



Research article

Identification of a novel splice variant for mouse and human interleukin-5



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ABSTRACT

Expression of interleukins and their receptors is often regulated by alternative splicing. Alternative isoform of IL-5 receptor α -chain is well studied; however, no data on functional alternative splice variants of IL-5 has been reported up today. In the present study, we describe a novel splice variant for the mouse and human IL-5. The new form was found during analysis of PCR-products amplified from different mouse lymphoid tissues with a pair of primers designed to clone full-length mIL-5 ORF. A single short isoform of mIL-5 was detected along with the canonical full-length mRNA in ConA-stimulated lymphoid cells isolated from spleen, thymus, lymph nodes and blood. It was 30–40 nt shorter, and less abundant than classical form. The sequence analysis of an additional form of mIL-5 revealed that it lacks exon-2 ($\delta 2$). Using RT-PCR with the splice-specific primers we obtained an additional evidence for $\delta 2$ form expression. To verify whether mIL-5 $\delta 2$ transcript is translated into protein, the coding sequences corresponding to full and $\delta 2$ forms of mIL-5 were cloned into an expression plasmid. After transfection into the human 293T cell line, we found that the short form of mIL-5 protein is expressed in cells and secreted into the supernatant, but at the reduced level than that detected for full isoform of mIL-5. Fluorescence microscopy examination revealed a partial translocation of mIL-5 $\delta 2$ into cytoplasm, whereas mIL-5 resided mostly within endoplasmic reticulum. This can explain why the level of $\delta 2$ protein expression was reduced. Using a similar set of experimental approaches, we received the evidence that the human IL-5 mRNA has the $\delta 2$ splice form (hIL-5 $\delta 2$) as well. It can be firmly detected by RT-PCR in PHA-activated mononuclear cells isolated from peripheral blood of healthy persons or patients with asthma. Altogether, our results showed that the human and mouse IL-5 have an alternative mRNA splice isoform, which loses exon-2, but nevertheless is expressed at protein level. However, more comprehensive studies will be required for evaluation of IL-5 $\delta 2$ expression, regulation, biological function and clinical significance.

1. Introduction

Cytokine IL-5 was first described by independent groups of scientists as T cell-replacing factor (TCR) [1, 2], B-cell growth factor II (BCGFII) [3] and eosinophil differentiation factor (EDF) [4, 5]. After mouse *il-5* (*mIL-5*) gene was cloned by Kinashi et al. in 1986 [6], it has been proved

that all described factors (TCR, BCGFII, EDF) were identical. In further studies sequence of mIL-5 was used as a reference gene for identification and cloning of human *il-5* (*hIL-5*) [7].

Both mouse and human *il-5* genes contain four exons and three introns. The similarity between mouse and human exons ranges from 70 to 85%. Moreover, nucleotide sequences of *il-5* and the other cytokines (IL-4, IL-3,

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IL-2, GM-CSF) are also homologous providing similarities in spatial structures of their protein forms [8, 9, 10]. Mouse IL-5 protein consists of 133 amino acid (aa) residues, including a signal peptide sequence (21 aa) and three sites for N-glycosylation. In contrast, human IL-5 consists of 134 aa residues, including a signal 22-mer peptide and two sites for N-glycosylation. Both mouse and human IL-5 exist as dimers linked via disulfide bonds [37]. Mouse IL-5 and hIL-5 have 71% amino acid homology [10, 11, 12]. However, while human IL-5 is able to stimulate mouse cells, mouse IL-5 is only weakly cross-reactive with human cells [13].

IL-5 acts as a homodimer and exerts its biological activity through a receptor consisting of two chains, IL5R α and IL5R β . IL5R α binds specifically to IL-5, while the IL5R β chain is common for interleukin 3 (IL-3) and colony stimulating factor 2 (CSF2/GM-CSF) receptors [14, 15].

Animal and clinical studies have shown that IL-5 is involved in pathogenesis of inflammatory diseases including bronchial asthma. This cytokine induces differentiation, recruitment and survival of eosinophils. Elevated IL-5 expression in broncho-alveolar lavage (BAL) was detected in asthma patients [14, 15] and IL-5 up-regulation was found in peripheral blood of asthmatics [16, 17, 18]. The studies using knock-out mice shed light on the role of the cytokine; no eosinophil infiltration in the BAL or lung tissue was observed in IL-5 deficient mice after induction of experimental bronchial asthma [19].

It is known that several cytokines are synthesized from a single gene in the form of alternatively splice variants. This increases the functional diversity of the cytokine network. Splice variants have been described for many cytokines, such as EGF, bFGF, TGF- β 1, TGF- β 2, IL-1 α , IL-2, IL-4, IL-6, IL-7, IL-15, IL-24, VEGF and others including mouse variants [20, 21, 22].

Alternative splicing is described for murine and human IL-5 receptor α -chain [23, 24, 25]. The structure and functions of the alternative soluble isoform of IL-5R α is well studied. However, little data available on alternative splice variants of IL-5 itself. Looking for alternative mouse *il-5* mRNA splice transcripts in different databases (UniProt and eEnsembl) returns no results [26, 27]. Nevertheless, in one report the possibility of alternative *il-5* mRNA splicing has been discussed [21] and there are described two mRNA transcripts for human IL-5. One of them (TranscriptID: ENST00000450655.1) contains three exons, lacking exon-4 and part of exon-1 and -3. As this transcript contains ORF it could code 51 aa protein. Another described transcript (TranscriptID: ENST00000462418.1) contains part of two exons and does not encode meaningful ORF [27].

Here, we describe a novel splice isoform for the mouse and human IL-5 (IL-5 δ 2), which is devoid of the exon-2, and, therefore, retains the leader sequence, but is 11 aa shorter than the full-length cytokine. The IL-5 δ 2 mRNA is expressed along with the canonical form in various activated murine lymphoid tissues and human PBMCs. Using ectopic expression of the mouse IL-5 and its δ 2 isoform in human cells, we demonstrated that the IL-5 δ 2 can be detected at a protein level suggesting that this alternatively spliced form of IL-5 may have a function.

2. Materials and methods

2.1. Animals and cells

All animal experiments in this study were conducted in accordance with the principles of EU Directive 2010/63/EU "Legislation for the protection of animals used for scientific purposes" and were approved by the Ethical Committee of the National Research Center - Institute of immunology of Federal Medico-Biology Agency of Russia, Moscow.

Female BALB/c mice of 19–20 g weight and 6-8-week-old (Pushchino, Russia) were sacrificed. Three intact mice for experiments were used. The spleen, thymus and mesenteric lymph nodes were surgically excised and placed in ice cold RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (PanEco, Russia), 300 mg/l L-glutamine (PanEco, Russia), 80 mg/l Gentamicin (Gibco, USA), 10mM HEPES (PanEco, Russia) and 0,05 mM 2-mercaptoethanol (Sigma-Aldrich, USA). To prepare cell suspensions, tissues were homogenized, sifted through the 40 μ m cell stainer and centrifuged for 5 min at 350 g. Cell pellets from

spleen, thymus and lymph nodes were resuspended in complete RPMI 1640 medium and cultured individually in 12-well plates (SP Laboratories) 5×10^6 cells/well in 1 ml/well.

