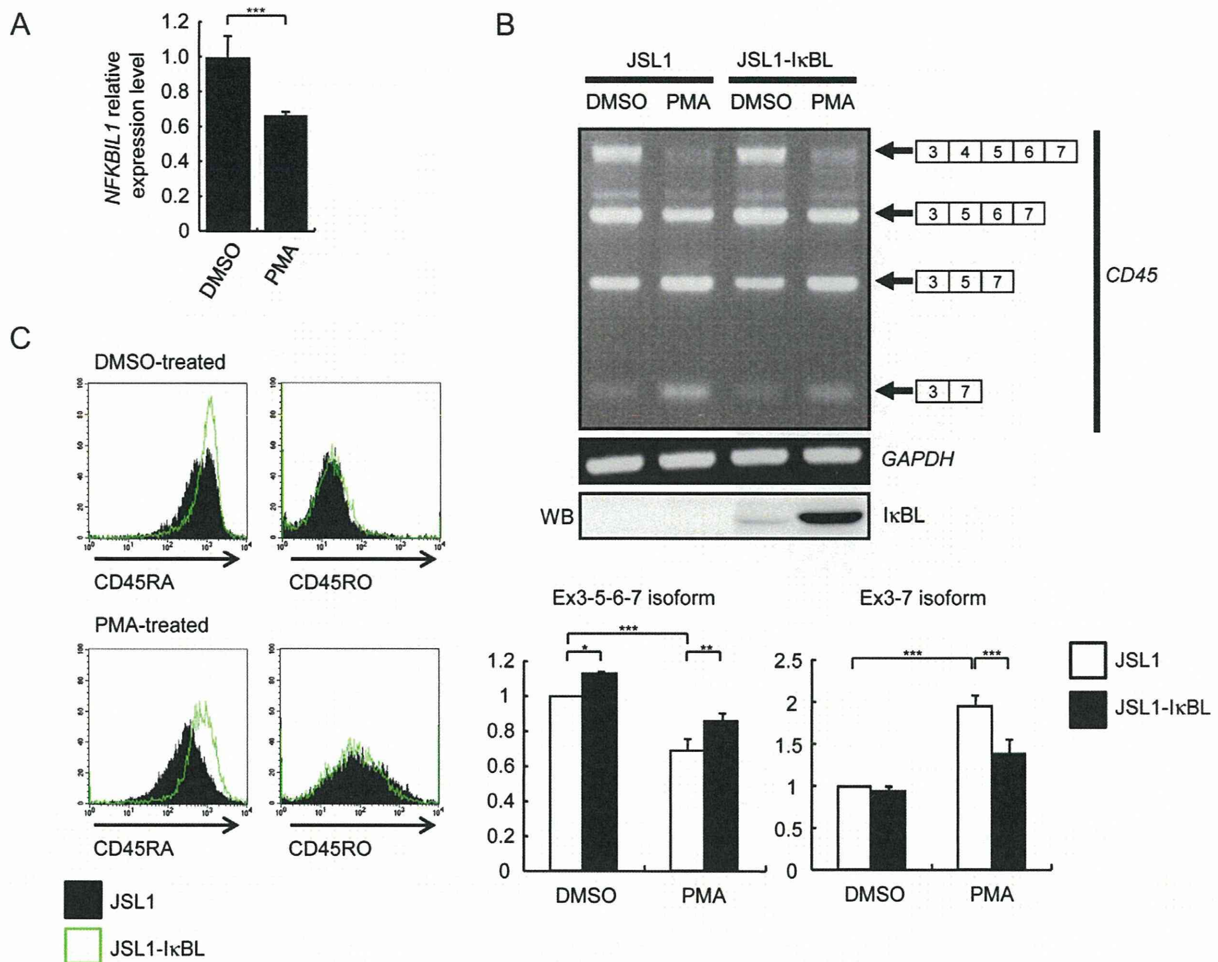

Fig. 5. Role of IκBL in the alternative splicing of endogenous *CD45* in JSL1. (A) Expression of endogenous *NFKBIL1* in JSL1 treated with DMSO (control) or PMA (10 ng/ml, 2 days) was analyzed by using real-time RT-PCR. *NFKBIL1* mRNA level was normalized to 18S rRNA. Data are shown as means ± SD of 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*,

