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Characterization of differentially spliced interleukin-7 and interleukin-7 receptor isoforms: paradigms for alternative splicing in the immune system

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Summary

Alternative splicing is a frequently occurring phenomenon in immunologically relevant molecules such as interleukin (IL)-2 (Vjacheslav et al., 1996), IL-4 (Atamas et al., 1996), IL-6 (O.P.Yatsenko et al., 2003), IL-10 (Shuling Wu et al., 2005), IL-15 (X Tan and L Lefrancois, 2006), and it also applies to IL-7 and its receptors (R). Due to alternative splicing more than one isoform of the wild type protein can be produced. In general alternative splicing may cover the spectrum of loss of function to the gain of function of the respective protein. Alternative spliced IL-7 isoforms differ in one, or more than one exon from the canonical protein. In the current study we cloned and expressed 6 different isoforms of the IL-7 in *E.coli*. The corresponding IL-7 isoforms were named based on the missing exons i.e IL-7 δ 4 lacks exon 4, IL-7 δ 5 lacks exon 5, IL-7 δ 4/5 lacks exon 4 and exon 5, IL-7 δ 3/4/5 lacks exon 3, 4 and 5, IL-7 δ 2 lacks a part (56bp) of exon 2, and IL-7 δ 4/2 lacks exon 4 and a part (56bp) of exon 2.

Genetic sequence comparison revealed that the cDNA of the IL-7 isoforms is identical to the cDNA of the originally described IL-7 canonical protein throughout the entire coding region except that of the omitted exons. IL-7 mediates (Signal transducer and activator of transcription) STAT-5 phosphorylation via JAK1 and JAK3. We measured STAT-5 phosphorylation induced by IL-7 in peripheral blood mono nuclear cells (PBMCs) by flowcytometry and used this method as a read out for the biological role of alternatively spliced IL-7 isoforms. We showed that some IL-7 isoforms do not phosphorylate STAT-5. IL-7 isoforms showed a dose- dependent increase in STAT-5 phosphorylation.

IL-7 signaling could be blocked using a monoclonal antibody directed against the IL-7R. We could also demonstrate alternative splicing of the IL-7R. In our study we cloned 2 different isoforms of the IL-7R alpha (IL-7R α). Two IL-7R isoforms are identical in the cDNA sequence as compared to the IL-7R except missing exons. Interestingly, a missing exon encodes for the transmembrane part of the R, which would lead to the retainment of the IL-7R in the cytoplasm. The IL-7R isoform, which lacks exon 6, is called IL-7R soluble. Sequence comparisons of the other IL-7R isoforms with the IL-7R revealed that it lacks exon 5 in addition to exon 6, results in the premature termination of translation such that cDNA would code for a R protein that contains a cytoplasmic domain of only 2 amino acids, which is known as IL-7R soluble δ 5.

Introduction

IL-7 plays a major role in survival, proliferation, differentiation and maturation of haematopoietic cells including B and T-lymphocytes. IL-7 stimulates the proliferation of anti-tumor reactive cells and a number of T and B-cell malignancies (Alexander Korte., et al 1999). IL-7 also activates natural killer (NK) cell precursors and promotes expression of CD56, production of TNF- α and peripheral monocytes, inducing their tumoricidal activity and secretion of secondary cytokines (Alderson et al., 1990). Recently it has been shown that IL-7 protects NK cells (Armant et al., 1995) and T-cells (Hernandezcaselles et al., 1995) from death by apoptosis.

Inactivation of IL-7 by gene targeting techniques leads to lymphopenic phenotype in mutant mice (U.Von Freeden et al., 1995). In contrast, targeted gene deletion of other cytokines such as IL-2, IL-4, or IL-10 revealed that these cytokines are not essential for development and proper function of either B-lymphocytes or T-lymphocytes (Schorle, H., T. Holtschke, et al., 1991, Kuhn, R., K. Rajewsky et al., 1991, Murray, R., T. Suda et al., 1989).

IL-7 was first discovered at Immunex Research and Development Corporation in 1988 (Namen et al., 1988). The production of IL-7 takes place in the thymus most likely by epithelial cells (Namen et al., 1988) and it is also produced by some other cells in the body such as bone marrow stromal cells (Funk et al., 1993), intestinal endothelial cells (Watanabe et al., 1995; Laky et al., 1998) and keratinocytes in skin (Heufler et al., 1993). The human IL-7 gene is located on chromosome 8q12-13 (Sutherland.,et al 1989) which contains 6 exons over 33Kb with an open reading frame of 534 nucleotides and the mature protein contains 177 aminoacids, including a signal peptide of 25 amino acids. The amino acid sequence predicts a molecular weight of 17.4 Kd, but glycosylation results in 25 Kd. Three dimensional structure modeling of human IL-7 (Fig.1) predicted, based on homology to other cytokines like IL-4, IL-2 and granulocyte macrophage cell stimulating factor (GM-CSF), that human IL-7 belongs to the 4 α -helix bundle family of cytokines (including IL-2, -4, -6, -9, -15 and -21). All four helices connect to each other with two long and one short loop (Larry Cosenza., et al 2000) in a up-up and down-down topology (Wlodawer et al., 1993). Amino acid sequence of IL-7 contains 6 cysteines and form 3 disulphide bridges (Romano et al., 1996).



Fig. 1 Predicted 3D structure of human IL-7 and positions of cysteine molecules, which form disulphide bridges. Four helices given in different colors, orange-helix A, yellow-helix B, green-helix C, blue- helix D. (Source: Romano T.Kroemer et al., 1996)

IL-7 signals through the IL-7 receptor (R) complex, which is composed of at least two subunits: IL-7Ralpha (IL-7R α) chain (CD127), and the common cytokine-R gamma (γ)-chain (CD132), which is commonly shared among other IL-receptors such as IL-2, IL -4, IL-9, and IL-15 (Noguchi et al., 1993). IL-7R α and γ -chain dimer formation is crucial for high affinity binding of IL-7 (Renata et al., 2007). Six extracellular and four intracellular cysteine residues are present in human as well as in the murine IL-7R α coding for a 439 amino acid protein with a calculated molecular weight of 49.5 KDa (Sugamura et al., 1996).The extracellular domain of IL-7R α contains 220 amino acids, transmembrane domain consists 25 amino acids and the cytoplasmic domain contains 195 amino acids.

IL-7R α has been detected on pre-B cells, thymocytes, some T-lineage cells (Park et al., 1990; Rich et al., 1993), on human intestinal cells (Reinecker and Podolsky, 1995), colorectal cancer cells, renal cell cancer cells, on bonemarrow derived macrophages and cutaneous T-cell lymphomas (Bagot et al., 1996) but not on mature B-cells (Foxwell., 1992). The human IL-7R α is expressed on both naive and activated memory CD4⁺ or CD8⁺ T-cells.