Mouse and human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on the gradient density of Ficoll (PanEco, Russia) at 350 x g for 30 min and washed once with PBS. Mouse PBMCs were purified from three animals and cultured individually in complete medium as described above. Human PBMCs obtained from three healthy subjects and three allergic bronchial asthma patients and incubated individually at a final concentration of 5×10^6 cells/well in 1 ml/well. Biomaterial from healthy subjects and asthma patients were collected in the bronchial asthma department of National Research Center - Institute of immunology of Federal Medico-Biology Agency of Russia. Participation in the study was voluntary. According to the Helsinki Declaration, patients gave their oral and written consent before being included in the study.

All indicated mouse cells were activated with 2 μ g/ml of Concanavalin A (ConA) (Sigma-Aldrich, USA). Human PBMC were activated with 20 μ g/ml of phytohemagglutinin (PHA) (PanEco, Russia). 24 hours later cells were pelleted, supernatants were harvested and stored at -70 °C until analysis.

Human embryonic kidney 293T and HeLa cell lines (obtained from ATCC, USA) were cultured in DMEM (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (PanEco, Russia), 300 mg/l L-glutamine (PanEco, Russia), 80 mg/l Gentamicin (Gibco, USA), 10mM HEPES (PanEco, Russia) at 37 °C and 5% CO₂ atmosphere conditions.

2.2. RNA isolation, cDNA synthesis, PCR amplification and sequencing

Activated cells were lysed in cell lysis buffer (Qiagen, Netherlands). The total RNA was purified using RNeasy Mini Kit (Qiagen, Netherlands) according to manufacturer's recommendations. cDNA synthesis was performed using (dT)₁₈ primer and First Strand cDNA Synthesis Kit (Thermo Scientific, USA). After that individual cDNA samples from three mice were pooled in one cDNA library for each cell type (spleen, thymus, lymph nodes and PBMC).

Mouse *il-5* ORF was amplified from cDNA with using specific primers illustrated in Figure 1B. Additionally, short splice-specific primers to detect full-length and truncated mRNA transcript were designed. Forward primer (F_{1,2}: CAATGAGACGATGAGGCTT) covered exons-1 and -2 junction, forward primed (F_{1,3}: GACAAGCAATGAGCACCA) covered junction between exon-1 and -3 and reverse primer (R₃: GACA-TACTGAAGAATCAAAGTGTCCG) targeted to exon-3. A schematic representation of designed primers is shown in the Figure 3A.

Human *il-5* was PCR amplified with designed forward primer hIL5-F₁ (ACCTTGGCACTGCTTTCTAC) and reverse hIL5-R₃ (ACAGTTT-GACTCTCCAGTGTG) targeted to exons 1 and 3, respectively. Additionally, splice-specific primers to detect full-length and truncated mRNA transcript of human IL-5 were designed. Forward primers hIL5-F_{1,2} (GCCAATGA-GACTCTGAGGAT) and hIL5-F_{1,3} (CTGATAGCCAATGAGCACCC) covered junction between exon-1/2 and exon-1/3, respectively; common reverse primer hIL5-R₃ (ACAGTTT-GACTCTCCAGTGTG) targeted exon-3.

PCR amplification was set up using Tersus PCR Kit (Eurogen, Russia) and the following parameters: (95 °C 2 min) x 1 cycle, (95 °C 30 s, 56 °C 30 s, 72 °C 40 s) x 45 cycles. PCR products were run on 1,5% agarose gel (AppliChem, Germany) with ethidium bromide (Sigma-Aldrich, USA), and visualized using Gel Doc XR + instrument (BioRad, USA). DNA bands were excised from the gel and extracted with GeneJET Gel Extraction Kit (Thermo Scientific, USA). Purified PCR products were cloned into pAL-TA vector (3 kb) (Eurogen, Russia) or pJet1.2 (ThermoFisher, USA) and sequenced by Sanger's method on an automated DNA Analyzer (Applied Biosystems 3730xl, USA) using standard M13 forward and reverse primers.

2.3. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed using Mini-PROTEAN Tetra Handcast Systems (Bio-Rad, USA) with 12% gel

