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Signal-peptide-peptidase-like 2a is required for CD74 intramembrane proteolysis in human B cells

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A B S T R A C T

The invariant chain (CD74) mediates targeting of the MHCII complex to endosomal compartments, where CD74 undergoes degradation allowing MHCII to acquire peptides. We demonstrated recently that intramembrane proteolysis of the final membrane-bound N-terminal fragment (NTF) of CD74 is catalyzed by Signal-peptide-peptidase-like 2a (SPPL2a) and that this process is indispensable for development and function of B lymphocytes in mice. In *SPPL2a*$^{-/-}$ mice, homeostasis of these cells is disturbed by the accumulation of the unprocessed CD74 NTF. So far, evidence for this essential role of SPPL2a is restricted to mice. Nevertheless, inhibition of SPPL2a has been suggested as novel approach to target B cells for treating autoimmunity. Here, we characterize human B cell lines with a homozygous microdeletion on chromosome 15. We demonstrate that this deletion disrupts the *SPPL2a* genomic locus and leads to loss of *SPPL2a* transcript. Lymphoblastoid cell lines from patients with this deletion exhibit absence of SPPL2a at the protein level and show an accumulation of the CD74 NTF comparable to B cells from *SPPL2a*$^{-/-}$ mice. By this means, we present evidence that the role of SPPL2a in CD74 proteolysis is conserved in human B cells and provide support for modulation of SPPL2a activity as a therapeutic concept.

1. Introduction

The intramembrane protease Signal-peptide-peptidase-like 2a (SPPL2a) resides in lysosomes and late endosomes [1] and has been implicated in the processing of type 2 transmembrane proteins [2] including TNFα [3,4], the Fas ligand [5], the Bri2 protein [6] and the invariant chain (CD74) of the MHCII complex [7]. Among these, only the latter has been confirmed *in vivo*. We recently demonstrated that the intramembrane cleavage of CD74 by SPPL2a is an essential process for development and functionality of B lymphocytes in mice [7]. This was based on a distinct phenotype of *SPPL2a*$^{-/-}$ mice that is characterized by a developmental arrest of B cells at the transitional stage 1 (T1) which was recovered to a significant degree by additional ablation of CD74 in SPPL2a-CD74 double-deficient mice [7]. This clearly identified the N-terminal fragment (NTF) of CD74, which accumulates in the absence of SPPL2a, as the causative element of this B cell phenotype. Key results from this work were independently confirmed by two other laboratories [8,9].

Based on these studies, SPPL2a was suggested to represent a putative therapeutic target. Apart from an impairment of tooth enamel generation [10] in addition to the described B cell phenotype, absence of SPPL2a appeared to be well tolerated in mice. Thus, pharmacological inhibition of SPPL2a may represent a novel small-molecule based approach to deplete and/or modulate B cells. This concept would require that the described conclusions on the importance of SPPL2a for proteolysis of CD74 and homeostasis of B cells are also valid in humans. However, all experimental data available to date are derived from mice.

Abbreviations: CMA, chromosomal microarray analysis; ICD, intracellular domain; MHCII, MHC class II complex; NTF, N-terminal fragment; RIP, regulated intramembrane proteolysis; SPP, Signal peptide peptidase; SPPL, Signal-peptide-peptidase-like.

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Here, we provide initial data on the role of SPPL2a in human B cells. We made use of cell lines derived from two siblings with a 192 kb homozygous deletion on chromosome 15q21.2 [11]. Chromosomal microarray (CMA) analysis indicated that the deletion included the exon 1 of the *SPPL2a* genomic locus. We show here that this homozygous 15q21.2 deletion disrupts SPPL2a expression. Using lymphoblastoid cell lines derived from these patients, we demonstrate that SPPL2a-deficiency leads to a massive accumulation of CD74 NTF in lysosomal/late endosomal compartments thus confirming that the requirement of SPPL2a for CD74 intramembrane proteolysis is conserved in humans.