The IL-7R α chain is also used by thymic stromal-derived lymphopoietin (TSLP) as a part of a complex that contains a second R chain (TSLPR), which is exclusively used by TSLP (Park et al., 2000). TSLP is an epithelial cell derived cytokine highly expressed by bronchial epithelial cells, muscle cells, lung fibroblasts and primary skin keratinocytes (Z.Shamim et al., 2006). The function of the TSLP-IL-7R α pathway is still under investigation. In general,

activation of IL-7R α in T-cells appears to be from IL-7 itself; the phenotypes of knockout mice that lack IL-7R α or IL-7 are similar but are distinct from the phenotypes of mice that lack TSLPR (Renata Mazzucchelli et al., 2007). Development of early stages of lymphocytes is highly dependent on IL-7R signaling. Almost complete elimination of pre, immature and mature B-cells in the IL-7R $^{-/-}$ mice demonstrates the great importance of IL-7R and IL-7 in lymphocyte differentiation and proliferation (Peschon et al., 1994, von Freeden-Jeffry et al., 1995).

IL-7R signalling is initiated when IL-7 binds to the extracellular domains of IL-7R α which leads to dimerization with γ_c (Fig. 2). Janus kinase (JAK) 3, associated with γ_c , phosphorylates tyrosine residues in the cytoplasmic portion of the IL-7R α , and leads to the recruitment of JAK1. JAK1 and JAK3 mutually phosphorylates each other. JAK proteins phosphorylate IL-7R α , and create a docking site for other signalling molecules. A key signalling molecule is STAT-5, (signal transducer and activator of transcription) 5, which is phosphorylated and interacts with an area spanning the tyrosine residue 409 at the c-terminal end of the IL-7R α . Phosphorylated STAT-5 then dimerizes and translocates to the nucleus where it induces specific target gene transcription (Terry J. Fry et al., 2002, Renata Mazzucchelli et al., 2007). The requirement of the γ_c is crucial due to the lack of intrinsic tyrosine kinase activity in IL-7R α . JAK3 is necessary to trigger phosphorylation of IL-7R α -associated proteins (Lai SY, XU W, Gaffen SL, et al 1996). A chimeric erythropoietin R containing JAK2 can substitute for γ_c - associated JAK3 (Lai SY, Molden et al., 1997).

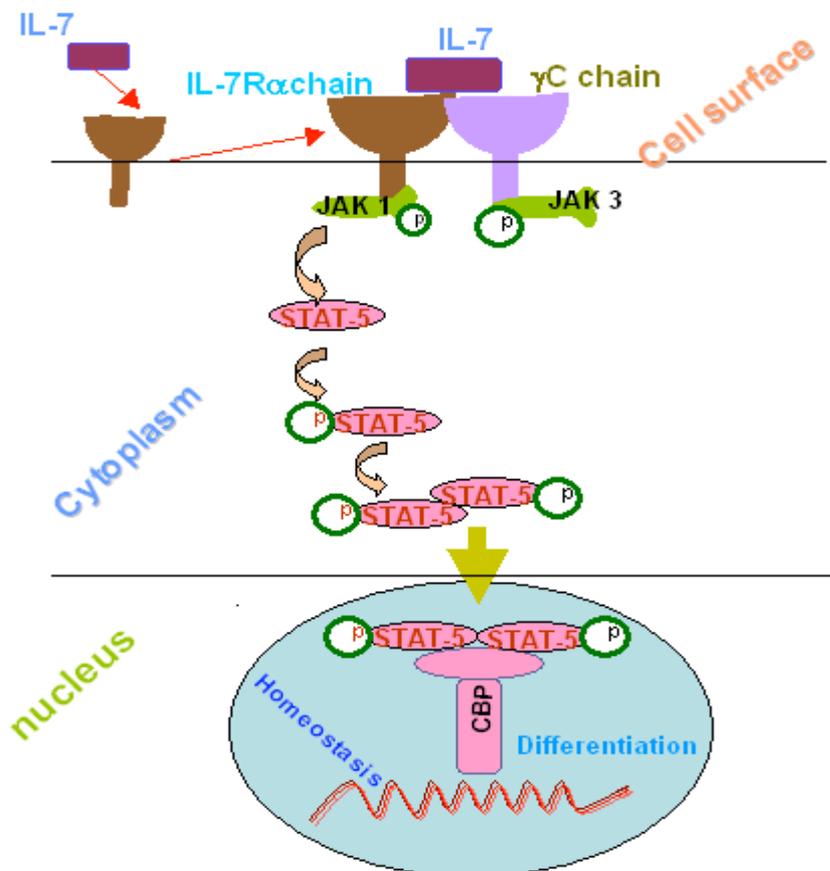


Fig. 2. JAK-STAT signaling cascade: Attachment of IL-7 to the R leads to the start of the signaling cascade. IL-7R α heterodimerizes with the common γ -chain, which in turn recruits the signal molecules JAK1 and JAK3. They phosphorylate each other and subsequently the STAT molecule. The phosphorylated STAT molecule dimerizes and signals to the nucleus.

Defective IL-7R signaling is one of the principal causes of severe combined immunodeficiency disease (SCID) in mice and humans. A novel mutation of the IL-7R in a Korean SCID patient has been described with greatly diminished T-cell count but normal numbers of B-cells and NK cells (Eun-Kyeong Jo, Hoon Kook et al., 2004).

Alternative splicing is a very common phenomenon seen in cytokines. Alternative splicing of IL-4 mRNA lacking exon 2, (IL-4 δ 2) has been reported (Sergei p. Atamas et al., 1996). Similar to IL-4 δ 2, which inhibits IL-4 to bind to its R and blocks T-cell proliferation, two isoforms of IL-2, IL-2 δ 2 and IL-2 δ 3, which lack exon 2 and exon 3 respectively inhibit IL-2 from binding to its high affinity R (Vjacheslav N. Tsytsikov et al., 1996). Alternative splicing of similar effects were also found in IL-7. Until now approximately 7 isoforms of IL-7 were found (A. Korte et al., 1999) but no biological role of these isoforms has been assigned.

Similar to the IL-7, its R also produces differentially spliced IL-7R α isoforms (A. Korte et al., 2000). A differential splicing event in mRNA encoding for the secreted form of the IL-7R capable of binding IL-7 in solution, a form that may be important for binding circulating IL-7 (Raymond G. Goodwin., 1990) has been reported. These transcripts have been identified in human acute lymphoblastic leukemia and apparently lack a part of cytoplasmic domain, but they are still capable of binding IL-7, similar to the intracellularly truncated erythropoietin R that fails to mediate proliferation and is capable of inhibiting the functions of the wild-type R in a dominant negative fashion (Nakamura and Nakauchi, 1994).

Aims

The aim of the present project was to characterize differentially spliced IL-7R isoforms and to test the biological activity of the different IL-7 isoforms using the STAT-5-assay.

Materials and Methods

Subjects

Peripheral heparinized blood from healthy donors was obtained from the blood bank, Karolinska Hospital, Stockholm, Sweden.

PBMC's

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by Ficoll-paque separation (Amersham Pharmacia, Uppsala, Sweden) and frozen in liquid nitrogen.

Isolation of RNA

Total RNA was isolated from PBMCs (Donor 1- 3×10^6 cells, Donor 2- 1.6×10^6 cells, and Donor 3- 4×10^6 cells) using RNeasy kit (Qiagen, VWR AB, Stockholm) according to the manufacturers directions.

