RESEARCH ARTICLE

Characterization of a long isoform of IL-1R8 (TIR8/SIGIRR)

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ABSTRACT. IL-1R8, also known as SIGIRR or TIR8, is a trans-membrane protein belonging to the IL-1 receptor family. The human gene includes ten exons, and alternative splicing can result in different isoforms. We, herein, characterized a longer isoform of IL-1R8 containing an in-frame additional sequence between the TIR domain and the C-terminal portion of the protein. IL-1R8 Long (IL-1R8L1) mRNA was specifically expressed and regulated in distinct cell lines, in a manner similar to the classic isoform. Overexpression of IL-1R8L1 resulted in the production of a corresponding protein that showed a pattern of cell localization similar to the classic isoform. An antibody directed against an IL-1R8L1 specific peptide, detected this novel isoform in different cell lines and tissues where this protein may complement the anti-inflammatory functions of classic IL-1R8.

Key words: interleukin-1 receptor, Toll-like receptor, IL-1R8, TIR8, SIGIRR, splicing

The interleukin-1 receptor family (IL-1R) groups several proteins that share a common modular structure made up by an extracellular portion containing three Ig-like domains, and an intracellular TIR domain (**T**oll/**I**nterleukin-1/**R**esistance domain) [1]. This same TIR domain is also shared by Toll-like receptors (TLR); however, TLR differ in the extracellular ligand-binding domain that is characterized by several leucine rich repeats [2].

Interleukin-1 (IL-1) is a prototypic inflammatory cytokine; two different genes encode for either IL-1α or IL-1β that share sequence homology and binding to a cell surface hetero-complex made of IL-1 receptor type 1 and IL-1 receptor accessory protein [3]. After interleukin-1 binding to the IL-1R1 and IL-1RacP, a proximal signaling complex is assembled around the TIR domain that recruits the corresponding TIR domain of the adapter protein MyD88 and that subsequently binds to distinct IRAK kinases; a signaling cascade is rapidly triggered leading to NF-kB activation and production of pro-inflammatory mediators [4, 5]. A tight control of the activity of IL-1 is exerted by different molecular mechanisms including specific IL-1 receptor antagonists, a decoy type II IL-1 receptor and the inhibitory receptor IL-1R8 [6].

IL-1R8, also known as SIGIRR or TIR8, is a trans-membrane protein belonging to the IL-1R family [7]. Accordingly, IL-1R8 has a modular structure with a single Ig-like domain followed by a trans-membrane domain and a TIR domain [8]. IL-1R8 is functionally implicated in the negative regulation of IL-1R and TLR signaling pathways; it cannot induce the MyD88-dependent signaling cascade likely, because its TIR domain lacks key conserved amino acids. In contrast, IL-1R8 inhibits NF-kB activation triggered by the IL-1R and distinct TLR (i.e. TLR4 and TLR7) [9-12]. In detail, based on computational modeling, it was suggested that IL-1R8 might exert its inhibitory effect through blocking the molecular interface of TLR4, TLR7, and the MyD88 adaptor mainly via its BB-loop region in the TIR domain [13]. In mouse models, lack of IL-1R8 exacerbates inflammation and inflammation-associated tumors including colon cancer and chronic lymphocytic leukemia [7, 14-16].

Even though IL-1R8 has long been considered incapable of binding to any extracellular ligand, it was shown that IL-1F5 mediates anti-inflammatory activity in the brain through interaction with SIGIRR/TIR8 [17]. More recently, it has been demonstrated that IL-1R8 is assembled into the signaling complex of IL-37 together with IL-18R, and IL-1R8 plays a functional role in mediating the anti-inflammatory effects of this cytokine [18-20].

A lot of already predicted, alternatively spliced isoforms of IL-1R8 mRNA, are present in the NCBI database, and a recent paper reported the expression of a short isoform of IL-1R8 in colon cancer [21]. We, herein, identified, cloned and characterized a longer isoform of human IL-1R8, herein, called IL-1R8L1; by using a novel specific antibody, we demonstrated that IL-1R8L1 protein is specifically expressed in distinct human cells and tissues.