2. Materials and methods

2.1. Cell culture

Primary skin fibroblasts and peripheral blood mononuclear cells were obtained from the two siblings with homozygous deletions and their family members, who either carried a heterozygous deletion or did not have the deletion [11], after obtaining written informed consent and approval by the institutional review board. Epstein–Barr virus (EBV) transformation of primary lymphocytes was performed according to standard procedures. Fibroblasts were maintained in DMEM (PAA) with L-glutamine supplemented with 10% (v/v) FBS (PAA), 100 units/ml penicillin (PAA) and 100 µg/ml streptomycin (PAA). The human B cell line Raji and the lymphoblastoid cell lines were grown in RPMI-1640 with L-glutamine (PAA) containing 10% (v/v) FBS and penicillin/streptomycin as well as 50 µM β-mercaptoethanol (Gibco) and 1 mM sodium pyruvate (Sigma–Aldrich). All cell lines were cultured at 37 °C in a humidified 5% CO_2/95% air atmosphere. Inhibitor treatment of Raji cells with $(Z\text{-}LL)_2$-ketone (Pepta Nova) or inhibitor X (Tocris) was performed for 16 h at final concentrations of 10 µM and 1 µM, respectively.

2.2. PCR

Lysates were prepared from harvested cells using the DirectPCR Lysis reagent (Peqlab) and used for PCR amplification with Dream Taq Polymerase (Fermentas). The following primer pairs were utilized: Exon 1: Fw, 5′-CGAGTGAGCTGCGCCGCAC-3′; Rv, 5′-GGAAAGAGGAGTGCGAGAGCAG-3′; Exon 2: Fw, 5′-GGCAAGCCATAACATTGCAAGT-3′; Rv, 5′-CCAGCCTCCTTCTTCACTA-3′; Exon 6: Fw, 5′-AGTTCCTGTGTGTTTGTACAG-3′; Rv, 5′-AATATGACTTCTTACCCAACC-3′; Exon 11: Fw, 5′-CACACTTGAATAGTGGCAGAT-3′; Rv, 5′-CAGTGAATTACACAGGAAGGC-3′; Exon 15: Fw, 5′-AGTGCTCCAGAAGGAGTGCTCA-3′; Rv, 5′-TGTAACTGTCAGTACCAGCTC-3′.

2.3. RT-PCR

Total RNA was isolated with the NucleoSpin® RNA II kit (Macherey–Nagel). Reverse transcription was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) and random hexamer primers. For PCR amplification with Dream Taq Polymerase (Fermentas), the following oligonucleotides (marked in Fig. 2B) were employed: Exon-1-Fw: 5′-TACTCTGGGGCTTCCTGCTCCA-3′, Exon-2-Fw: 5′-GGAAGCAATCTTGCATGCGTC-3′, Exon-9/10-Fw: 5′-AGTTGCCCAACTTCAAGTCATG-3′, Exon-4/5-Rv: 5′-CTCCTAGAGTCTGGTTCATATC-3′, Exon-14/15-Rv: 5′-CCAAATGGTCCATCATCTGATAG-3′. In parallel, a fragment of β-actin (fw: 5′-CTGGGACGACATGGAGAAAA-3′, rv: 5′-AAGGAAGGCTGGAAGAGTGC-3′) was amplified as a control.

2.4. Western blotting

Cells were harvested and total lysates prepared in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1.0% (v/v) Triton X-100, 0.1% (w/v) SDS and 4 mM EDTA supplemented with protease inhibitors as described before [16]. For isolation of total cellular membranes, cells were mechanically disrupted in 250 mM sucrose, 10 mM Hepes–NaOH, pH 7.4 and 1 mM EDTA by 5 passages through a 25G cannula and a post-nuclear supernatant recovered after centrifugation for 10 min at 1000×*g*. Membranes were sedimented for 1 h at 100,000×*g* and, after resuspension, incubated in the presence of 100 mM $NaCO_3$, pH 11.5, for 1 h on ice in order to enrich integral membrane proteins. After re-sedimentation (1 h, 100,000×*g*) and one step of washing in 10 mM Hepes–NaOH, pH 7.4, the membrane suspension was used for further analyses. Protein concentrations were determined with a BCA protein assay (bicinchoninic acid, Thermo Scientific). SDS–PAGE and semi-dry transfer to nitrocellulose were conducted as described [16,17]. To allow detection of human SPPL2a, a polyclonal antiserum against a C-terminal epitope of this protein (ATNEENPVISGEQIVQQ, residues 504–520) was raised in rabbits and affinity-purified against the immobilized peptide (Pineda Antikörper-Service). For CD74, the established monoclonal antibody PIN1 (StressMarq Biosciences) that is directed against an N-terminal epitope of the protein was employed. To confirm equal protein loading, antibodies against the α-1 subunit of the Na^+/K^+-ATPase (clone C464.6, Millipore), elongation factor 2 (EEF2, Abcam) or β-tubulin (E7, Developmental Studies Hybridoma Bank) were used.