Preparation of cDNA

Total RNA was converted into cDNA using superscriptTMIII First strand synthesis system for RT-PCR (Invitrogen life technologies) according to the user's guidelines. RT-PCR was performed in a total volume of 10 μ l. The RNA/primer reaction mixture containing 8 μ l of RNA (up to concentration of 5 μ g), 1 μ l of 50 μ M oligo(dT) and 1 μ l of 10 mM dNTP mix, incubated at 65°C for 5 min and at 4°C for 1 min. Ten μ l of cDNA synthesis mix containing 2 μ l of 10x RT buffer, 4 μ l of 25 mM MgCl₂, 2 μ l of 0.1 M DTT, 1 μ l of RnaseOUTtm (40 U/ μ l) and 1 μ l of superscripttmIII RT (200 U/ μ l) was mixed with RNA/primer Mixture, vortexed gently and incubated at 50 °C for 50 min and finally the reaction was terminated at 85 °C for 5 min and 1 min at 4 °C. One μ l of RnaseH was added to the above mixture and incubated at 37 °C for 20 min.

Amplification of IL-7 and IL-7R cDNA by PCR

PCR primers were designed based on the gene sequence of the IL-7R from the literature. One sequence is provided as a template (Fig. 3).

1 ctctctctct atctctctca gaatgacaat tctaggtaca acttttgcca tggtttttc
 61 tttacttcaa gtcgtttctg gagaagtgg ctatgetcaa aatg.gagact tggaagatgc
 121 agaactggat gactactcat tctcatgcta tagccagtgg gaagtgaatg gatcgagca
 181 tteactgacc tgtgcttttg aggaccaga tgtcaacacc accaatctgg aattgaaat
 241 atg.tggggcc ctcgtggagg taaagtgcct gaatttcagg aaactacaag agatatattt
 301 catcgagaca aagaattct tactgattgg aaagagcaat atatgtgtga aggttgaga
 361 aaagagtcta acctgcaaaa aaatagacct aaccactata g.ttaaactg aggtccttt
 421 tgacctgagt gtcatctatc gggaaggagc caatgacttt gtggtgacat ttaatacatc
 481 acactgcaa aagaagtatg taaaagtttt aatgcatgat gtagcttacc gccaggaaaa
 541 ggatgaaaaac aaatggagc.c atgtgaattt atccagcaca aagctgacac tctgcagag
 601 aaagctccaa cggcagcaa tgtatgagat taaagttcga tccatcctg atcactattt
 661 taaaggcttc tggagtgaat ggagtccaag ttacttctc agaactccag agatcaataa
 721 tagctcag.gg gagatggatc ctatcttact aaccatcagc atttgagtt tttctctgt
 781 cgctctgttg gtcatcttgg cctgtgtgtt atggaaaaaa ag.gattaagc ctatcgtatg
 841 gcccagtctc cccgatcata agaagactct ggaacatctt tgtaagaaaac caagaaaa.aa
 901 tttaaatgtg agtttcaatc ctgaaagttt cctggactgc cagattcata ggggtgatga
 961 cattcaagct agagatgaag tggaaggttt tctgcaagat acgtttctc agcaactaga
 1021 agaactctgag aagcagagcc ttggagggga tgtgcagagc cccaactgcc catctgagga
 1081 tgtagtctgc actccagaaa gctttggaag agattcatcc ctacatgcc tggtgggaa
 1141 tgtcagtgca tgtgacgccc ctattctctc ctcttcagg tcctagact gcagggagag
 1201 tggcaagaat gggcctcatg tgtaccagga cctctgctt agccttggga ctacaaacag
 1261 cacgtgccc cctccatttt ctctccaate tggaatcctg acattgaacc cagttgctca
 1321 gggtcagccc attcttactt cctgggac aaatcaagaa gaagcatatg tcaccatgtc
 1381 cagcttctac caaaaccagt gaagtgtgaa aaaccagac tgaacttacc gtgagcgaca
 1441 aagatgattt aaaaggaag tctagagttc ctagtctccc tcacagcaca gagaagacaa
 1501 aattagcaaa acccaactac acagtctgca agattctgaa acattgcttt gaccactctt
 1561 cctgagtcca gtggcactca acatgagtca agagcatcct gcttctacca tgtggatttg
 1621 gtcacaaggt ttaaggtgac ccaatgatcc agctattt

Fig.3. Coding sequence of the IL-7R showing different exons. Highlighted sequence represents the primer sequence. The forward sequence is the same for both canonical and soluble Rs but contains two different reverse primer sequences. Different colours above indicates different exons of the sequence.

The cDNA was used as a template for amplification of the IL-7R soluble and canonical receptor. PCR was performed in a 50 µl reaction mixture containing 10 µl cDNA, 5 µl 10x per buffer, 4 µl of 2.5 mM dNTP mix, 1.5 µl of 50 mM MgCl₂, 5 units of 1.5µl of Taq DNA polymerase and 2.5 µl of 10 µM specific forward and reverse primers (sense 5'TTGG CCATGgaaagtgg ctatgetcaa aatg3' and anti sense 5'GCGC AAGCTT CTA (GTG)₆ ttaaatttttcttgg₃, for IL-7R soluble, sense 5'TTGG CCATGgaaagtgg ctatgetcaa aatg3 and anti sense 5' GCGC AAGCTT CTA (GTG)₆ ctactggttttgg₃) for full length. Cycling conditions are: 94 °C for 3 min, followed by 35 cycles at of 94 °C for 45 sec, 55 °C for 30 sec and 72 °C for 1 min 30 sec and an elongation step at 72 °C for 20 min and finally termination at 4 °C in a Invitrogen thermocycler. PCR products were analysed by electrophoresis using 1% agarose gel. Molecular weight marker was run on the same gel and bands were visualized by gel red.

Cloning and sequencing of IL-7R variants

Amplified PCR products were excised under trans illumination and appropriate bands were purified using a QIAquick gel extraction kit followed by manufacturers instructions (QIAGEN, VWR AB, Stockholm).

TA cloning

The individual purified products were cloned into the PCR2.1-TA cloning vector (TOPO cloning kit, Invitrogen) according to the users guidelines. The resulting constructs were transformed into *E.coli* strain DH5 α .cells (one shot TOP 10F', Invitrogen) following manufacturers instructions. Transformed *E.coli* cells were applied on Luria-Bertani (LB) agar and ampicillin plates containing 40 μ g/ml X-gal. Colonies containing recombinant vector were selectively isolated using the blue and white colony principle. Single colonies with white and light blue colour were picked up and grown separately. Colony PCR was run using 5 ul of bacterial culture as the template and the sense 5'TTGG CCATGgaaagtgg ctatgctcaa aatg3' and anti sense 5'GCGC AAGCTT CTA (GTG)₆ ttaaatttttcttgg₃ forward and reverse primers were used to identify the correctly oriented colonies for IL-7R soluble. Recombinant plasmids were then isolated and plasmid mini preps were carried out using the QIAprep Miniprep kit (QIAGEN, VWR AB, Stockholm).

Sequencing of IL-7R Isoforms

To confirm that the insert present in the PCR2.1 was correctly oriented bidirectional sequence was run using a multi color fluorescence based DNA analyse system (AB1 3130 XL genetic analyzer) and vector specific primers M13 forward and M13 reverse primers were used to amplify the insert.