METHODS

Cells

The following human cell lines were analyzed: Hek293, Hela, Jurkat, MEC1, Ramos, Daudi, Namalwa, and THP1;

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the cell lines were obtained from ATCC or DSMZ cultured *in vitro* and routinely screened for genotyping to assess their identify.

Reagents and antibodies

Mouse monoclonal anti-FLAG antibody (Sigma); Goat polyclonal anti-IL-1R8 (Santa Cruz); Rabbit polyclonal anti-IL1R8 (Proscience). The rabbit polyclonal anti-IL-1R8L isoform antiserum was produced by PRIMM Biotech Srl (Milano, Italy) after peptide immunization.

PCR and cloning

Total RNA was purified from cells and cell lines by using Trizol according to the manufacturer's instructions. First strand cDNA was transcribed from total RNA (for gene expression analysis) or poly-adenylated RNA (for cloning) using a high capacity cDNA synthesis kit (Fermentas). Gene Pool cDNA from human placenta was purchased from Invitrogen.

The following primers were used for RT-PCR analysis:
– IL-1R8 FOR: GGACCCCATGCTGATTCTTCG;
– IL-1R8 REV: TCATCCTTGGACACCAGGCAG;
– IL-1R8L1 FOR: AGCAGGGGGTGTCCGGGGGC;
– IL-1R8L2 FOR: GGTGAGCAGGGGCAGCCCCT.

The following primers were used for cloning into pCR2.1 vector (TA-cloning kit; Invitrogen):
– FOR: GTGACCCTGGAGCCCAGCCT;
– REV: CTGGCCCCCGCTGTCCCTAT.

The following primers were used for sub-cloning into pUNO expression vector (Invivogen):
– BAMH1-IL-1R8-FOR:
GTCGACGGATCCGCCACCATGCCAGGTGTCTGTG
ATAGGGCC;
– NHE-FLAG-IL-1R8-REV:
TGTCGAGCTAGCCTACTTGTCATCGTCGTCCTTGT
AGTCCATATCATCCTTGGACACCAGG;
– IL-1R8L2-FLAG-NHE-REV:
TGTCGAGCTAGCCTACTTGTCATCGTCGTCCTTGT
AGTCTCCTTGGACACCAGGCAGTAGAAG.

DNA sequencing of the amplified PCR products and the inserts into the plasmids was performed by an external service (Primm and GATC Biotech).

Transfection

Hek293 cells and Hela cells were transfected with Lipofectamine 2000 reagent according to the manufacturer's instructions. Twenty-four hours after transfection, cells were analyzed by Western blot or immunofluorescence analysis as detailed below.

Western blot

Protein lysates (RIPA buffer with protease and phosphatase inhibitors) were run onto SDS-PAGE mini-gels (4–12%, Invitrogen); proteins were electro-transferred onto nitrocellulose membranes (Promega) and incubated with the indicated antibodies at 4 °C over night. The immunoreactivity was detected by further incubation with secondary antibodies conjugated to HRP, followed by ECL reaction (Thermo Scientific Pierce) and exposure on photographic film or using the C-DiGit Chemiluminescent Western Blot Scanner (LI-COR). Cell lines Blot and Tissue Blot were purchased from Proscience and Novus Biologicals, respectively.

Immunofluorescence analysis

Hela cells, previously transfected with the indicated plasmids for 48 h (see transfection paragraph), were seeded on uncoated glass slides and incubated at 37 °C for 8 h. Cells were fixed with 4% PAF (Paraformaldehyde) and permeabilized. Primary antibodies diluted in blocking solution (1mg/mL BSA, 10% FBS in PBS) were added to the slides and incubated overnight at 4 °C and stained with fluorochrome-conjugated secondary antibodies for 1–2 hours at room temperature in the dark. Following this, slides were washed with PBS and incubated with DAPI for ten minutes and mounted on a coverslip (ProlongGold antifade, Life technologies). Images were taken by using a Leica SP2 confocal scanning microscope. The antibodies and reagents used were as follows: Monoclonal anti-FLAG M2 antibody produced in mouse (Sigma); Secondary antibody, anti-mouse 488; Alexa Fluor 594 phalloidin; and DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride).