2.5. Immunocytochemistry

Lymphoblastoid cells were adhered to coverslips coated with poly-L-lysine [7]. Fixation with 4% (w/v) paraformaldehyde in PBS

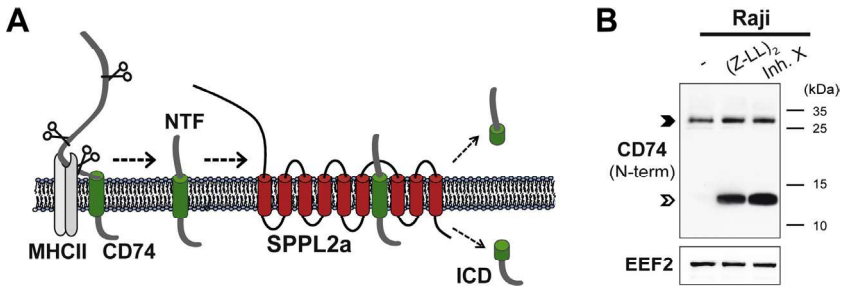

Fig. 1. The CD74 NTF is subjected to intramembrane proteolysis in human B cells. (A) Scheme of CD74 NTF turnover by SPPL2a as deduced from previous studies in mice. After mediating trafficking of MHCII to specialized endosomal compartments, degradation of CD74 starts from the luminal domain. The remaining membrane-bound NTF is then subjected to intramembrane proteolysis by SPPL2a leading to the liberation of an intracellular domain (ICD) into the cytosol. (B) Raji cells were cultured in the presence of 10 µM $(Z\text{-}LL)_2$-ketone ($(Z\text{-}LL)_2$), 1 µM inhibitor X (Inh. X) or an equivalent amount of DMSO (−) for 16 h. Endogenous CD74 was analyzed by Western blotting with an antibody directed against an N-terminal epitope of CD74 detecting CD74 full-length protein and the CD74 NTF marked by closed and open arrow-heads, respectively. Equal protein loading was confirmed by detection of elongation factor 2 (EEF2).

and immunocytochemical staining was performed as described [18]. CD74 was visualized using the monoclonal antibody PIN1 detecting the full-length protein and the CD74 NTF. A polyclonal antibody against the lysosomal/late endosomal protein LIMP-2 [19], a kind gift of Dr. Michael Schwake, University of Bielefeld, was used for co-staining. Secondary antibodies conjugated with Alexa Fluor 488 or 594 were obtained from MoBiTec. Image acquisition was performed with a FV1000 confocal laser scanning microscope (Olympus).

2.6. Flow cytometry

To determine surface expression of the class II antigen HLA-DR and the BAFF receptor (BAFF-R) in the different EBV-transformed cell lines, cells were suspended in MACS buffer (PBS containing 0.5 % [w/v] bovine serum albumin and 2 mM EDTA, pH 7.4) and stained for 30 min at 4 °C with anti-HLA-DR (clone L243, BioLegend) or anti-BAFF-R (CD268, clone 11C1, BioLegend) conjugated to APC and PE, respectively. For detection of total HLA-DR, cells were fixed and permeabilized with Cytofix/Cytoperm™ Solution (BD Biosciences) for 20 min at 4 °C and washed twice with Perm/Wash™ (BD Biosciences) prior to staining with anti-HLA-DR for 30 min at 4 °C. Flow cytometry was performed using a FACSCanto flow cytometer (BD) and data were analyzed using FlowJo (Tree Star) software.

3. Results and discussion

3.1. CD74 NTF undergoes intramembrane proteolysis in human B cells

In previous studies in mice [7–9] SPPL2a was found to process an NTF of CD74 that comprises about 80 residues and remains from the sequential degradation of the luminal domain by different endosomal proteases (Fig. 1A). To assess if also in human B cells intramembrane proteolysis serves as degradation route for the CD74 NTF, we incubated the human B cell-derived Burkitt lymphoma cell line Raji in the presence of the SPP/SPPL inhibitors $(Z\text{-}LL)_2$-ketone or inhibitor X and analyzed endogenous CD74 with