Cloning into expression vector

The insert from a low expression-cloning vector was transformed to a high expression vector. Two restriction sites NcoI at 5' and HindIII at 3' flank the insert in the PCR2.1 vector. A different expression vector (pET 24d+) contains the respective insert flanked by the same restriction sites. The insert in PCR2.1 was then amplified using the (insert) specific primers, flanked by the same restriction sites NcoI at 5' and HindIII at 3', and gel purified. IL-7R soluble inserts and the expression vector were cut with the corresponding restriction enzymes (NcoI and HindIII, Fermentas, Ontario, Canada) and gel purified. The vector band without any insert and IL-7R soluble insert were ligated with T4 DNA ligase over night at room temperature and plated on LB agar and Kanamycin (25mg/ml), colonies were screened by colony PCR. Positive clones were identified by plasmid miniprep using the QIAprep Miniprep kit. The recombinant plasmids were amplified by sequence PCR using T7 promoter and T7 terminator as forward and reverse primers and then sequenced by a multi color fluorescence based DNA analyse system (AB1 3130 XL genetic analyzer). The recombinant plasmids were transformed into BL21 Star (DE3) cells according to the manufacturers instructions.

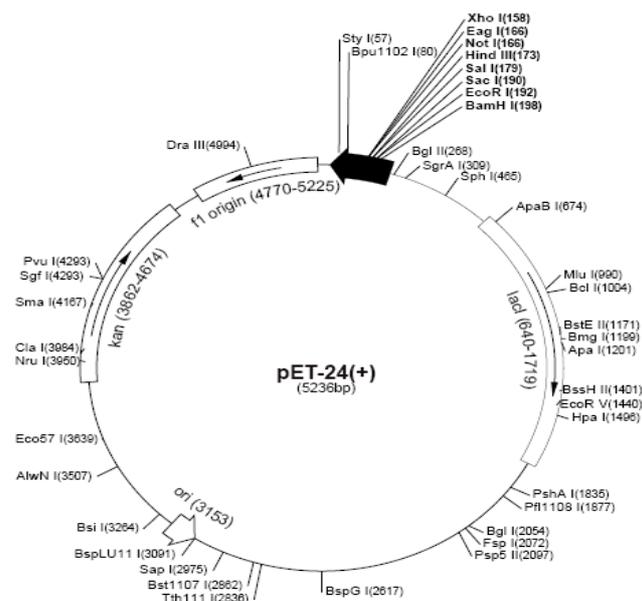


Fig. 4. pET-24+ represents the expression vector used to produce recombinant proteins. The picture shows different restriction sites and the origin of replication. The highlighted arrow indicates the position of the insert. (Source: Novagen pET-24a-d (+) vectors)

Expression and purification of IL-7 isoforms and IL-7R isoforms

All the IL-7 isoforms, including canonical IL-7 and the soluble IL-7R were transformed and expressed in BL21 Star (DE3) cells (Invitrogen, Carlsbad, California). A single colony was inoculated aseptically into LB medium containing 25 mg/ml kanamycin and cultured overnight at 37 °C, on a shaking incubator at 200 rpm. The over night culture was inoculated into 300 ml LB medium containing kanamycin and cells were further cultured for 6-7 hours at 37 °C, on a shaking incubator at 200 rpm. Expression was induced with 1 mM (Isopropyl thio galactosidase) IPTG final concentration and cultured over night at room temperature (22 °C), on a shaking incubator at 200 rpm. The culture was spun down using centrifuge (Beckman) at 4 °C, 4000 rpm. The cell pellet was resuspended in lysis buffer pH-8 containing 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM Imidazole with protease inhibitor cocktail (40 U/ml) and lysozyme (1 mg/ml) and kept on ice for 30 min. Cells were disrupted with the help of a sonicator using 15 sec bursts at maximum wave length with a 15 sec cooling period interval for 15 times. The sonicate was centrifuged at 10,000xg at 4 °C for 30 min to separate cell debris as pellet, the clear lysate was saved and mixed with 1 ml of 50 % Ni.NTA slurry for 4 ml of lysate followed by mixing gently on a rotary shaker for 1hour at 4 °C. The lysate-Ni-NTA mixture was then loaded into a column and was washed for twice with washing buffer pH-8 containing 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM Imidazole and finally the protein was eluted 6 times with 0.5 ml elution buffer pH-8 containing 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM Imidazole. The samples were then stored at -20 °C.

Total protein analysis

Concentrations of the purified protein samples were determined using the protein Coomassie blue assay kit, a standard Bradford Assay method (Bio Rad) according to the manufacturers instructions. Bovine serum albumin (BSA) was used as a reference protein.

SDS-PAGE

Purified protein samples were analyzed by SDS-PAGE using 16 % Tris-glycine gels (Invitrogen, Carlsbad CA). Samples were diluted 1:2 with Nupage sample buffer and 20 μ l of each sample was loaded on to the gel. Molecular weight marker was run on the same gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed for 45 min at 200 V and there after gels were stained with page blue protein staining solution (Fermantas, Ontario, Canada).

Cytokine Assay (ELISA)

Commercially available enzyme linked immuno sorbent assay (ELISA) was used to detect differentially spliced isoforms (IL-7 Eli-pair kit, DIACLONE) following the manufacturers instructions.

In brief, the plates were coated with the capture antibody and incubated overnight at 4 °C. Next morning the plates were washed twice with a washing buffer containing PBS-Tween 0.05 % and immediately plates were blocked with saturation buffer containing PBS 5 % BSAw/v. During the incubation time samples and standards were diluted according to manufacturers instructions. Samples and standards were added to the plates together with the detection antibody. Plates were incubated 2 hours at room temperature and washed there after three times with washing buffer. Horse radish peroxidase (HRP)-Strep was added to each well and plates were incubated 20 min at room temperature. After washing three times with washing buffer toulidine methyline blue (TMB) was added and incubated for 15 min in dark at room temperature to develop the color. The reaction was stopped by adding 1M H₂So₄ and read the plate at 450 nm with a reference filter set to 630 or 650 nm.

Flow cytometric analysis of STAT-5 phosphorylation

PBMCs from healthy donors were thawed, washed in RPMI standard medium (Invitrogen Corporation, Carlsbad, USA), and starved overnight in serum free AIMV standard medium (Invitrogen Corporation, Carlsbad, USA) and analyzed for IL-7 down stream signaling. Flow cytometric analysis was performed using the following monoclonal antibodies anti-CD3 ECD (clone UCHT1), anti-CD8 α phycocyanin (PC) 7 (clone SFC121Thy2D3), Anti CD4 PC5 (clone 13B8.2) obtained from Beckman coulter Inc. (BCI), Fullerton, USA. Anti p-STAT-5 antibody (y694) conjugated with Alexa 488 was obtained from BD-Biosciences.

IL-7, IL-7 Isoform induced and constitutive phosphorelated STAT-5 (p-STAT-5) expression was evaluated in CD4⁺ and CD8⁺ T-cells. PBMC's were thawed in RPMI and starved overnight in AIMV serum free medium. Cells were then incubated with recombinant human IL-7 (provided by Dr. Micrel Mars, Cytheris, France) 100 ng for 10⁵ cells and also with different IL-7 isoforms (1ul for 10⁵ cells). The cells were stained with the cell surface markers, anti-CD3 (clone UCHT1), anti CD4 (clone 13B8.2), anti-CD8 α (clone SFC121Thy2D3) (Beckman coulter Inc. (BCI), Fullerton, USA), for 15 min at 4 °C and fixed with 2% para formaldehyde at 37 °C for 10 min. Cells were then washed twice with staining buffer (BD Biosciences), and centrifuged at 800 rpm for 5 min without break. Cells were then permeabilized with 90 % methanol for 30 min on ice and immediately washed twice in staining buffer and incubated with anti p-STAT-5 antibody conjugated with Alexa 488 for 1 hour at room temperature in the dark. Samples were analyzed by flow-cytometry using a FACSaria (BD-Biosciences).