Real-time PCR

Total RNA was extracted from 10^6 cells and eluted in a volume of 20 μL H_2O DEPC using the ReliaPrep RNA cell Miniprep System according to the manufacturer's recommendations (Promega). First strand complementary DNA (cDNA) was synthetized from about 1 to 2 μg RNA using iScript adv cDNA kit for RT-qPCR (BIO-RAD). The gene expression analysis was performed using ABI PRISM 7900HT and Fast SYBR Green Master Mix (Applied Biosystems). The thermal cycling conditions were 20 sec at 95 °C followed by 40 cycles of 1 sec at 95 °C and 20 sec at 60 °C. The SDS software version 2.4 was used for the data collection, and relative gene expression levels were calculated using the 2^{-ddCt} method.

PRIMERS:
– B-ACTIN Forward CCAACCGCGAGAAGATGA;
– B-ACTIN Reverse CCAGAGGCGTACAGGGATAG;
– TIR8 Forward GCCTCATCGTGGTGCTTT;
– TIR8 Reverse GGTGATGAAGATGGGTCTGC;
– L1 Forward AGCAGGGGGTGTCCGGGGGC;
– L1 Reverse TCATCCTTGGACACCAGGCAG.

RESULTS

Identification and cloning of a longer alternatively spliced isoform of IL-1R8

Searching the public NCBI databases suggested the existence of distinct transcripts encoding for different isoforms of human IL-1R8 (NCBI database: http://www.ncbi.nlm.nih.gov/gene/59307). In detail, two longer isoforms generated by alternative splicing between exon 9 and exon 10 were predicted as isoform X1 (Accession: XP_005253103.1; XP_005253102.1; XP_005253101.1; 504 aa predicted protein) and isoform X2 (Accession: XP_016873588.1; 479 aa predicted protein) in which a 289 bp long intron was either unspliced or partially spliced into the mature mRNA (*figure 1A*); the

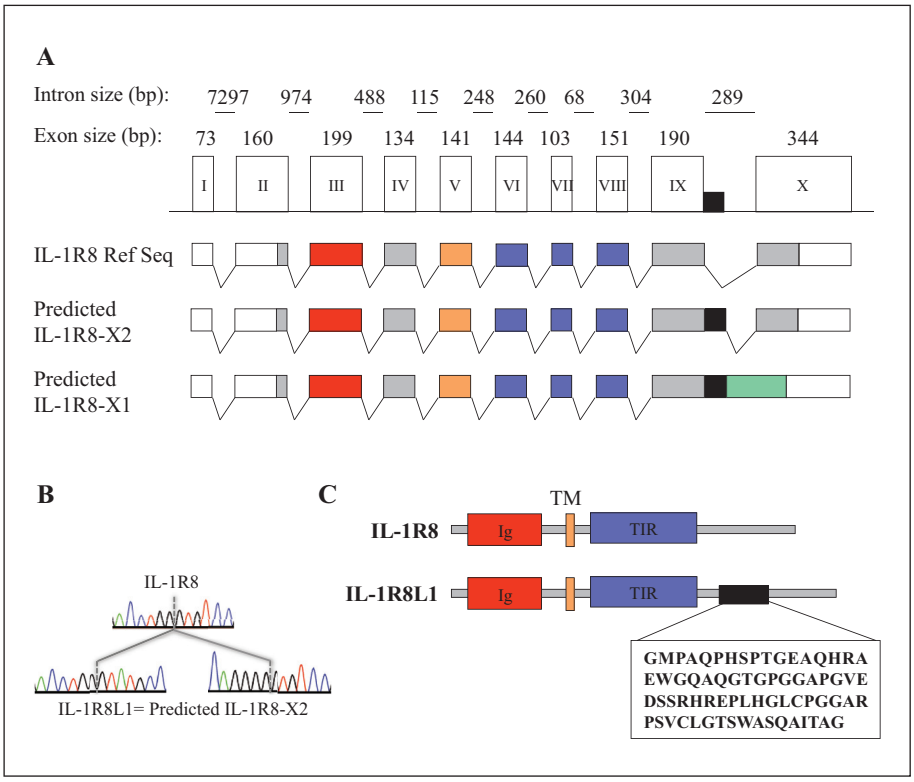

Figure 1

Alternative splicing of IL-1R8 mRNA. A. Schematic representation of the human IL-1R8 gene and corresponding mRNAs. Color codes indicate sequences encoding for different domains of the protein including Ig-like (red), transmembrane (orange) and TIR (blue). **B.** Partial cDNA sequence of IL-1R8 and IL-1R8L1 (around the alternatively spliced region). **C.** Schematic representation of IL-1RL1 and the classic isoform including the in-frame extra sequence before the C-terminal portion of the protein.