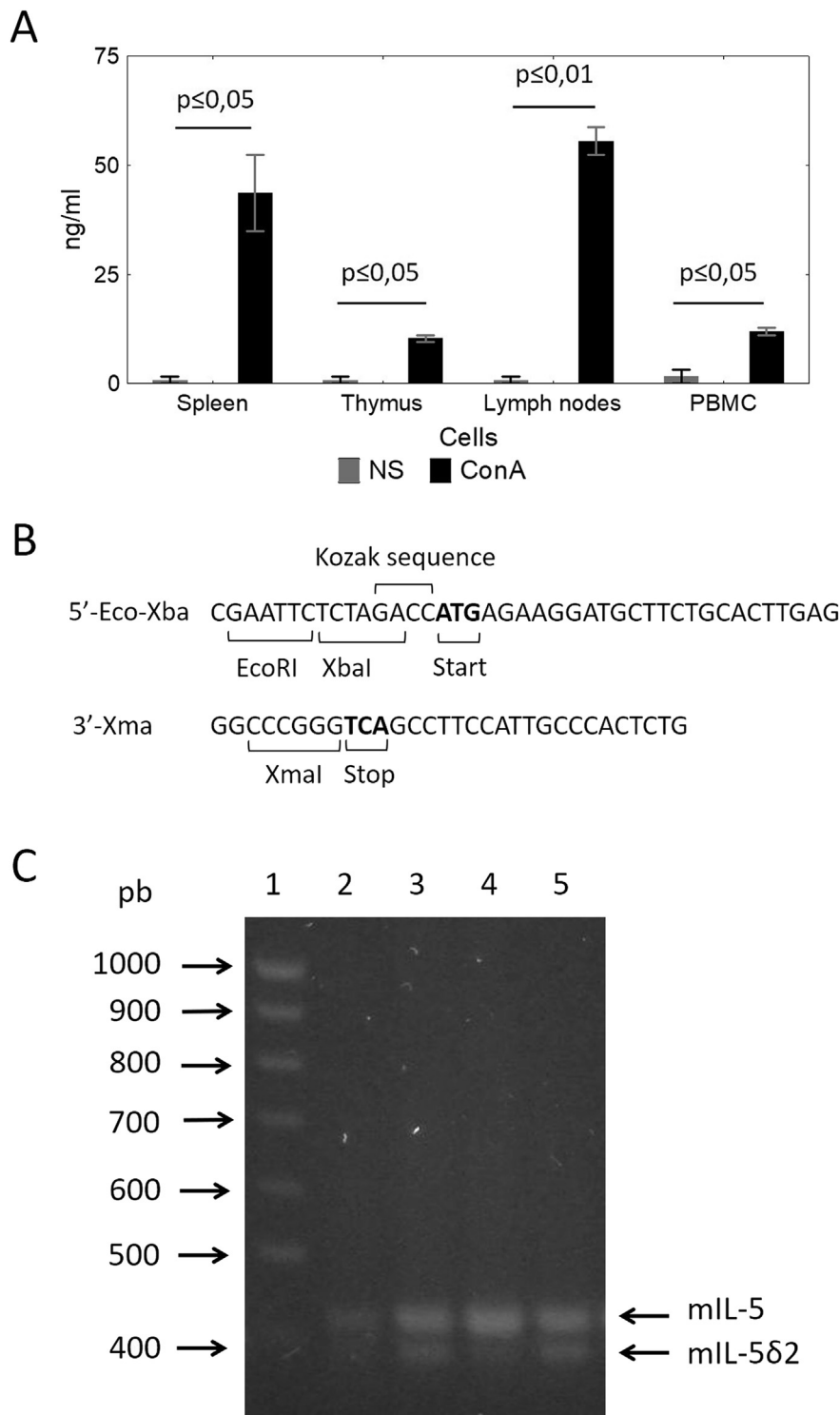


Figure 1. Expression of IL-5 in lymphoid cells isolated from different mouse tissues. **A.** ELISA quantification of mL-5 in resting and ConA-activated cells. Cells were isolated from indicated tissues of female BALB/c mice as described in Methods and activated with ConA for 24 h of left non-stimulated (NS). The supernatants were harvested and evaluated for mL-5 expression using ELISA. The data are representative of three independent experiments (N = 3) and shown as average \pm standard deviation. The differences are significant at indicated value of *p* calculated by Student's *t*-test. **B.** Schematic representation of primers used for *mIL-5* cDNA amplification and subsequent cloning into expression vector. **C.** Expression of two *mIL-5* transcripts in different mouse lymphoid tissues. The mRNAs of lymphoid cells isolated from spleen (2), thymus (3), PBMC (4) and lymph nodes (5) were purified, converted to cDNA, PCR amplified with mL-5 specific primers (B), and run on 1.5% agarose gel. Representative image of gel is shown. Full image is provided as supplementary material Fig S1.

according to the manufacturer's protocol. Fifteen μ l of DNA samples were placed on the gel with 6X DNA Loading Dye (Thermo Scientific, USA). Electrophoresis was carried out in TBE buffer at 70 V for 3 h. After electrophoresis, gels were removed from the cassette and stained for 15 min in water with 0.5 μ g/ml ethidium bromide (Sigma-Aldrich, USA), then rinsed with water and visualized using Gel Doc XR + instrument (BioRad, USA).

2.4. Plasmid construction for interleukin-5 expression

The coding sequences for full-length and δ 2 variants of *mIL-5* were PCR amplified from cDNA samples with primers containing Kozak and

flanking restriction sites, as indicated above. PCR products were first cloned into pAL-TA vectors, then sequence verified, subcloned into pCMVpA expression vector [28] using restriction sites XbaI and XmaI. pCMVpA vector is designed for gene expression in mammalian cells under control of CMV promoter.

2.5. Immunofluorescent microscopy

One day before transfection, 10^5 HeLa cells were seeded on coverslips in 24-well plate in complete medium. Cells were transfected overnight with 0.5 μ g of pCMVpA plasmid DNA encoding either full-length or δ 2 form of

mouse *il-5*. Transfection was carried out with Lipofectamine LTX (Invitrogen, USA) in accordance to manufacturer's instruction. Next day, samples were washed with PBS, fixed with 4% paraformaldehyde (Sigma, USA) in PBS for 15 min, washed with PBS again and permeabilized in PBS solution containing 0.1% saponin (Sigma, USA) and 1% FCS for 30 min. Full-length and short forms of *mIL-5* were stained sequentially with the goat polyclonal anti-mIL-5 antibody (R&D Systems, USA) and the secondary Alexa 488 anti-goat Ab (Molecular Probes, USA). Endoplasmic reticulum (ER) and Golgi complex were stained simultaneously in serum-free permeabilization solution containing rhodamine-labeled ConA (Invitrogen, USA) at a concentration of 50 µg/ml and wheat germ agglutinin Alexa 350 conjugate (Invitrogen, USA) at a concentration of 10 µg/ml, respectively. After washing, coverslips were transferred to the slides and maintained in Dako Cytomation Fluorescent Mounting Medium. Slides were analyzed with Olympus IX-71 inverted fluorescence microscope. Z-stacks were captured using Olympus cellSens Dimension software. The images were deconvolved with AutoQuant X3 software. Colocalization of interleukins with ER was estimated through Z-stacks of individual cells and from different areas of the samples as the Pearson Coefficient of Correlation (1.0 is full colocalization, 0 is no colocalization) using Fiji image processing software.

2.6. Flow cytometry

Sixteen hours prior transfection, 293T cells at a concentration of 2×10^5 per well were grown in 12-well plate. Cells were transfected with 1 µg of pCMVpA plasmid encoding one of the isoforms of *mIL-5* or empty plasmid (control) and 2 µl of Lipofectamine 2000 (Invitrogen, USA) in accordance to manufacturer's protocol. 24 hrs later, cells were trypsinized, washed with PBS and fixed with 4% paraformaldehyde. Cell permeabilization and intracellular staining with anti-IL-5 antibody was carried out as described above for HeLa cells. Samples were analyzed by FACScan flow cytometry instrument (Becton Dickinson) and Cell Quest software. Data files were presented using Win MDI 8.0 software.

2.7. ELISA

The concentration of murine IL-5 in the supernatants from ConA-activated primary mouse cells (see preparation in first paragraph of the Method section) were quantified using Mouse IL-5 ELISA Set (BD, USA) in accordance to manufacturer's protocol. To detect both $\delta 2$ and full-length *mIL-5*, 293T cells were transfected with plasmids as described above for flow cytometry analysis. Sixteen hours later, cells were washed once with PBS and incubated in serum-free culture medium. 48 hrs after transfection supernatants were harvested. To remove microvesicles and cell debris supernatants were centrifuged at 100 000 x g for 2 h. The clarified supernatants were sorbed overnight on the ELISA-treated plastic surface in 96-well plate. After removal of supernatants the wells were blocked with PBS containing 2% BSA, washed with PBST and incubated sequentially with the goat polyclonal anti-mIL-5 antibodies (R&D Systems, USA) and the HRP-conjugated anti-goat IgG (Sigma, USA). Peroxidase activity was developed with TMB substrate and analyzed on Microplate reader MPR 1 (SCO Diagnostics, Germany) at 450 wavelength.

3. Results

3.1. Discovery of a novel mouse *IL-5* transcript variant by DNA gel electrophoresis

During the earlier studies on the role of IL-5 in allergic disease models in mice, we aimed to clone *mIL-5* cDNA from mouse tissues for *ex vivo* experiments. Prior to cDNA preparation, we determined the levels of murine IL-5 protein produced by lymphocytes that were derived from different mouse tissues such as spleen, thymus, lymph node cells and blood. To induce cytokine production, cells were stimulated with 2 µg/ml ConA for 24 h prior supernatant harvest. The collected supernatants were

used to quantify the levels of *mIL-5* by ELISA commercial sandwich kit. As shown in Figure 1A, only activated cells produced IL-5.

In order to clone cDNA of mouse *il-5* gene into expression plasmid, we designed primers, where the appropriate restriction sites and Kozak sequence were added for cloning and optimal expression purposes, respectively (Figure 1B). The total RNA was isolated from ConA-activated cells, reverse transcribed with (dT)₁₈ primer, and PCR-amplified using designed primers. Surprisingly, after PCR products were resolved on agarose gel, not one, but two clear DNA bands with the size of approximately 430 and 390 bp were detected in all mouse tissue samples (Figure 1C). The analysis of the mouse *il-5* gene structure suggested that the heavy bands can represent a full-length *il-5* ORF with a size of 402 bp, whereas the light bands corresponded to a splice isoform, which is most likely omitting the shortest exon-2 of IL-5 with a length of 33 nt. The slightly higher size of upper band than the size of *il-5* cDNA is explained by the inclusion of Kozak sequence and three restriction sites in PCR product.