IL-7R blocking

To see if the IL-7R mediated STAT-5 signaling could be blocked by anti IL-7R α antibody. The cells were pre incubated with anti-IL-7R α monoclonal antibody (clone R34.34, Beckman coulter) at 10 μ g/ml and stimulated with different IL-7 isoforms. Phosphorylated STAT-5 signaling was determined by FACS as mentioned above.

Results

RT-PCR analysis of soluble IL-7R

To determine the expression of soluble IL-7R, PBMCs from healthy donors were analyzed by RT-PCR using primers that allow entire IL-7R sequence. Surprisingly PCR revealed amplification of novel IL-7R transcripts. As shown in Fig 5, in addition to the expected IL-7R soluble product, a number of different IL-7R transcripts were observed in PBMCs after gel electrophoresis. Besides, the expected amplified product corresponding to IL-7R soluble (800 bp), various lengths of PCR fragments were obtained ranging from 600 bp to 900 bp. This data suggested that the detected variety of amplification products represents the previously undetermined IL-7R soluble variants produced by alternative splicing.

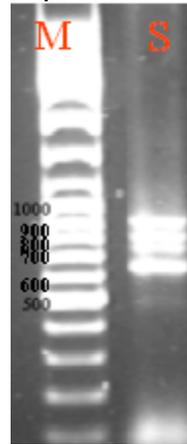


Fig. 5. PCR amplification of soluble IL-7R from PBMCs from healthy donors and size separation of resulting products by agarose gel electrophoresis. M is a standard DNA marker in bp. 'S' corresponds to the sample.

Sequence analysis of novel soluble IL-7R variants

The amplified products were characterized after isolation by TA cloning into PCR 2.1-TA, and subsequent bidirectional sequencing. The IL-7R soluble sequence was obtained from the NCBI gene bank database. As shown in Fig. 6 comparison of the cDNA sequence of the previously described IL-7R revealed that the soluble IL-7R lacks the sequence which codes for exon 6, which in turn encodes the transmembrane part. Deletion of this exon 6 results in the premature termination of translation, making it freely available in the cell. The cDNA for soluble IL-7R codes for a R protein, which contains a cytoplasmic domain of only 27 amino acids. The cDNA sequence analysis of the soluble R $\delta 5$ was similar to that of the cDNA of the described soluble R, except that it lacks exon 5. This isoforms also lacks the transmembrane part of the receptor, an alternatively spliced isoform of a soluble IL-7R.

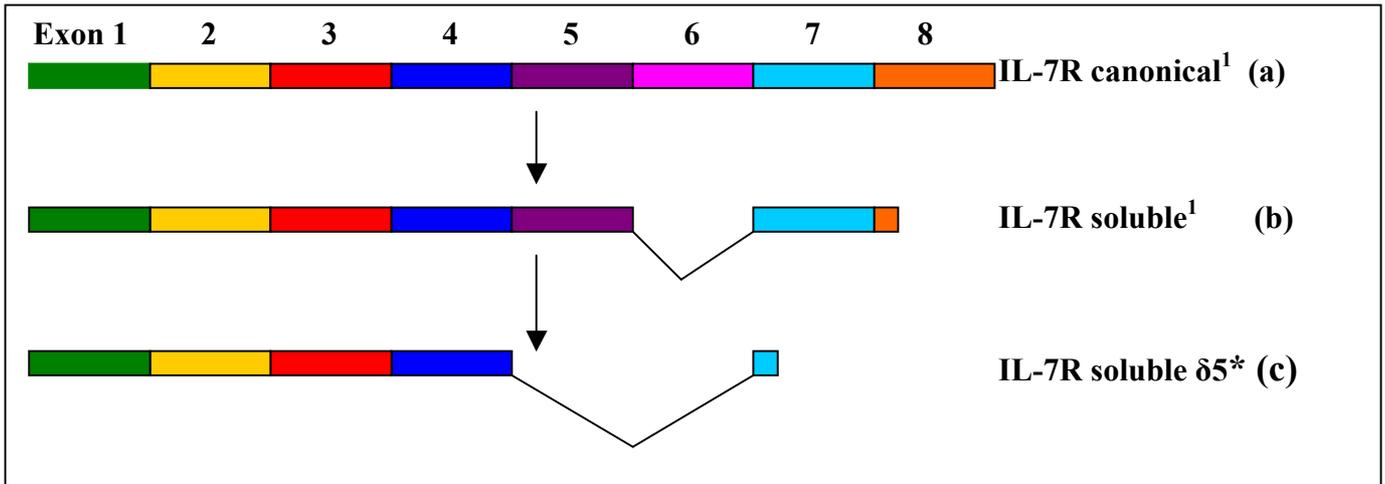


Fig. 6. Human IL-7R cDNA. Schematic representation of different IL-7R isoforms produced by differential splicing. (a) Canonical IL-7R, (b) IL-7R soluble, lacks exon 6 because of which terminates prematurely in exon 8. (c) soluble IL-7R $\delta 5$ (1 already published sequence, * = not yet published sequence (Korte et al., 2000). Due to alternative splicing soluble IL-7R $\delta 5$ terminates prematurely in exon 7.

Unlike IL-7 soluble R, IL-7 soluble R $\delta 5$ completely lacks the cytoplasmic domain, it contains only two amino acids because of the frame shift at the time of translation, which can also be called IL-7 extracellular receptor.

Expression of the canonical IL-7 and alternatively spliced IL-7 isoforms in E.coli

Recombinant human IL-7 (canonical IL-7; IL-7c) and its isoforms (IL-7 $\delta 2$, IL-7 $\delta 4/2$, IL-7 $\delta 5$, IL-7 $\delta 4$, IL-7 $\delta 4/5$, IL-7 $\delta 3/4/5$) were individually transformed and expressed in bacterial *E.Coli* strain BL 21 Star (DE3) cells. Expressed proteins were characterized by SDS PAGE analysis.

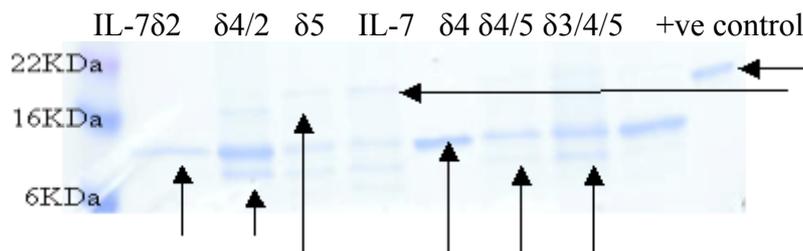


Fig. 7. SDS PAGE analysis: Different lanes correspond to different isoforms of IL-7 expressed as recombinant proteins. Arrows indicate the protein bands of the above-indicated isoforms. +Ve control = recombinant human IL-7 from Cytheris Company.

Concentration of the expressed IL-7 isoforms measured by ELISA

Each IL-7 Recombinant protein was tested for reactivity using a commercially available IL-7 specific ELISA kit. Only two out of seven isoforms were detected in the ELISA.