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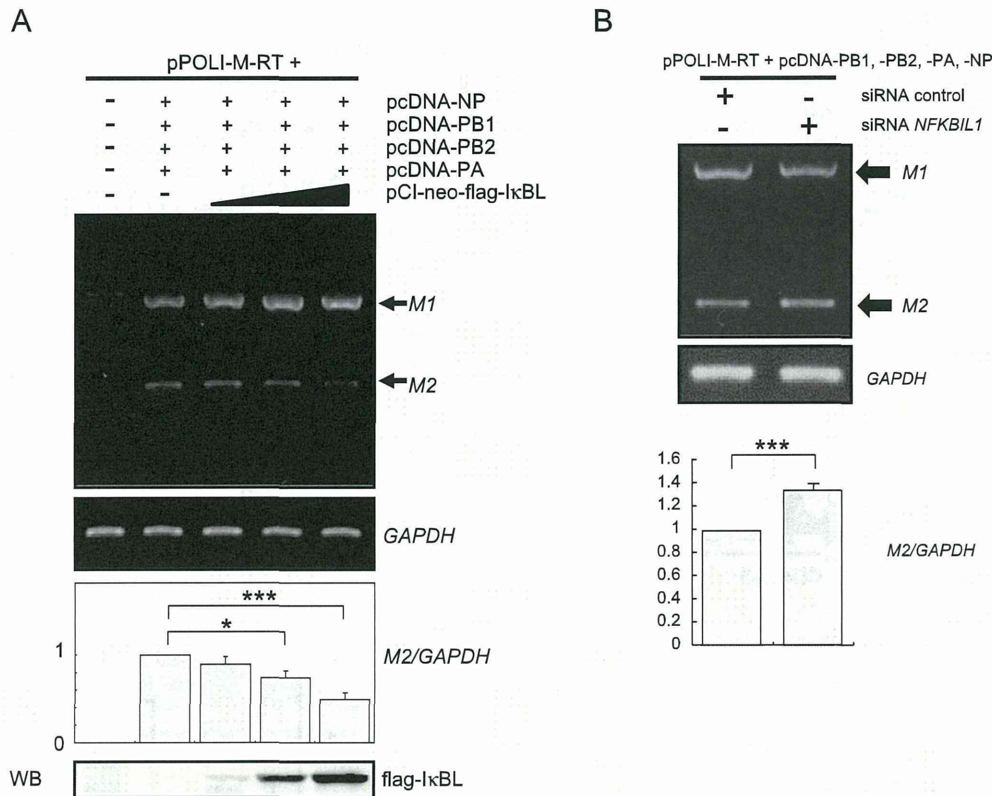

Fig. 6. Regulation of alternative splicing in influenza A virus *M* gene by IκBL. (A) Influenza A virus *M* gene plasmid was co-transfected with plasmids encoding viral RNA polymerase complex factors, i.e. PB1, PB2, PA and NP along with different dose of flag-IκBL into COS7 cells. Expression of flag-IκBL 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.

Acknowledgments

We thank Dr. Kristen W. Lynch (Perelman School of Medicine, University of Pennsylvania) for giving us the JSL1 T cells. We also thank Dr. George G. Brownlee and Dr. Ervin Fodor (Sir William Dunn School of Pathology, University of Oxford) for providing us with the constructs used for plasmid-based rescue system for the influenza A virus. This work was supported by Grants-in-Aids for Scientific Research from the Japan Society for the Promotion of Science and research grants from the Ministry of Health, Labour and Welfare, Japan.

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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Major Histocompatibility Complex 2013; 20 (3): 191–197

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

Received: November 6, 2013, Accepted: November 13, 2013
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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)[1], 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.