3.2. Sequence analysis of the novel mouse *IL-5* transcript variant

In order to analyze DNA sequence of alternative *il-5* transcript variant, both light and heavy PCR fragments were excised from the gel, purified, cloned into pAL-TA vector, and sequenced. Sequence analysis of cDNA fragments obtained from spleen, thymus, lymph nodes cells and PBMCs demonstrated that all heavy fragments have a sequence corresponding to the full-length *il-5* ORF, while all light DNA bands demonstrated the lack of exon-2 (33 bp) of *mIL-5* (Figure 2 A-B). The loss of exon-2 in the short fragments can be explained by alternative splicing that may occur during *mIL-5* mRNA maturation. Thus, the sequencing results indicated that the novel *il-5* mRNA splice variant represents the exact deletion of exon-2. Consequently, this mRNA splice variant of cytokine was designated as IL-5 $\delta 2$.

3.3. Identification of the mouse *IL-5 $\delta 2$* transcript using splice-specific primers and polyacrylamide gel electrophoresis

To gain an additional evidence that IL-5 $\delta 2$ transcript is produced, we designed a number of short splice-specific primers to detect full-length and truncated mRNA transcripts. Forward primers F₁₋₂ and F₁₋₃ covered exon-1/2 and exon-1/3 junction, respectively; reverse primer R₃ targeted to exon-3 (Figure 3A).

First, we checked the specificity of designed primers using pAL-TA plasmids, where full or short form was cloned. As demonstrated in Figure 3B, PCR amplification with F₁₋₂ and R₃ pair of primers from pAL-TA-*mIL-5* template gave a rise of a 97 bp product, while the use of F₁₋₃ instead of F₁₋₂ resulted in no product. Conversely, amplification from the pAL-TA plasmid carrying *mIL-5 $\delta 2$* resulted in generation of 70 bp PCR product with F₁₋₃ and R₃ primers, but not with a F₁₋₂ and R₃ combination of primers. Thus, the obtained results were expected and confirmed the specificity of splice form detection.

Second, using splice-specific primers (F₁₋₂ + R₃ and F₁₋₃ + R₃) we set up PCR reaction with cDNA samples purified from ConA-stimulated spleen, thymus and lymph node cells, and resolved DNA products by polyacrylamide gel electrophoresis. As shown in Figure 4A-C, the PCR products corresponding to both variants of mRNA transcripts (*mIL-5* and *mIL-5 $\delta 2$*) were clearly detected in mouse tissues samples.

In summary, we identified a new splice variant for murine *il-5* gene that lacks exon-2. This 33 bp truncated *mIL-5 $\delta 2$* mRNA is expressed in all tested murine tissues together with full-length IL-5, as demonstrated by using RT-PCR with splice-specific primers.

3.4. The $\delta 2$ protein isoform of murine *IL-5* is expressed and secreted

In order to understand how relevant is *mIL-5 $\delta 2$* mRNA transcript detection for cytokine production and function, we aimed to determine the expression of $\delta 2$ isoform of *mIL-5* at the level of protein in cells and

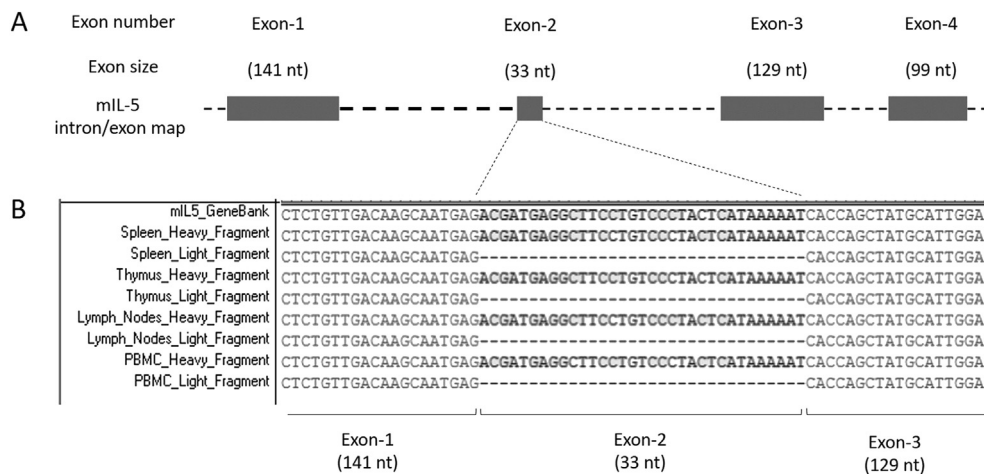


Figure 2. Sequence analysis of mouse IL-5 transcripts. **A.** Intron/exon map of mouse *il-5* gene. **B.** Alignment of reference mouse *il-5* coding sequence (GeneBank accession number X06270.1) with sequences of two (heavy and light) *il-5* cDNA fragments obtained from ConA-stimulated spleen, thymus, lymph nodes cells and PBMC.

supernatants. Since there are no antibodies that would specifically detect full-length and $\delta 2$ isoforms of mIL-5, it was not possible to distinguish these isoforms in primary mouse cells, which expressed both full and short transcripts of *il-5*.

To solve this problem, we cloned the coding sequences for *mIL-5* and *mIL-5 $\delta 2$* into expression plasmid under CMV promoter. Then we transfected 293T cells with one of the plasmids, which encodes either full-length or $\delta 2$ form of mouse *il-5*. To detect mIL-5 protein in the supernatants or inside the cells, we initially probed the different available anti-mIL-5 monoclonal antibodies (mAbs) and commercial ELISA Kit. However, none of the tested mAbs reacted with mIL-5 $\delta 2$ protein (data not shown). This suggests that deletion of exon-2 in mIL-5 is critical for the binding of mAb with cytokine or that the mIL-5 short form is not expressed as a protein. Unlike monoclonal, polyclonal antibody recognizes many epitopes that are present in an antigen. This prompted us to try goat anti-mIL-5 polyclonal antibody

available on the market to detect both the intracellular and secreted cytokine. To estimate intracellular expression of full-length and $\delta 2$ forms of mIL-5, transfected 293T cells were fixed, permeabilized, stained sequentially with primary and secondary Alexa488-conjugated antibodies, and analyzed by flow cytometry. As demonstrated in Figure 3A, the $\delta 2$ isoforms was expressed, albeit at the lower level than full-length mIL-5. This difference was observed by measuring two parameters, the percentage and the mean fluorescence intensity (MFI) of positive cells (Figure 5AB).