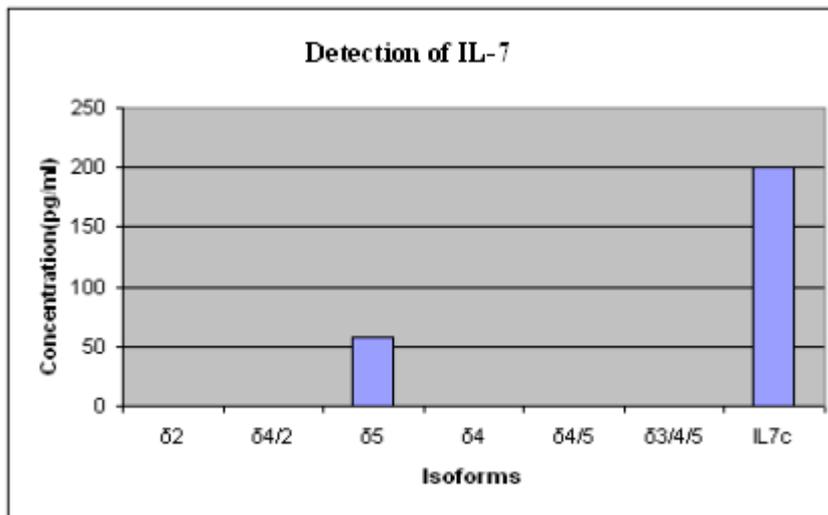


Fig. 8. Concentration of IL-7 isoforms measured by commercially available ELISA. Exclusively the canonical IL-7 and the IL-7 isoform lacking exon 5 are detectable.

Biological activity of IL-7 isoforms

The biological role of different IL-7 isoforms was determined by a STAT-5 phosphorylation assay, a method that measures the rate of phosphorylated STAT-5 upon stimulation with recombinant human IL-7 or IL-7 isoforms (Fig. 9c). Phosphorylation of STAT-5 in CD4+ and CD8+ T-cells obtained from healthy blood donors was measured constitutively and after stimulation with IL-7 or IL-7 isoforms. IL-7 δ 2, IL-7 δ 4, IL-7 δ 4/2, IL-7 δ 4/5 did not show any biological activity as determined by STAT-5 phosphorylation (Fig. 9c). Cells stimulated with IL-7 δ 5 or the IL-7 δ 3/4/5 showed significant STAT-5 phosphorylation, which is comparable to the effects of the canonical IL-7 protein (Fig. 9c).

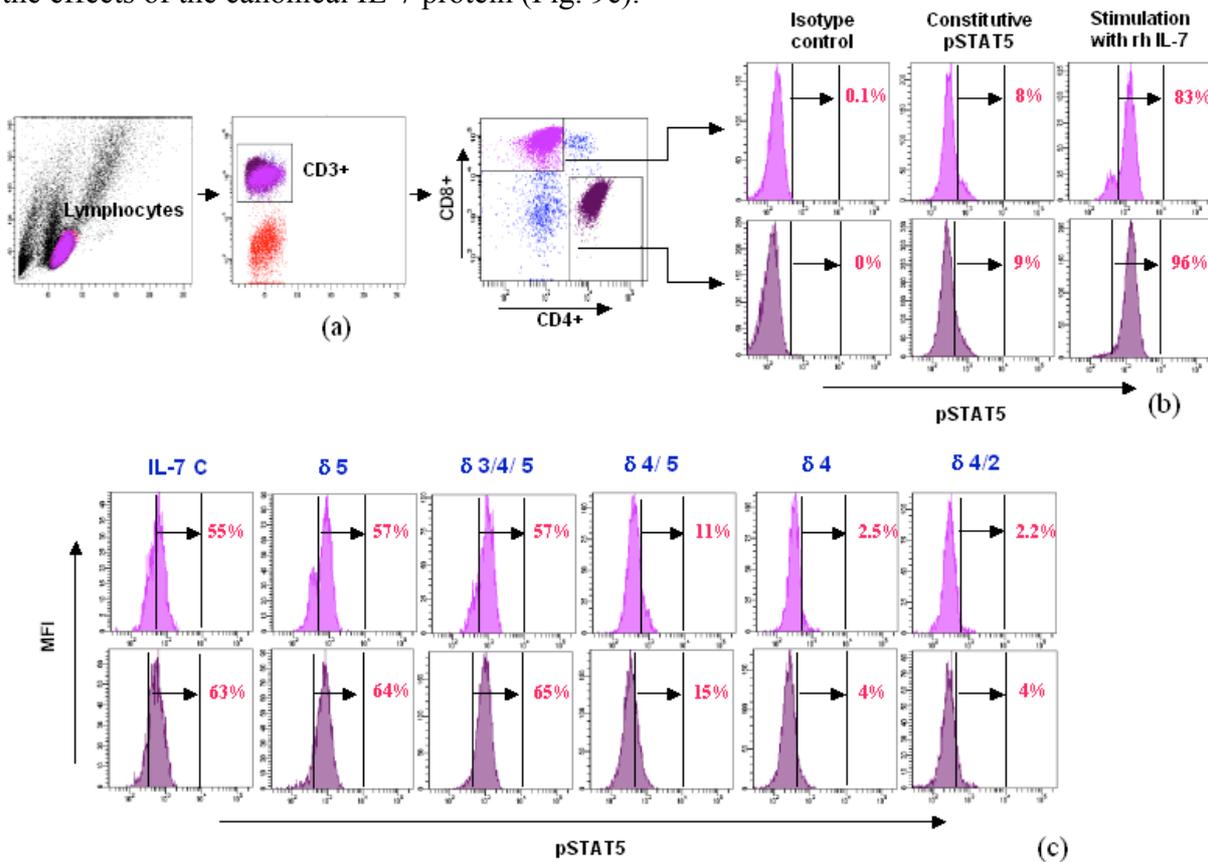


Fig. 9. FACS analysis of STAT-5 phosphorylation (p-STAT5) in unstimulated and stimulated T-cells from healthy blood donors. **a.** Gating strategy: Sequential gating of CD3+ T-cells, followed by CD4+, CD8+ T-cells **b.** STAT-5 phosphorylation constitutively and after stimulation with recombinant human IL-7. **c.** Evaluation of different IL-7 isoforms in the p-STAT5 assay. Note that IL-7, IL-7 δ 5, IL-7 δ 3/4/5 lead to STAT-5 phosphorylation.

Dose dependent signaling of IL-7 isoforms

Next we evaluated dose-dependent effects of IL-7 proteins, which showed activity (i.e. IL-7, IL-7 $\delta 5$, IL-7 $\delta 3/4/5$) delivered by STAT-5 phosphorylation. PBMCs were stimulated with different concentrations of the different IL-7 isoforms. Phosphorylation of STAT-5 was increased in a dose dependent manner.