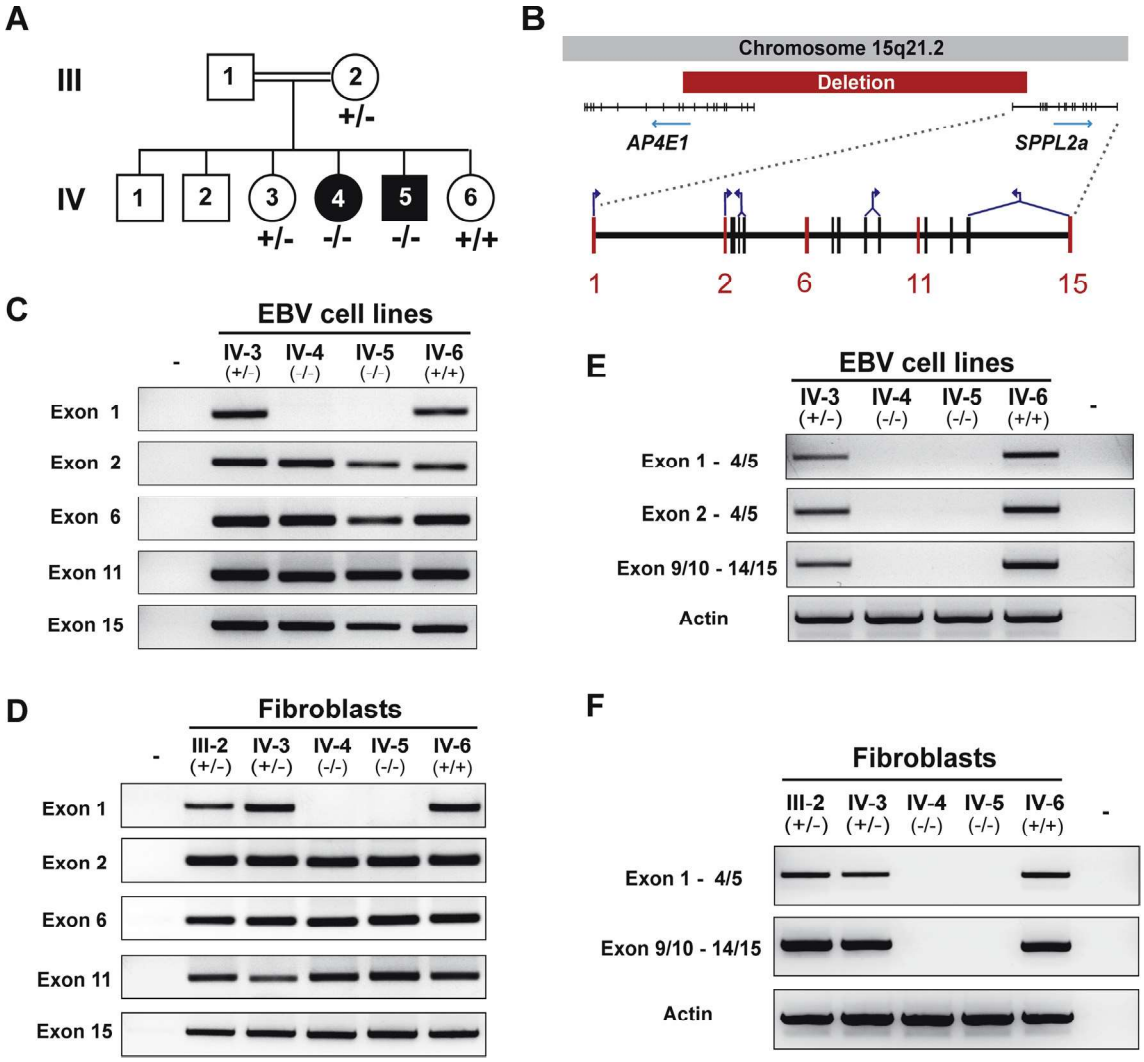

Fig. 2. A 15q21.2 deletion disrupts the *SPPL2a* gene. (A) Pedigree indicating the genetic status of the family members regarding the 15q21.2 deletion as far as known and reported in [11]: −/−, homozygous deletion; ±, heterozygous deletion; +/+, no deletion. Individuals IV-4 and IV-5 were reported to be affected by a cerebral palsy syndrome (black symbols). (B) Reported positioning of the 15q21.2 deletion as determined by chromosomal microarray analysis in relation to the genomic regions of *AP4E1* and *SPPL2a* and detailed view of the exon–intron structure of the human SPPL2a gene. Arrows indicate oligonucleotides employed for detection of SPPL2a transcript by RT-PCR. (C, D) In genomic DNA isolated from lymphoblastoid cell lines (C) or fibroblasts (D) of the indicated individuals presence of exons 1, 2, 6, 11, 15 (red in (B)) of SPPL2a was determined by PCR with flanking oligonucleotides. (E, F) SPPL2a transcript was analyzed by RT-PCR using different combinations of forward and reverse primers for amplification of different parts of the SPPL2a ORF. As indicated, several of the employed oligonucleotides annealed to exon–exon interfaces (e.g. 4/5). cDNA prepared from total RNA of lymphoblastoid cell lines (E) or fibroblasts (F) derived from the indicated individuals was used as template. Actin was employed as positive control. In (C–F) negative controls (–) without the addition of template were included. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

an antibody directed against an N-terminal epitope (Fig. 1B). Treatment with both compounds was found to stabilize the CD74 NTF thereby confirming the requirement of SPP/SPPL proteases for CD74 NTF turnover in these cells. However, currently no inhibitors with specificity for individual SPP/SPPL proteases are available.

3.2. A 15q21.2 deletion disrupts the SPPL2a gene

In order to specifically scrutinize a role of SPPL2a for CD74 NTF proteolysis in human B cells, we characterized cell lines with a microdeletion on chromosome 15q21.2. In a previous study, this 192 kb deletion was identified by CMA analysis of a family with two siblings affected by an idiopathic neurological condition with severe disability [11]. The two affected individuals (IV-4 and IV-5) were found to be homozygous for this deletion, other, unaffected family members were heterozygous carriers (Fig. 2A). By CMA, the 