We also measured levels of two forms of mIL-5 in cell supernatants. Specifically, the next day after transfection, 293T cells were washed and incubated in culture medium without serum for 24 h. Supernatants were harvested, and sorbed on a plastic surface. Then wells were blocked with BSA, incubated with the primary goat and then the secondary anti-goat HRP-conjugated antibody, developed with substrate, and the results were

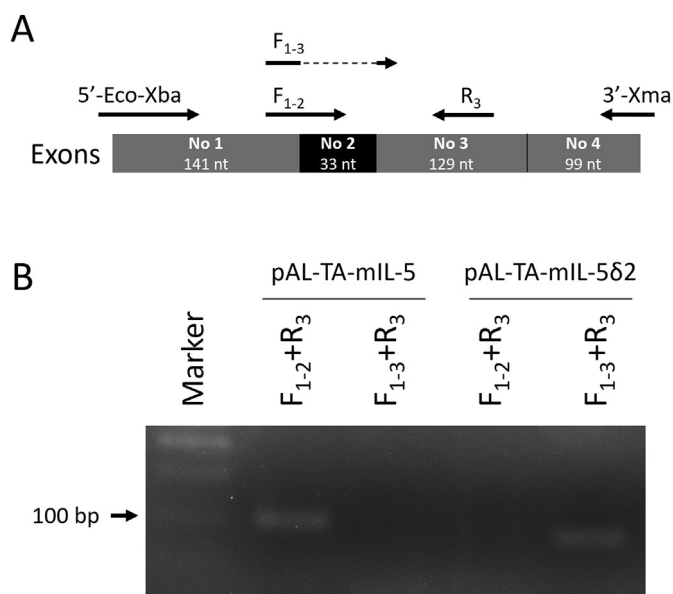


Figure 3. Schematic representation of mIL-5 and mIL-5 $\delta 2$ splice specific primers (A) and specificity PCR amplification on plasmid template (B). pAL-TA plasmid carrying mIL-5 or mIL-5 $\delta 2$ transcript were PCR amplified using two primer pairs F₁₋₂ + R₃ and F₁₋₃ + R₃ followed by agarose gel electrophoresis separation of the amplicons. One of the representative gel photographs is presented. Full image is provided as supplementary material Fig S2.

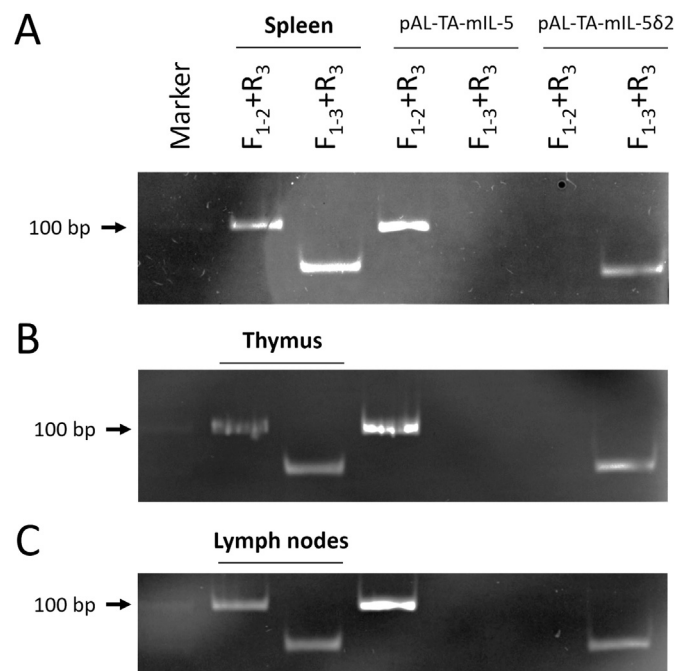


Figure 4. Expression of two *mIL-5* transcripts in different mouse lymphoid tissues. The mRNAs of lymphoid cells isolated from spleen (A), thymus (B) and lymph nodes (C) were purified, converted to cDNA, PCR amplified using splice-specific pairs of primers (B), and run on polyacrylamide gel. Representative images of gel are shown. Full image is provided as supplementary material Fig S3, S4, S5.

recorded on ELISA reader. As shown in Figure 5C, the level of IL-5 δ 2 protein was about 1.3-fold lower than the level of full-length cytokine. Together, these data demonstrate that mIL-5 δ 2 can be expressed at the level of protein, although at a relatively lower level than canonical form of cytokine.

3.5. The intracellular localization of mIL-5 and mIL-5 δ 2 proteins are different

To clarify the mechanisms involved in the expression of δ 2 isoform, we determined localization of the full-length and the short forms of mIL-5 protein within the intracellular compartments. HeLa cells were transfected overnight with the expression plasmid encoding one of the isoforms. The cytokine was stained with goat polyclonal antibody as described above. The endoplasmic reticulum (ER) and Golgi complex were labeled with rhodamine-conjugated ConA and Alexa350-conjugated wheat germ agglutinin (WGA), respectively. As shown in Figure 6A, the full-length IL-5 (green) colocalized very well with the ER (red); the Pearson coefficient of correlation (R) as a measure of colocalization was 0.59 ± 0.05 . The δ 2 form of mIL-5 colocalized with ER at the lower level than the full-length form; $R = 0.38 \pm 0.06$ (Figure 6B). This difference was statistically significant at $p < 0.01$. Neither δ 2 nor full-length isoform of mIL-5 accumulated in the Golgi complex (blue). Normally cytokines accumulate in this compartment, when secretion of protein is blocked with monensine or other chemicals. However, initially cytokines pass the ER and then transported to the Golgi complex. Our data suggest that the mislocalization of mIL-5 δ 2 (out of ER) can be responsible for its partial degradation and/or impaired secretion in a conditioning medium.

3.6. Identification of human IL-5 δ 2 transcript

It was logical to hypothesize that the human IL-5 mRNA may also undergo to alternative splicing. To check this possibility, first, we compared intron/exon structures of the mouse and the human *il-5* genes and found them quite similar. Importantly, the exon-2 of the human gene

has the same length (33 bp) as the corresponding mouse exon-2 [5] (Figure 8AB). In order to identify the human *IL-5 δ 2* transcript, we isolated PBMCs from 3 healthy subjects and 3 patients with bronchial asthma and cultured them individually with or without PHA stimulation. 24 hours later, non-stimulated cells produced significantly less quantity of hIL-5 compared to stimulated ones, as was assayed by qPCR (Figure 7A). Hence, we isolated total RNA from only stimulated PBMC of 3 healthy subjects and 3 bronchial asthma patients (totally 6 RNA samples were received) and converted to cDNAs as described for murine cells. Three cDNAs from PHA-stimulated PBMC of healthy subjects were pooled in one cDNA sample. Similarly, three cDNAs from bronchial asthma patients were pooled, as well. Totally two cDNA libraries were received.