alternatively spliced introns were delimited by both the consensus sequences GT and AT.

TBlastN sequence analysis identified several human Expressed Sequence Tags (ESTs) corresponding to either X1 isoform or X2 isoform, thus confirming the expression of both of these mRNAs in human cells. In detail, X1 specific junction sequence (GLCPGGARPSVCLGTSWASQAITAGGEQGQPLAVG LGQGCGWPPQASRSPHP) was found in BM981752.1, BU683133.1, AW001375.1, and BI765555.1 clones from primary lung cystic fibrosis epithelial cells, lung tissue, and colonic mucosa from 3 patients with Crohn's disease and pooled colon, kidney, stomach, respectively. X2 specific junction sequence (GLCPGGARPSVCLGTSWASQAITAGGVRGPVFGEP SAPPHTSGVSLGESRSS) was found in CB243174.1, CB243183.1, and BG287953.1 clones from Human Lung Epithelial cells and transitional cell papilloma cell line, respectively.

Protein Blast sequence analysis using the X1 specific sequence against the non-redundant database identified an open reading frame sequence previously reported by bioinformatics assessment as GenBank accession number AAQ88708 [22]; on the contrary, no complete protein sequence was reported for the X2 isoform except for the aforementioned predicted isoforms.

As expected, when we performed RT-PCR amplification followed by Reverse transcription and cDNA cloning, using 2 primers spanning the start and stop codons, we cloned the classic IL-1R8 isoform; in addition, a higher molecular weight cDNA corresponding to the predicted isoform X2 was cloned from different cell lines (i.e. Jurkat, MEC1 and Daudi) and placenta cDNA. Sequencing of the cDNA confirmed that the predicted X2 isoform mRNA was indeed expressed, and encoded for a 479 aa IL-1R8 protein containing an in-frame insertion of 69 aa (NCBI gene bank accession number: MF095124); *figure 1B*).

In contrast, we could not amplify any additional longer isoforms from the cell lines analyzed; nevertheless, the open reading frame of X1 isoform mRNA was amplified by PCR from the EST clone number 3047195 (GeneBank: AI925221). The predicted X1 isoform resulted into a longer cDNA encoding for a 504 aa IL-1R8 protein with an out-of-frame addition of a different C-terminal portion; therefore, we named these two isoforms Long-1 or L1 and Long-2 or L2, respectively (*figure 1C*).

Of note, IL-1R8L1 and IL-1R8L2 cDNAs contained 3 distinct base changes previously reported as Single Nucleotide Polymorphisms (SNP); of which one was changing the Glutamine (Q) in position 312 into an Arginine (R). Even though the official reference sequence of IL-1R8 did not show this variation, we previously found a high frequency of this variant in different cell lines (not shown).

Blastp sequence analysis using the novel aa sequence specific of L1 and L2 against non-human sequences (nr database) did not retrieve any similarity to previously characterized proteins.

These data support the existence of a novel alternatively spliced IL-1R8 mRNA encoding a longer isoforms in humans only.