15q21.2 deletion was shown to delete part of the AP4E1 gene coding for the epsilon 1 subunit of the adaptor protein complex 4 (AP4) (Fig. 2B). This was considered to underlie the clinical condition since other cases with deficiency of adapter protein complex-4 (AP-4) subunits and similar symptomatology had been described in the literature [12,13]. However, the published mapping also showed loss of the first exon of the adjacent *SPPL2a* gene (Fig. 2B). This finding was not followed up further in the previous study [11]. Therefore, we analyzed the effect of the *SPPL2a* deletion in more detail in fibroblasts and EBV-transformed lymphoblastoid cell lines derived from the individuals indicated in Fig. 2A. We performed PCR amplification of exons 1, 2, 6, 11 and 15 of the *SPPL2a* gene using primers annealing in the adjacent introns. In agreement with published results, we were unable to amplify exon 1 from genomic DNA of cell lines derived from probands IV-4 and IV-5 that were homozygous for the deletion. All other exons of the *SPPL2a* locus that were tested were not affected by the deletion (Fig. 2C and D). We assumed that this constellation may disrupt generation of functional *SPPL2a* mRNA. Thus, we performed RT-PCR employing different primer combinations for the detection of *SPPL2a* transcript. As expected no product was obtained with a forward primer annealing in exon 1 in cell lines carrying the homozygous deletion (IV-4, IV-5) since this exon was lost from the genomic locus (Fig. 2B, E and F). However, also with forward primers in exon 2 or 9 that were preserved at the genomic level no *SPPL2a* cDNA could be detected in these cell lines (Fig. 2E and F). Presumably, this indicates that the loss of exon 1 and the 5′ upstream genomic sequences completely disrupts transcription of this locus.

3.3. In human B cells SPPL2a is indispensable for CD74 NTF turnover

Based on our inability to detect *SPPL2a* mRNA in the homozygous cell lines, we sought to analyze the effects at the protein level by Western blotting. We were able to reveal endogenous SPPL2a in fibroblast (Fig. 3A) and lymphoblastoid cell lines (Fig. 3B) derived from the individuals IV-3 and IV-6. However, in the patients' homozygous cell lines IV-4 and IV-5 no SPPL2a protein was detected (Fig. 3A and B), thus confirming that the 15q21.2 deletion leads to SPPL2a deficiency at the level of the protein. In the cell