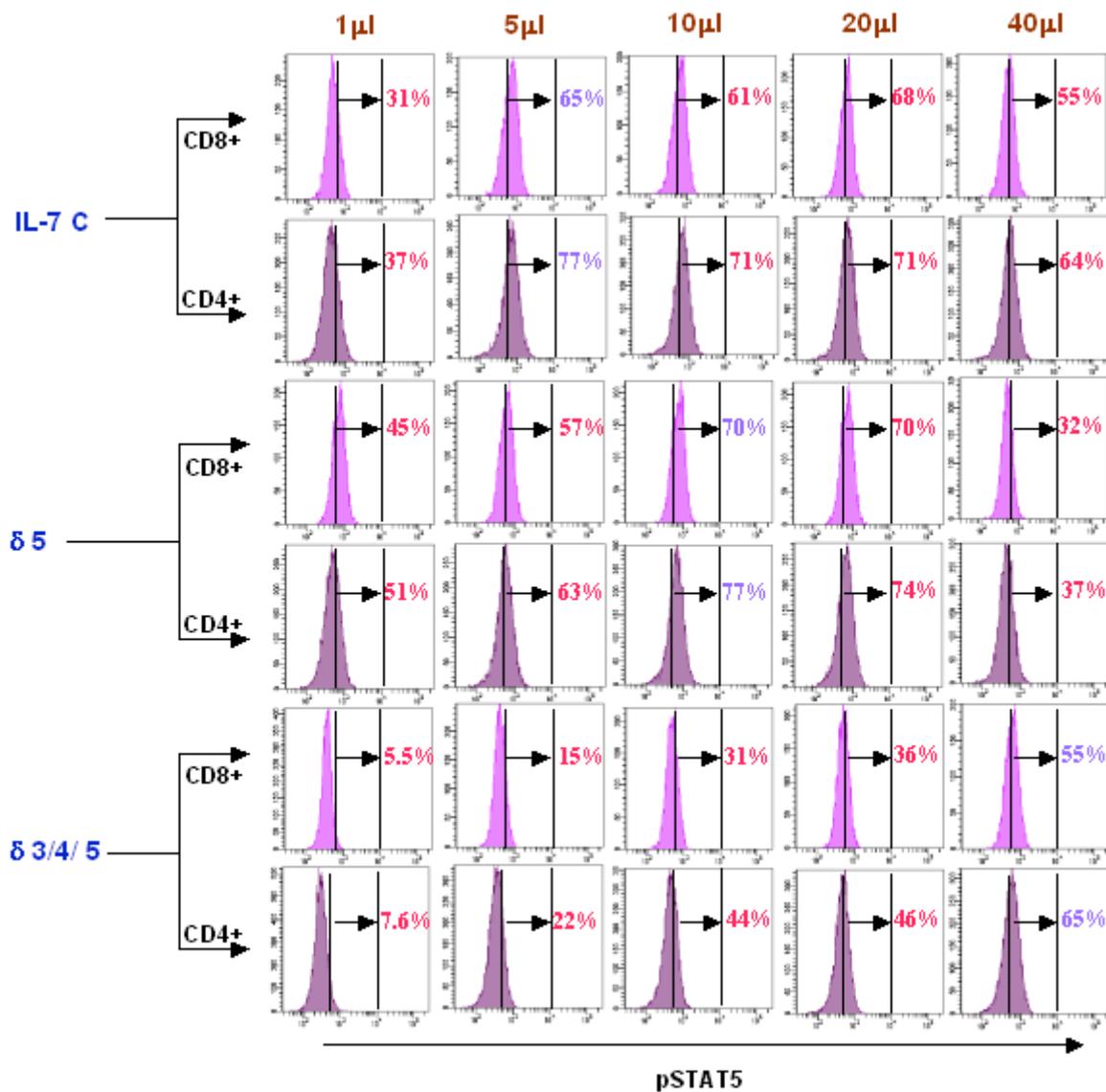


Fig. 10. FACS analysis of STAT-5 phosphorylation upon stimulation of T-cells from healthy blood donors with different concentrations of IL-7 isoforms. Since the recombinantly produced IL-7 isoforms did contain protein contaminations after purification, we used dilutions of the purified proteins to test their biological activity in a dose-dependent manner. IL-7c and IL-7 $\delta 5$ protein concentration. The concentration of the IL-7c and IL-7 $\delta 5$ isoforms could be determined by ELISA and the concentration of IL-7 $\delta 3/4/5$ was estimated based on SDS-PAGE staining along with the known concentration of IL-7c.

IL-7 isoforms signal via the IL-7R α chain

T-cells were blocked with a monoclonal antibody (R34.R34) directed against the IL-7R α chain. Incubation of T-cells with the respective isoforms leads to STAT-5-phosphorylation, which can be ablated in the presence of the blocking monoclonal antibody.