The human cDNAs were PCR-amplified using primers hIL5-F₁ and hIL5-R₃ specific to exon-1 and -3, respectively. After a separation of products by polyacrylamide gel electrophoresis we have revealed two bands (140 and 107 bp) corresponding to full-length and truncated mRNA transcript presumably lacking exon-2 (Figure 7B). To gain an additional evidence that the human IL-5 has an alternative splice mRNA variant, we set up 45 cycles PCR amplification with cDNA templates using the splice-specific primers. Forward primers hIL5-F_{1,2} and hIL5-F_{1,3} covered junction between exon-1/2 and exon-1/3, respectively; common reverse primer hIL5-R₃ targeted exon-3. Amplification using primer pairs hIL5-F_{1,2}/hIL5-R₃ and hIL5-F_{1,3}/hIL5-R₃ followed by agarose gel electrophoresis revealed the specific DNA bands of 102 bp and 74 bp, respectively, in cDNA samples from patients with bronchial asthma and healthy donors. As shown in Figure 7C, the size of the heavy band corresponded to the full-length hIL-5 and the size of the light band matched the exon-2(-) alternative mRNA transcript (hIL-5 δ 2). Additionally, we performed PCR amplification with splice-specific primers under reduced numbers of cycles (40, 35 and 30). We determined that both hIL-5 δ 2 and full-length transcripts can be clearly detected even at 35 cycles (Figure 7D). Next, both size PCR products were gel purified, cloned into pJet1.2 vector, and DNA-sequenced by Sanger method. As illustrated in Figure 8, the exon-intron structure of the human and mouse *il-5* genes are

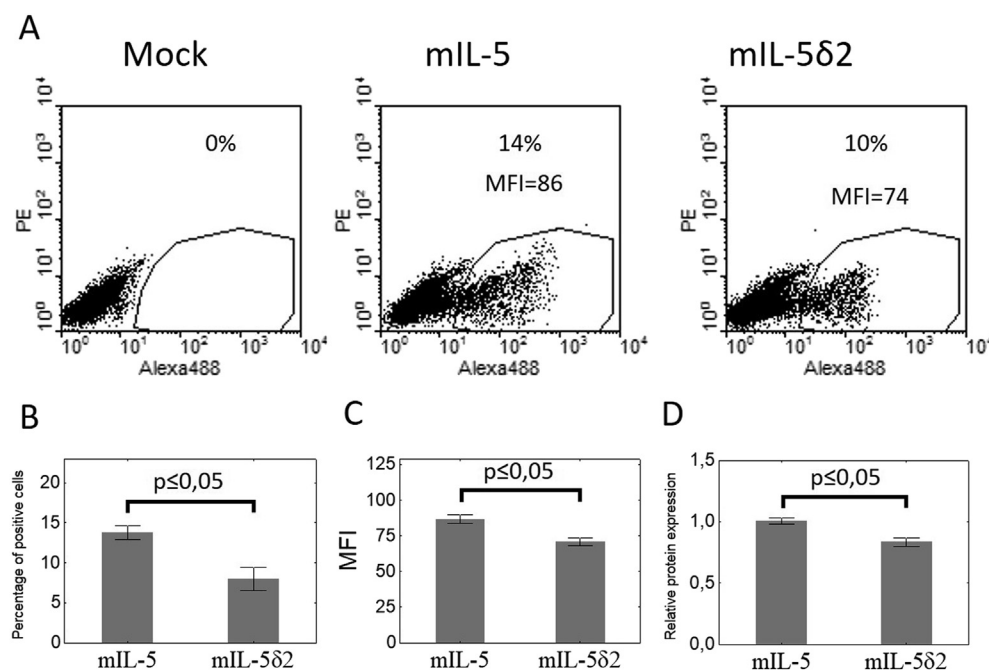


Figure 5. Protein analysis of mIL-5 δ 2 isoform expression. A. Flow cytometry graphs demonstrating intracellular expression of full-length and δ 2 forms of mIL-5. Human 293T cells were transfected with an equal amount of pCMVpa expression plasmid encoding one of the variants of mIL-5 or with empty plasmid (Mock). 24 hrs later, cells were fixed, permeabilized, and stained for mIL-5 with polyclonal antibody. The percentage and mean fluorescence intensity (MFI) values for mIL-5 expressing cells (gated on DotPlots) are shown. The magnitudes of these parameters obtained from four independent experiments were calculated and presented in respective histograms (B and C). D. The levels of mIL-5 isoform expression in the supernatants. 293T cells were transfected as described in A, then incubated in serum-free medium for 24 h. The levels of cytokines were quantified as indicated in Results and Methods sections. The absorption values obtained for the full-length IL-5 was set at 1, and the expression of δ 2 isoform was recalculated relative to that of the full-length protein. The data are representative of at least four independent experiments and shown as average values with standard deviations. The differences are statistically significant at indicated p values calculated using Mann-Whitney U-test.

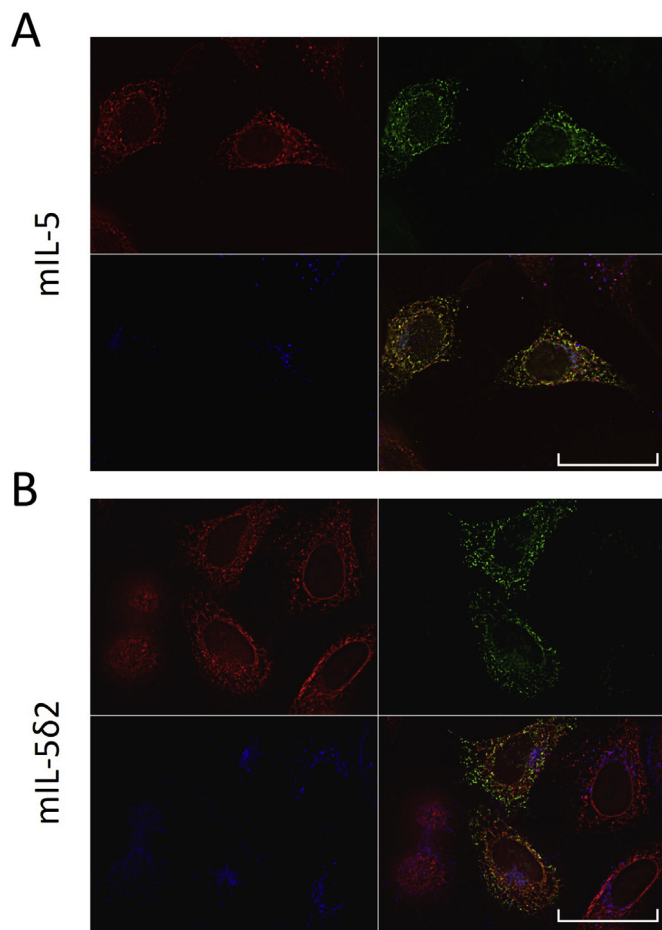


Figure 6. Intracellular localization of full-length and $\delta 2$ isoforms of mIL-5. HeLa cells were adhered on glass coverslips and transfected overnight with expression plasmid encoding full protein (A) or $\delta 2$ isoform (B) of mIL-5. After fixation and permeabilization cells were stained for mIL-5 first with the primary goat polyclonal and then with the secondary Alexa488-conjugated antibodies (green). Endoplasmic reticulum (ER) (red) and Golgi complex (blue) were labeled with rhodamine-ConA and Alexa350-WGA (wheat germ agglutinin) conjugates. Z-stacks of cells were captured with fluorescence microscope and deconvolved. Representative images of the cells with optical section adjacent to the plastic surface are shown. Scale bar, 20 μm .

very similar (A-B) and the shorter form of cytokine lacks precisely 33 nt corresponding to exon-2.

In conclusion, we have identified the new splice variant for the human *il-5* gene, which, like mouse *il-5* gene, lacks exon-2. The new $\delta 2$ isoform of the human IL-5 mRNA along with the full-length cytokine mRNA was detected in PHA-stimulated PBMCs isolated both from healthy subjects and patients with allergic asthma.