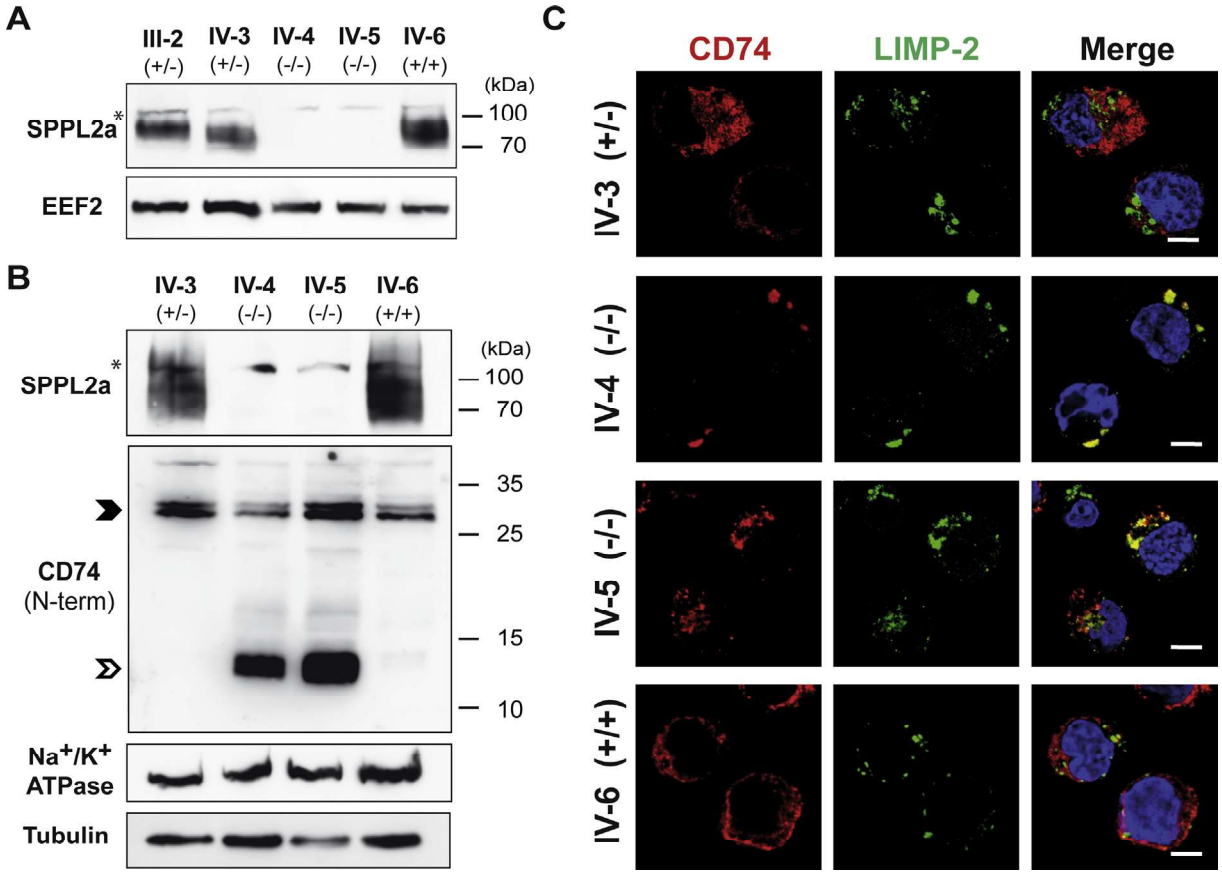

Fig. 3. In human B cells SPPL2a is indispensable for CD74 NTF turnover. (A, B) Total membrane preparations or total lysates from fibroblasts (A) or lymphoblastoid cell lines (B) derived from the indicated individuals were analyzed for SPPL2a (A, B) and CD74 (B) by Western blotting, respectively. Detection of Na^+/K^+-ATPase (membranes) and EEF2 or tubulin (lysates) was included to confirm equal protein loading. The employed CD74 antibody was directed against an N-terminal epitope, thus detecting CD74 full-length protein and the NTF that were marked by closed and open arrow-heads, respectively. * Unspecific band. (C) Immunocytochemical visualization of CD74 in the same lymphoblastoid cell lines. CD74 was detected using the same antibody as in (B) recognizing full-length CD74 and the NTF. Co-staining with the lysosomal/late endosomal membrane protein LIMP-2 was performed. Scale bars, 5 µm.

lines from heterozygous carriers, levels of SPPL2a appeared to be reduced in comparison to IV-6 with two functional SPPL2a alleles.