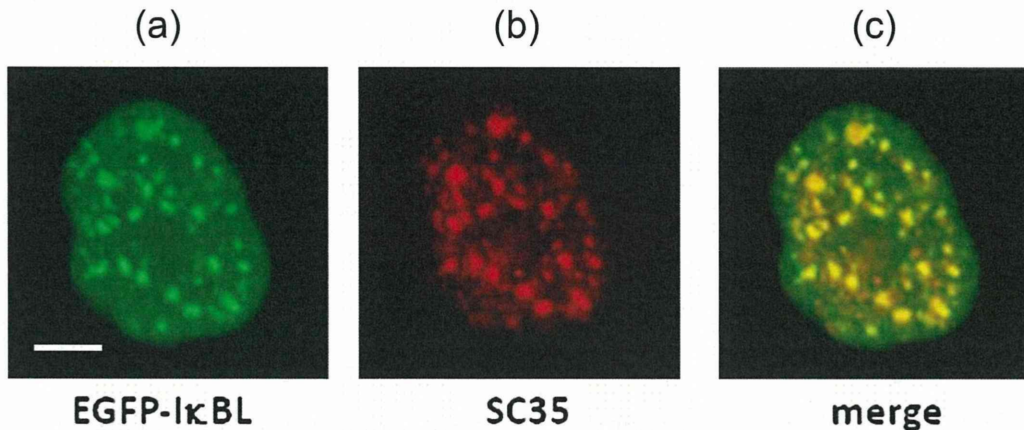

Figure 1 Subcellular localization of IκBL

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

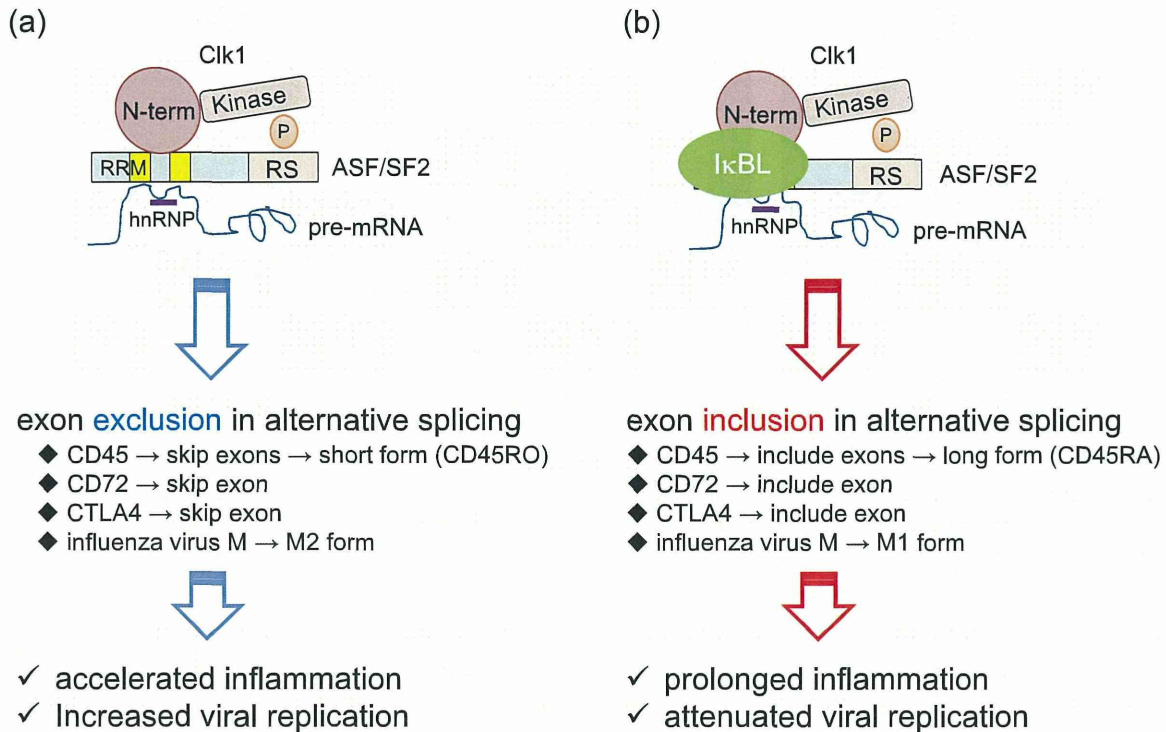

Figure 2 Involvement of IκBL in the Clk1-mediated alternative splicing

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 1 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.