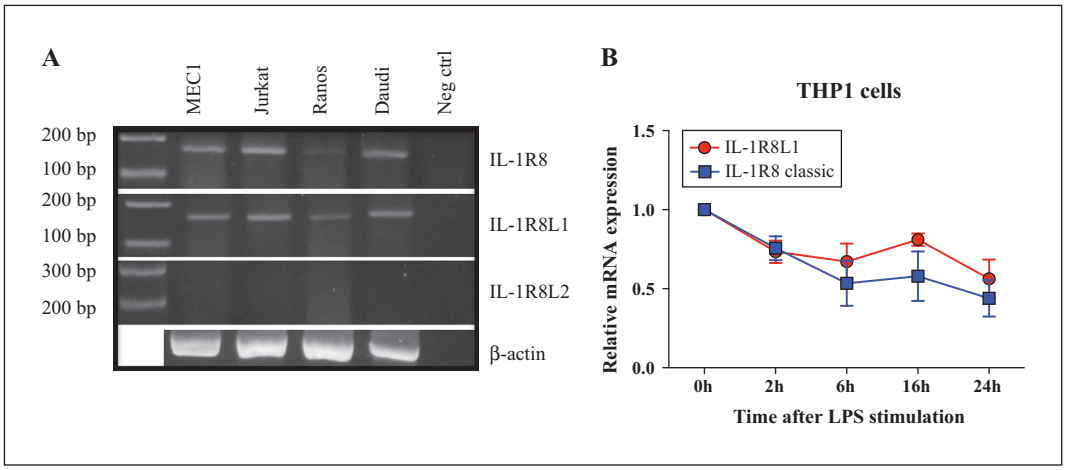

Figure 2

Expression and regulation of IL-1R8L1 mRNA. A. Reverse transcriptase PCR analysis of IL-1R8, IL-1R8L1 and IL-1R8L2 in human cell lines including MEC1 (chronic lymphocytic leukemia), Jurkat (T-cell leukemia), Ramos (Burkitt's lymphoma) and Daudi (Burkitt's lymphoma). **B**. LPS treatment of THP1 cells (acute monocytic leukemia) reduces the mRNA levels of both IL-1R8 and IL-1R8L1; data are the average of 3 independent experiments and are expressed as ratio of IL-1R8/β-actin after real-time PCR analysis.

Expression pattern and regulation of IL-1R8L1 mRNA

We first designed different pairs of primers to specifically amplify by PCR, the cDNA of classic IL-1R8, IL-1R8L1, or IL-1R8L2 (see methods section for primer sequence). We analyzed human lymphoid cell lines previously characterized for IL-1R8 expression including Jurkat (T-cell leukemia), MEC1 (chronic lymphocytic leukemia), and the B-cell lymphoma Ramos and Daudi [23]. Jurkat cells, and to a lesser extent also B-cell lymphoma cell lines, expressed both IL-1R8 and IL-1R8L1; in contrast, none of them expressed IL-1R8L2 (*figure 2A*).

Next, we focused our attention on IL-1R8L1, and we designed additional PCR primers for real-time PCR analysis to accurately quantify the levels of expression and regulation of IL-1R8L1. We analyzed THP1 cell line (acute monocytic leukemia) that was previously reported to express high levels of classic IL-1R8 mRNA and protein [23,24]; we stimulated the cells with LPS to characterize any possible regulation upon activation, and we observed that both classic IL-1R8 (as previously described) [24] and novel IL-1R8L1 mRNA levels partially decreased over time after LPS stimulation (*figure 2B*).

These results demonstrated that IL-1R8L1 mRNA is expressed in different human cell types, and it can be regulated by LPS stimulation in a manner similar to the classic isoform.

Characterization of IL-1R8L1 protein

To understand if novel mRNA isoforms could lead to different proteins, we transfected HEK-293 cells with different FLAG-tagged constructs schematically represented in *figure 3A*. Western blot analysis of cell lysates transfected with the classic isoform of IL-1R8 confirmed that several immunoreactive proteins including the predicted 45.6 kDa molecular weight were detected with different molecular weight as previously reported [8] (*figure 3B*; anti-FLAG and anti-IL-1R8 antibodies in the upper panel and lower panel respectively). As expected, the same bands were detected in cells transfected with the vector encoding a polymorphic variant (IL-1R8-SNP). In contrast, transfection of IL-1R8L1 expression vector resulted in the overexpression of higher molecular weight proteins, which is consistent with the calculated molecular weight of 52.5 kDa with or without the addition of potentially glycosylated forms. When the IL-1R8L2 expression vector was used, the appearance of a faint immunoreactive band at the same molecular weight as IL-1R8L1 suggested that this isoform is less expressed and/or stable (*figure 3B*); the size of the bands are again consistent with the calculated molecular weight of 55.2 kDa.