Since lymphoblastoid cell lines represent transformed B lymphocytes, we analyzed the biochemical consequences of SPPL2a deficiency on CD74 intramembrane proteolysis in these cell lines. Upon Western blot analyses with an antibody against an N-terminal epitope of CD74, we could demonstrate the presence of CD74 full-length protein in all four lymphoblastoid cell lines irrespective of their genotype (Fig. 3B). In addition, cells homozygous for the 15q21.2 deletion exhibited a very prominent band representing an NTF of CD74 with an apparent molecular weight between 10 and 15 kDa. The CD74 NTF accumulating in the two SPPL2a-deficient lymphoblastoid cell lines (IV-4, IV-5) was detected in lysosomal/late endosomal compartments based on its co-localization with the protein LIMP-2 (Fig. 3C) equivalent to the CD74 NTF accumulation seen in B cells from *SPPL2a*$^{-/-}$ mice [7]. In the heterozygous cell line (IV-3), no increase of CD74 NTF levels and, thus, no impairment of CD74 NTF turnover was observed in agreement with the preserved and only slightly reduced expression of SPPL2a in these cells.

3.4. Putative effects of CD74 NTF accumulation

In primary splenic B cells from SPPL2a-deficient mice, the accumulating CD74 NTF provokes several distinct cellular changes including a reduction of the surface expression of the BAFF receptor (BAFF-R) and an increase of total and surface levels of MHCII as we reported previously [7]. Therefore, we analyzed expression of the BAFF-R and the class II antigen HLA-DR in the different proband-derived lymphoblastoid cell lines by flow cytometry (Fig. 4). Whereas no significant differences in BAFF-R surface levels between the different cell lines could be ascertained (Fig. 4A and B), the HLA-DR expression was significantly lower in the cell lines derived from the affected patients (IV-4, IV-5) and the heterozygous carrier (IV-3) as compared to the unaffected control (IV-6) (Fig. 4C and D). This applied to total as well as surface levels of HLA-DR. In the SPPL2a-deficient cell lines IV-4 and IV-5, the heterogeneity of HLA-DR expression was increased within the analyzed cell population as depicted in the histograms (Fig. 4C). Similar findings were obtained upon analysis of another class II antigen, HLA-DP (not shown). Thus, in these human, patient-derived cell lines absence of SPPL2a was associated with a decrease rather than an increase of MHCII as it had been observed in the murine system [7]. Thus, CD74 NTF-associated effects on BAFF-R and MHCII expression were not recapitulated in these cell lines in the way they had been observed in primary murine SPPL2a-deficient B cells possibly indicating intrinsic differences between primary and immortalized B cells. Whether the reduction of HLA-DR observed especially in the homozygous cell lines IV-4 and IV-5 is indeed causally linked to SPPL2a-deficiency, cannot be conclusively deduced from the available data since a role of the concomitant disruption of the AP4E1 gene or simply clonal differences between the different cell lines cannot be excluded.

3.5. SPPL2a – a putative new locus in hereditary immunodeficiencies?

The presented data clearly show that the essential role of SPPL2a in CD74 proteolysis described in mice is conserved in human B cells. In both cases, degradation of CD74 is halted at the stage of the CD74 NTF in the absence of SPPL2a. Thus, this finding strongly supports the assumption that in humans the loss of SPPL2a could also lead either to a depletion or at least a major functional deficit of B cells. Clinically, this might present as primary Common Variable Immunodeficiency (CVID) characterized by an isolated impairment of B cell function [14,15]. It seems therefore justified to consider *SPPL2a* as a putative disease-associated gene in this type of disorder and to systematically analyze this locus in cohorts of CVID patients with a currently unidentified