4. Discussion

In this study, we describe a novel mRNA transcript for the mouse and the human *il-5* gene. This transcript has a deletion of the entire exon-2 (*mIL-5 $\delta 2$*) that was detected in all tested types of murine cells. The sequence analysis of cDNA obtained from different murine cells and human PBMCs showed that *IL-5 $\delta 2$* lacks 33 nucleotides matching the exon-2. Thus, the short variant of *IL-5* mRNA represents an alternatively spliced variant of the cytokine.

Despite that IL-5 had been discovered a long time ago, no alternatively spliced forms for this cytokine has been reported. One possible explanation is that the majority of primary murine cells express both full and $\delta 2$ mRNAs of *il-5* (Figure 1C), and it is difficult to distinguish two

forms of *mIL-5* at the level of protein without generation specific detection reagents, such as antibodies.

Unlike *il-5*, the $\delta 2$ isoform has been described previously for human [29] and mouse [30] Th2-cytokine IL-4. This IL-4 $\delta 2$ form has a certain similarity to IL-5 $\delta 2$; both have a deletion of the second exon (16 and 11 amino acids, respectively) and the overall architectural similarity with a four-helix bundle motif [31]. The human *IL-4 $\delta 2$* is naturally expressed at the protein level [32, 33, 34, 35], interacts specifically with the hIL-4R receptor and competes effectively with the full-length IL-4 for the receptor binding site, which is common for both variants [29, 36]. Thus, the affinity of IL-4 $\delta 2$ to its natural receptor is not affected dramatically due to deletion of 16 aa [25, 26].

The length of protein, which is translated from the newly described *mIL-5 $\delta 2$* mRNA should be 122 aa. The short protein isoform of mIL-5 can be structurally very similar to the full-length mIL-5 (133 aa). However, in our study, mIL-5 $\delta 2$ protein was detected with a polyclonal antibody and not with any monoclonals that we tested. This suggests that 11 aa corresponding to exon-2 of mIL-5 can be important for the formation of epitope structure of cytokine. Whether mIL-5 $\delta 2$ binds to the IL-5R or other types of cytokine receptors and transduces signaling in the recipient cells remains to be investigated. A certain assumption can be made based on X-ray data available for the complex of mIL-5 with receptor chain IL-5R α [25, 26]. These structural studies showed that full-length IL-5 protein is composed of four α -helices ($\alpha 1$ – $\alpha 4$), connected by three loops containing β -strands and disordered regions. Importantly, the described mIL-5 $\delta 2$ isoform retains conserved cysteine residue at position 62 (see Figure 9). This residue is critical for the formation of functional homodimer of mIL-5, because it is involved in the formation of two intermolecular disulfide bonds, Cys62–Cys104 and Cys104–Cys62. Eleven aa that are absent in mIL-5 $\delta 2$ (Figure 9) located entirely in the loop $\alpha 1$ – $\alpha 2$ of mIL-5. These residues are involved in the formation of interchain hydrogen bonds to stabilize the IL-5 dimer structure and form the correct orientation for interaction with the cohesive receptor [25, 26]. Hence, the deletion of this fragment can potentially affect the folding of mIL-5 $\delta 2$ protein and its interaction with the receptor. In addition, it has been demonstrated that not only loop $\alpha 3$ – $\alpha 4$, but also the loop $\alpha 1$ – $\alpha 2$ is directly involved in the interaction of mIL-5 with the receptor IL-5R α [38].

In our study, we have demonstrated that the novel isoform of *mIL-5 $\delta 2$* could be expressed as a protein inside the cells, and secreted outside of cells, but at the levels that are lower than that detected for the full-length mIL-5. The fluorescence microscopy examination showed that the level of $\delta 2$ form colocalization with ER was moderately lower, than the same parameter measured for the full-length mIL-5 protein. This altered compartmentalization of mIL-5 $\delta 2$ protein suggests that the short form of mIL-5 may have some folding problems, which may enhance intracellular degradation of protein inside the cells, impair its transition from the ER to the Golgi apparatus and ultimately reduce the level of its secretion. Nevertheless, the observed differences in the expression and secretion of $\delta 2$ form compared to the full form of mIL-5 were not drastic.

It was interesting to know whether the IL-5 mRNA from different species undergoes the similar alternative splicing. Currently, there are two alternative transcripts for the human IL-5 available on databases [27]. The first variant (TranscriptID: ENST00000450655.1) contains 3 exons (loss of exon-4) and could code 51 aa protein, and the second one (TranscriptID: ENST00000462418.1) contains 2 exons and does not possess ORF. In order to detect *hIL-5 $\delta 2$* transcript, we purified cDNAs from PHA-stimulated PBMCs, which were isolated from either healthy subjects or patients suffering from allergic bronchial asthma. Individuals with the indicated pathology were chosen because IL-5 play a significant role in allergic asthma [39]. Since it has been reported that IL-5 expression increases asthma symptoms [40], it was interesting to find the expression of canonical and $\delta 2$ forms of IL-5 in donor and patient samples. Using splice-specific primers we clearly detected both forms of IL-5 mRNA in healthy individuals and asthma patients (fig. 7BC), and confirmed the deletion of exon-2 in a short form by DNA sequencing

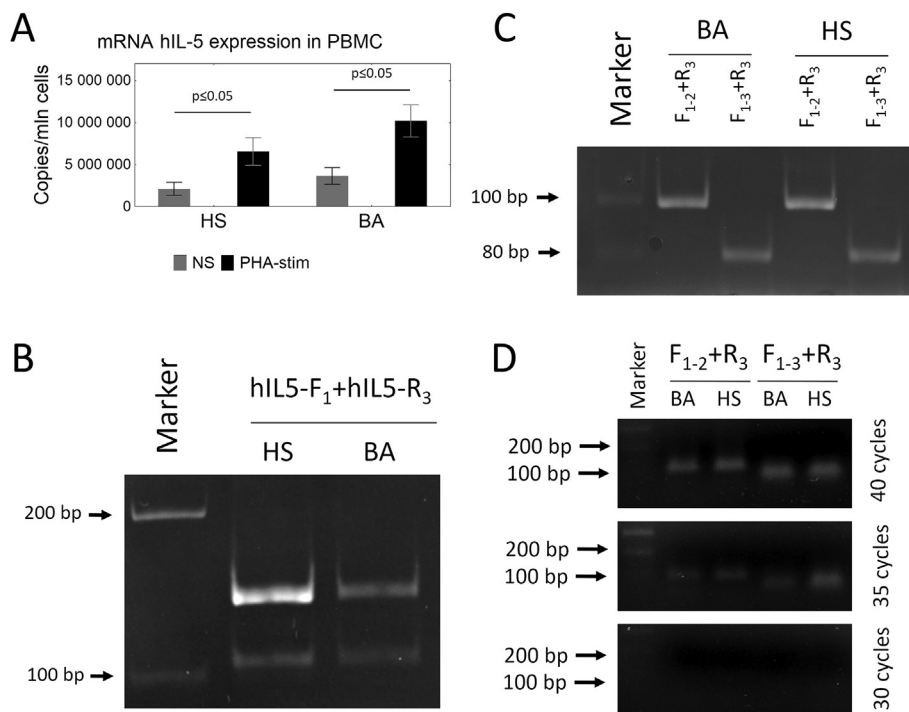


Figure 7. Analysis of human IL-5 transcripts expression in PBMC. A. qPCR quantification of hIL-5 in resting and PHA-stimulated PBMC. PBMCs were isolated from patients with bronchial asthma (BA) or from healthy subjects (HS) and activated with PHA for 24 h or left non-stimulated (NS). Afterwards, total mRNA was isolated, converted to cDNA followed by qPCR quantification. The data are representative of three independent experiments and shown as mean of copies hIL-5 mRNA per 10^6 cells \pm standard deviation. P values were determined by Mann-Whitney U-test. cDNAs from indicated samples were PCR-amplified (45 cycles) with a pair of primers targeting exon-1 and -3 (B) or a pair of splice-specific primers using 45 cycles of amplification (C) or lower numbers of cycles (D). Representative images of polyacrylamide gels (B and C) and of agarose gels (D) are shown. Full image is provided as supplementary material Fig S6, S7, S8.