Immunofluorescence analysis using an anti-FLAG antibody confirmed the presence of both IL-1R8 and IL-1R8L1 within sub-membrane and cytoplasmic regions with no marked difference between them (*figure 3C*).

To better characterize the endogenous IL-1R8L1 isoform, we developed a rabbit polyclonal antiserum directed specifically against a portion of the L1 specific sequence; however, it should be noted that this sequence is potentially shared by a predicted L2 isoform. Western blot analysis of HEK-293 transfected cells confirmed the specificity of the antiserum against IL-1R8L1 and to a lesser extent IL-1R8L2 (*figure 4A*). First, we tested this serum against a Western blot membrane containing lysates from different human cell lines, and we detected a long isoform of IL-1R8 specifically in HeLa (cervical cancer), HT-29 (colorectal adenocarcinoma), SK-N-HS (neuroblastoma) and PC3 (prostate cancer) but not in 293 (embryonic kidney), HepG2 (liver cancer) and A-549 (lung adenocarcinoma); low levels were detected in MCF7 cells (breast cancer) (*figure 4B*). When we tested a Tissue Blot including lysates from different human tissues, we detected IL-1R8L1 in heart, small intestine, kidney, liver, lung, stomach, spleen, ovary and testis, but not in the brain or skeletal muscle (*figure 4C*).

These data demonstrate the expression of a novel long isoform of TIR8 protein in distinct human samples suggesting tissue-specific regulation.