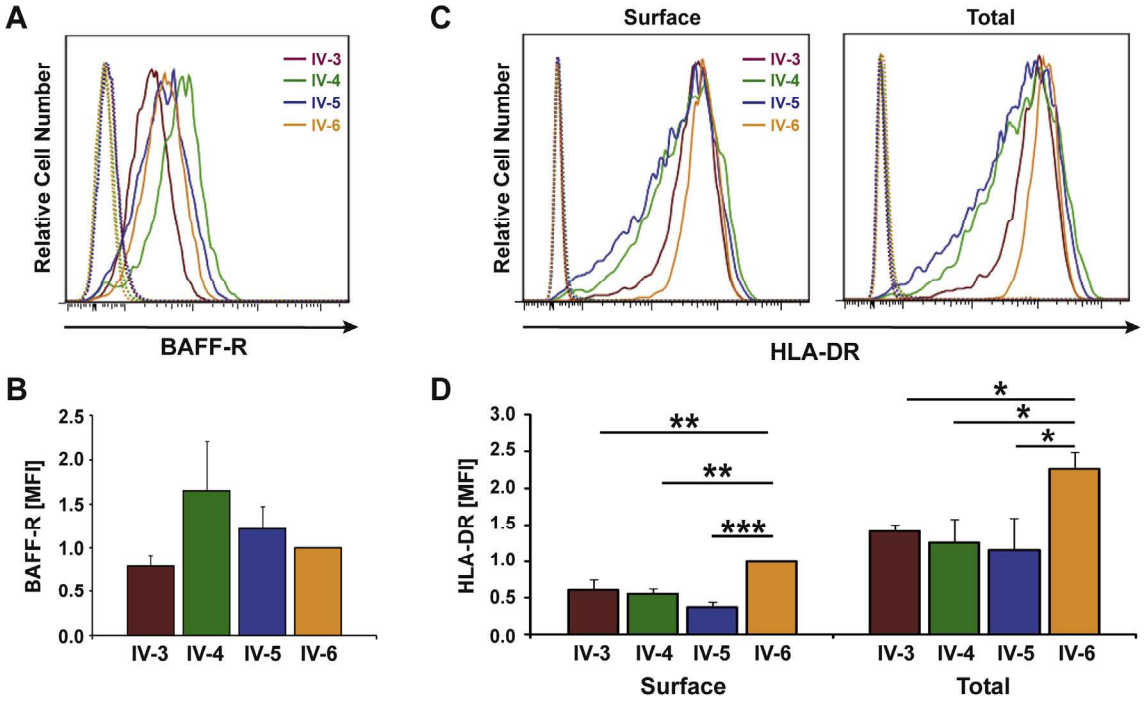

Fig. 4. Expression of BAFF-R and HLA-DR in SPPL2a-deficient lymphoblastoid cell lines. EBV-transformed cells derived from the probands IV-3 (±), IV-4 (−/−), IV-5 (−/−) and IV-6 (+/+) were stained for BAFF receptor (BAFF-R) or the class II antigen HLA-DR with or without preceding permeabilization and analyzed by flow cytometry. Surface BAFF-R expression (A, B) as well as surface and total HLA-DR levels (C, D) are shown as histograms from a representative of three independent experiments (A, C) or as mean of median fluorescence intensity (MFI) normalized to the surface expression of the unaffected control IV-6 (B, D). In A and C isotype controls are depicted as dotted lines. In B and D data represent mean ± standard deviation, $n = 3$ per cell line. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ as determined by one-way ANOVA with Bonferroni post hoc testing.

genetic basis. However, having confirmed here that the biochemical consequences of SPPL2a deficiency regarding CD74 NTF accumulation are equivalent in human and murine B cells, no final evidence is available yet that the phenotypic consequences of the CD74 NTF accumulation in human patients mirror that from mice. Interestingly, growth and proliferation of the SPPL2a-deficient lymphoblastoid cell lines were not detectably impaired compared to the control cell lines despite of the accumulating CD74 NTF arguing against a general cytotoxicity of this fragment. However, these cells had been transformed by EBV. Therefore, this may indicate that the maturation arrest of B cells in the *SPPL2a*$^{-/-}$ mice at a very distinct developmental stage [7] is caused by a selective interference of the CD74 NTF with survival pathways that are essential in primary B cells but dispensable in immortalized B cell lines. Unfortunately, no clinical information on the hematological and immunological status of the individuals giving rise to the SPPL2a-deficient cell lines analyzed here is available or could be obtained. Therefore, the phenotypic consequences of the SPPL2a-deficiency in these two individuals remain elusive.

In conclusion, the analysis of cell lines with the homozygous 15q21.2 deletion has provided unambiguous evidence that in human B cells the intramembrane protease SPPL2a is indispensable for cleavage and turnover of CD74 NTF. This is an important prerequisite for the proposed concept that modulation of SPPL2a activity may represent a novel approach for targeting B cells. Therefore, the current findings provide strong support and justification in order to pursue this concept further.

Acknowledgments

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