(Figure 8). If the human IL-5 δ 2 play any role in IL-5-mediated signaling and manifestation of allergic diseases in humans remains unknown and can be investigated in future more comprehensive and quantitative experiments.

In summary, we described a novel splice form for the mouse and the human IL-5 with identical deletion of the entire exon-2 (*mIL-5 δ 2* and *hIL-5 δ 2*) that has not been reported earlier. We believe that the δ 2 isoform can

have a function, since it keeps ORF and important elements for cytokine secretion and dimerization intact, and at least the mouse IL-5 δ 2 protein can be detected in cells and supernatants with a polyclonal antibody. However, the development of reagents for specific detection of IL-5 δ 2 protein, purification of recombinant protein, cytokine receptor studies, selective knockout of IL-5 isoforms and other methods will clarify the function of the earlier unknown isoform of the human and mouse IL-5.

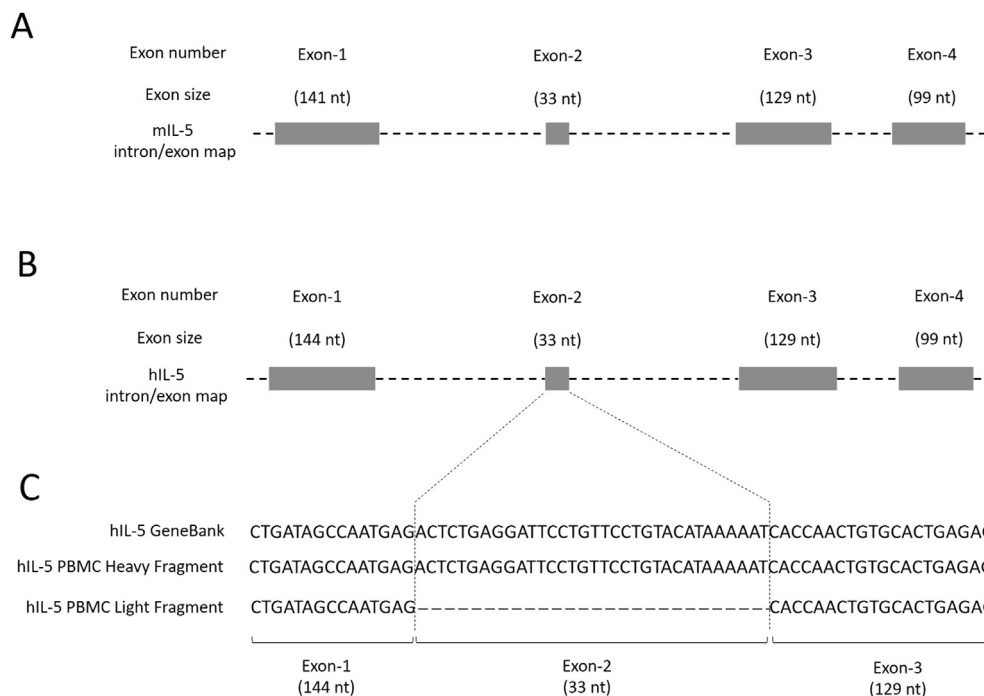


Figure 8. Sequence analysis of human IL-5 transcripts. Intron/exon structure of the mouse (A) and the human (B) *il-5* gene. C. Alignment of the reference human *il-5* mRNA sequence (GeneBank accession number NM_000879.2) with sequences of two (heavy and light) *il-5* cDNA fragments obtained by PCR from PHA-stimulated PBMCs. *mIL5* – mouse IL-5, *hIL5* – human IL-5, nt – nucleotides.

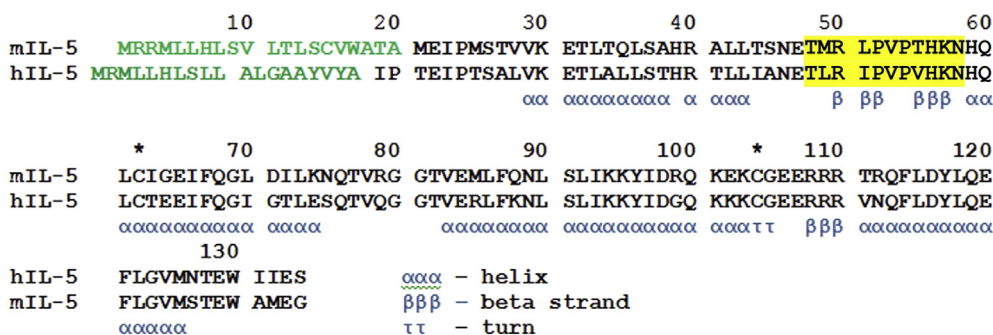


Figure 9. Amino acid sequence alignment of mouse and human IL-5. Alignment of mouse (top) with human IL-5 (bottom) was performed using ClustalW algorithm. The fragment deleted in $\delta 2$ splice isoform of mIL-5 is highlighted in yellow. Signal peptide is shown by green letters. The elements of protein secondary structure are depicted by Greek symbols below the sequence alignment. Cysteine (C) residues that are important for the formation of cytokine domains are indicated by asterisks.

5. Conclusions

1. We identified new splice variants for murine and human *il-5* genes that lack exon-2. mIL-5 $\delta 2$ mRNA is expressed in murine spleen, thymus, lymph nodes and PBMC; hIL-5 $\delta 2$ was observed in PBMC from healthy subjects and allergic asthma patients.
2. By cloning $\delta 2$ isoform of murine IL-5 and expressing it in the human 293T cells, we confirmed that mIL-5 $\delta 2$ protein can be expressed and secreted, although at a slightly lower level than mIL-5.
3. The $\delta 2$ form of mIL-5 was less colocalized with the ER than the full-length form. Neither $\delta 2$ nor full-length isoform of mIL-5 accumulated in the Golgi complex. The partial mislocalization of mIL-5 $\delta 2$ (outside of the ER) can be responsible for its enhanced degradation and/or reduced secretion into surrounding medium.

Declarations

Author contribution statement

Igor Shilovskiy, Musa Khaitov: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sergei Andreev: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Dmitriy Mazurov, Svetlana Bolotova, Alexander Nikolskii: Performed the experiments.

Ekaterina Barvinskaia: Performed the experiments; Wrote the paper.

Ilya Sergeev, Dmitrii Kudlay: Contributed reagents, materials, analysis tools or data.

Artem Maerle: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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