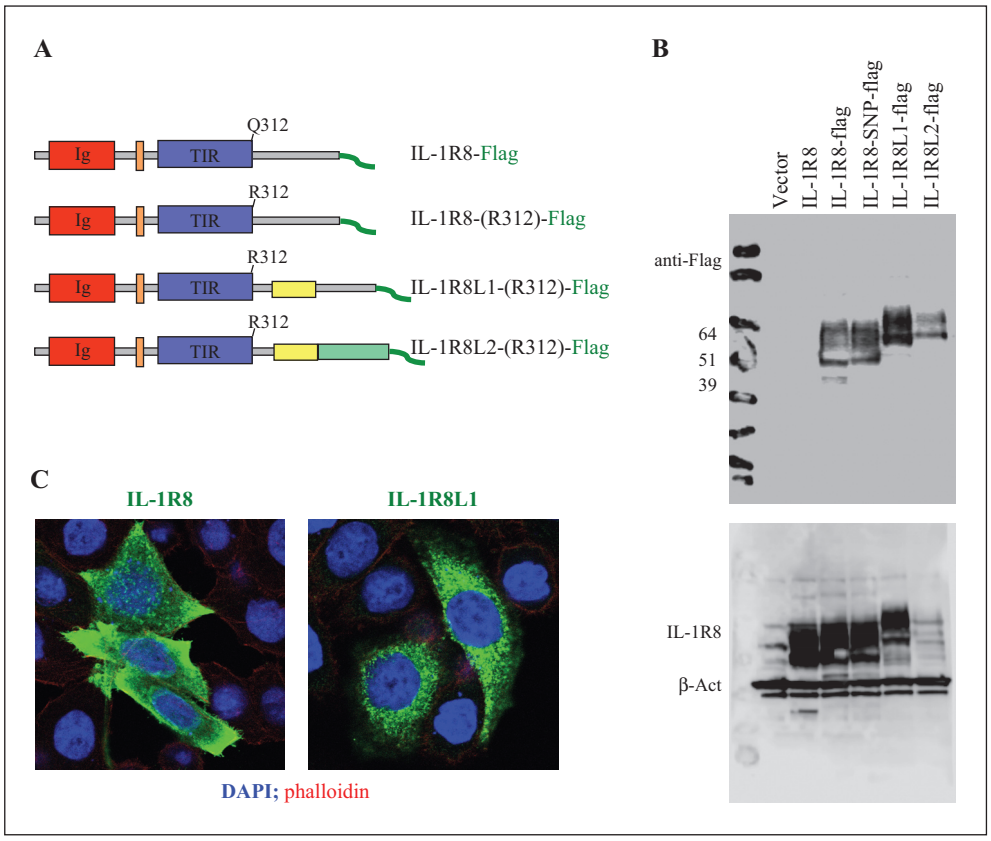

Figure 3

Ectopic expression of IL-1R8 isoforms. A. Schematic representation of the FLAG-tagged proteins encoding for the different IL-1R8 isoforms resulting from the corresponding plasmids as indicated. **B.** Western blot analysis of HEK293 cells transfected with the indicated plasmids reveals overexpression of the FLAG-tagged isoforms with different molecular weights as indicated (upper panel); the use of an anti-IL-1R8 antibody confirmed the detection of the same molecular weight proteins (lower panel). **C**. Immunofluorescence analysis of Hela cells transfected with the indicated plasmids reveals sub-membrane and cytoplasmic localization of both IL-1R8 and IL-1R8L1.

DISCUSSION

We herein identified, cloned and characterized a long isoform of IL-1R8 that is expressed in distinct cells and tissues at the levels of both mRNA and protein.

We analyzed the intracellular localization of IL-1R8L1, and we observed a sub-membrane cytoplasmic punctate staining, similar to the classic IL-1R8 protein. To note, IL-1R8 was previously demonstrated to be membrane associated by biochemical studies; nevertheless, the absence of a canonical "signal peptide" suggested that alternative routes of localization may exist [8]. Our observation that IL-1R8L1 shows both sub-membrane and cytoplasmic localization suggests that they may have additional intracellular functions. In fact, IL-1R8 was proposed to inhibit also the activity of TLR7 protein that is an endosome-associated intracellular molecule [13].

The functional role of IL-1R8 is rather complex, as it has been involved in several different inflammatory receptors including IL-1R, TLR4, TLR7, and IL-37 [25]. On the basis of the sequence analysis, we suggest that IL-1R8 and IL-1R8L1 may have similar functional activity in inhibiting signaling, because they share a full TIR domain; however, the functional role of the C-terminal domain of IL-1R8 has never been addressed, and the additional sequence of IL-1R8L1 may modify any molecular function of this portion of the protein. In our current study, we did not address the functional activity of IL-1R8L1, and additional investigations are required to assess its role in the context of distinct IL-1R family members.

Alternative splicing is emerging as a key molecular mechanism regulating biological responses in health and disease [26]. In particular, several alternatively spliced isoforms of IL-1R family members were previously described and characterized; bacterial LPS can induce incompletely spliced transcripts of IL-1RI [27] and a soluble inhibitory form of IL-1RacP can be induced in liver cells [28]. A truncated soluble IL-18R β mRNA was identified in the brain [29]. A distinct isoform of the IL-1RacP is expressed specifically into the central nervous system where it modulates neuronal responses to IL-1 [30]. Finally, a recent report demonstrated the existence of a short IL-1R8 isoform lacking exon 8 and specifically expressed in colon cancer; this isoform lacks inhibitory functions as compared to the classic IL-1R8 transcript [21]. Therefore, the addition of a novel IL-1R8 isoform to this family deserves further investigation in the context of normal and tumor cells.

We developed a specific anti-IL-1R8L1 antiserum that may help future studies of expression analysis of this novel IL-1R family member in physiological and pathological inflammation-associated conditions. While these antibodies can potentially cross-react with IL-1R8L2, the following observations suggest that IL-1R8L1 represents the most expressed long isoform recognized:

– we were not able to amplify IL-1R8L2 mRNA from any cell lines analyzed suggesting that, if expressed, this isoform is less abundant then IL-1R8L1 or strictly tissue restricted;

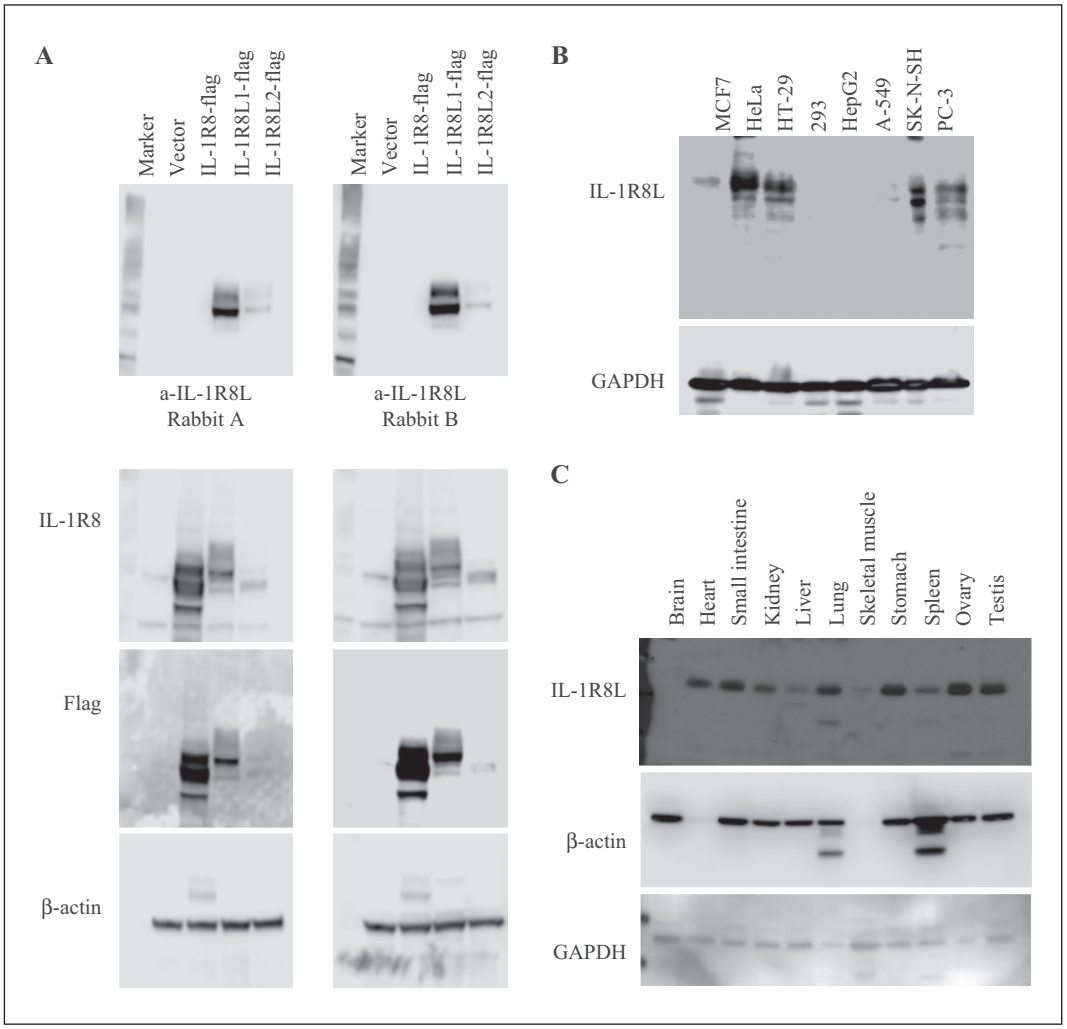

Figure 4

Expression of IL-1R8L1 protein. A. Western blot analysis of HEK293 cells transfected with the indicated plasmids; upper panel shows two different anti-IL-1R8L antisera from two different rabbits. Lower panels show the same blots with commercially available anti-IL-1R8 and anti-FLAG antibodies. β-actin was used as internal control. **B** and **C**. Anti-IL-1R8L antisera were used to detect the corresponding isoform in human cell lines and tissues. β-actin and GAPDH were used as internal controls.

– transfection of IL-1R8L2 cDNA in cells resulted in low levels of protein expression suggesting again that the IL-1R8L2 transcript, if expressed, can be translated into a less abundant protein as compared to the IL-1R8L1.

In conclusion, we identified, cloned and characterized a long isoform of IL-1R8; the use of a novel specific antibody directed against long IL-1R8 demonstrated the existence of this novel protein in different human tissues and cell lines. Future studies on the expression and function of IL-1R8 should include this novel long isoform.

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