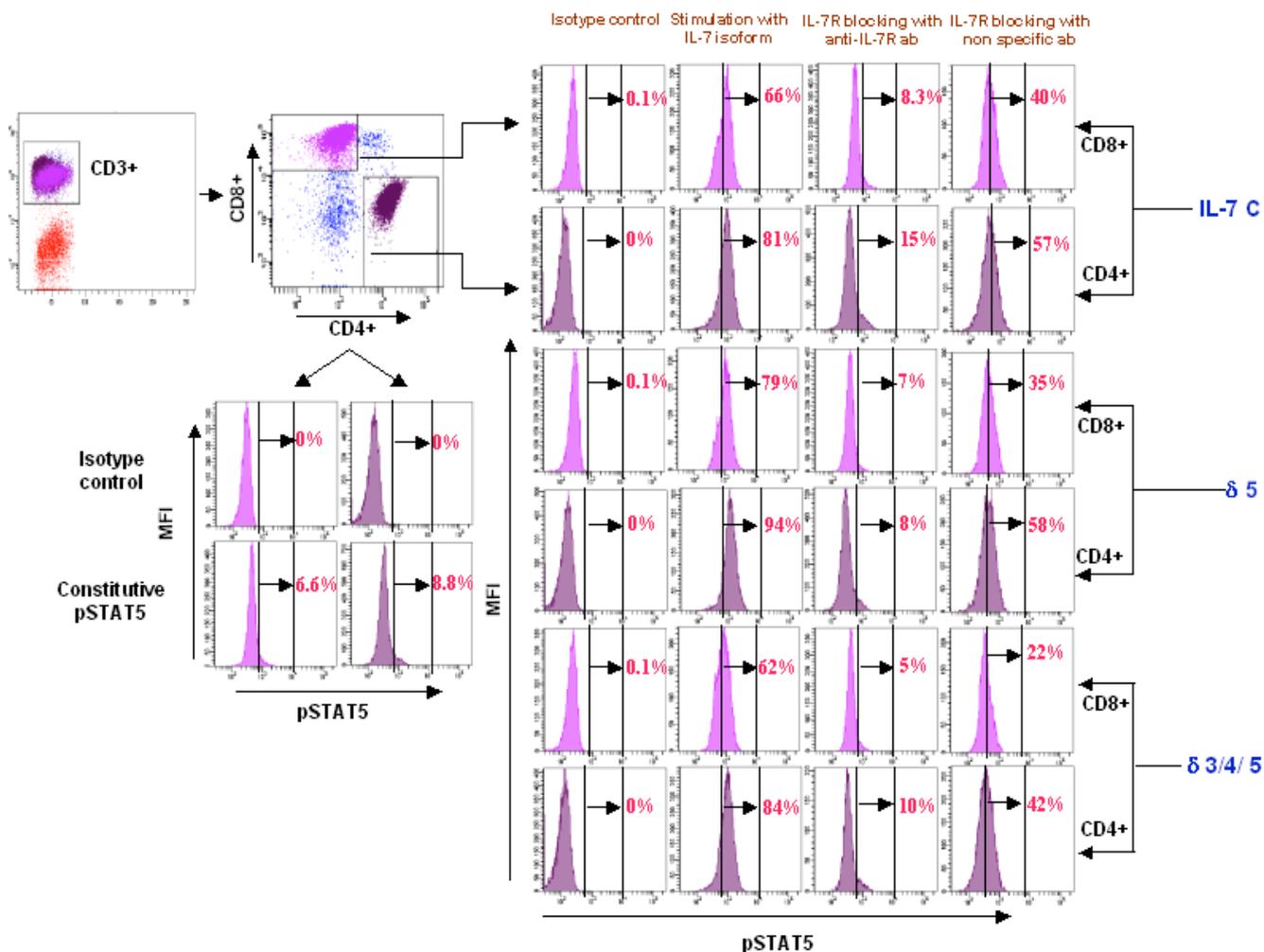


Fig. 11. IL-7 stimulated CD4+ and CD8+ T-cells show reduced STAT phosphorylation after blocking with an antibody against IL-7R α chain. A non-related monoclonal antibody (L243 directed against the human HLA-DR) did not show significant impact on the IL-7 mediated signaling.

Discussion

We report in the current study that alternative splicing of IL-7 and IL-7R produces differential splice variants. Alternative splicing of pre-mRNA is a frequently observed phenomenon that generates different protein isoforms derived from a single gene sequence. In the family of cytokines and their Rs, alternative splicing has been demonstrated to modify the expression of both interacting components. Splicing leads to R protein isoforms that present either soluble form, they may also differ in the cytoplasmic domain. We present in the current study the 'canonical' human IL-7R α : two alternatively spliced transcripts were described, in which either the cytoplasm domain has been completely deleted or else truncated. The latter IL-7R has already been described by Goodwin (Goodwin et al, 1990). Splicing results in the soluble form of the receptor (Fig.5b), which is still capable of binding IL-7. Interestingly, some of these soluble R isoforms have been demonstrated to be associated with several malignancies and leukemias (Richards JM et al., 1990, Pui CH et al., 1989). However, the physiological role of the soluble R and its signaling significance in leukaemic condition is unknown.

In our study, we identified a yet unknown alternatively splice variant, i.e. IL-7R soluble $\delta 5$ (Fig. 5c). We proved the existence of the specific splice variant by comparing the nucleotide sequence of variants with the sequence of the 'canonical protein' in which the nucleotide sequence is identical throughout the entire sequence except that for the specific exon skipping. This IL-7 soluble $\delta 5$ R isoform may exhibit divergent function that inhibits the function of full-length protein in a dominant negative fashion, as described earlier for the intracellular truncated erythropoietin R (Nakamura et al., 1992). The role of the IL-7 soluble $\delta 5$ R is not yet known and requires further investigation.

We expressed in *E.coli* 7 individual IL-7 isoforms (IL-7 $\delta 2$, IL-7 $\delta 4$, IL-7 $\delta 4/2$, IL-7 $\delta 5$, IL-7 $\delta 4/5$, IL-7 $\delta 3/4/5$ and IL-7 canonical) including the full-length protein and evaluated their biological function. Interaction of IL-7 with IL-7R activates the JAK/STAT pathway. JAK/STAT pathway plays a vital role in T-cell survival, maturation and B-cell development (Christine et al 2004). We examined the role of alternatively spliced IL-7 isoforms using the phosphorylated-STAT-5 assay. Interestingly, two isoforms i) IL-7 $\delta 3/4/5$, ii) IL-7 $\delta 5$ as well as the 'canonical' IL-7 resulted in STAT-5 phosphorylation. In contrast, other isoforms such as IL-7 $\delta 2$, IL-7 $\delta 4/2$, IL-7 $\delta 4/5$, IL-7 $\delta 4$ did not lead to STAT-5-phosphorylation. The exact role of IL-7 isoforms is still not clear and their biological role poorly understood. However, studies from alternate splice variants of IL-4 and IL-2 suggest that alternative splicing is used to generate inhibitory variants of the wild type protein (Atamas et al., 1996, Vjacheslav et al., 1996). Some of the IL-7 splice variants may very well represent such natural antagonists.

The putative role of alternative splice variants of IL-7 may be inferred from mRNA comparative analysis, the predicted three-dimensional structure of IL-7 and its interaction with IL-7R (recombinant human IL-7 has not yet been crystallized). IL-7 binds to a heterodimeric receptor containing IL-7R α chain and the common γ -chain. The predicted three-dimensional structure of IL-7 contains 4 alpha helices, connected in up-up-down-down topology. These helices are commonly called as A-D (Fig. 1) in an order (from exon 2 to exon 6). Exon 2 encodes amino acids starting from Asp1 to Leu24, which form the major part of helix A (11-24 aa). Exon 3 encodes Asp 25-Asn 51, which forms a connecting loop between helix A and helix B. Exon 4 encodes Lys 52-Gln 95, which forms helix B (53-68aa), helix C (78-98 aa) and a connecting loop. Exon 5 encodes Val 96 to Leu 113, which forms a long connecting loop between helix C and helix D. Exon 6 encodes Glu 114 to His 152, which

forms part of the connecting loop and helix D (128- 145 aa) (Korte et al., 1999). Taking into account the predicted 3D structure of different exons in IL-7, helix A and helix C binds to the alpha chain of IL-7R α , whereas helix D interacts with the γ -chain.

The human IL-7 full-length protein forms 3 disulphide bridges with 6 cysteine molecules, which are believed to be important for secondary structure formation. Due to the alternative splicing, different IL-7 isoforms miss individual exons and the corresponding cysteine molecules as well, which will disturb the secondary structure of the protein and in turn may affect the binding to the R. IL-7 $\delta 5$ missing exon 5, which encodes a loop between helix C and helix D, may still be capable of binding to the R. IL-7 $\delta 3/4/5$ misses three exons (i.e. exon 3, 4 and 5) that encode a loop between helix A and helix B, the complete helix B and the loop between helix B and helix C respectively. Despite lacking three exons, IL-7 $\delta 3/4/5$, it is apparently still capable of binding to the IL-7R which was evident by the STAT-5 phosphorylation in T-cells and that STAT-5 phosphorylation was blocked specifically with a monoclonal antibody against the IL-7R α chain. IL-7 $\delta 2$, IL-7 $\delta 4$, IL-7 $\delta 4/2$ and IL-7 $\delta 4/5$ contain 'more exons' as compared to IL-7 $\delta 3/4/5$, but they do not lead to phosphorylation of STAT-5. More three-dimensional structural analysis is needed to study in detail the structural constraints of differential interactions of the IL-7 isoforms with its nominal R. The next step would represent affinity and binding analysis of the recombinant IL-7 isoforms using a Biacore platform, which allows studying ligand-R interaction(s).

IL-7 and its isoforms (IL-7 $\delta 5$, IL-7 $\delta 3/4/5$) exhibit dose dependent STAT-5 phosphorylation. Differences in cytokine isoform signaling have also been reported in experiments using IL-2 isoforms, which show dose dependent inhibition of T-cell proliferation (Vjacheslav et al., 1996). Blockade of IL-7R with a monoclonal antibody against IL-7R inhibits naive T-cell survival (Yoh-ichi et al., 2007), this suggests that the reduction in STAT-5 signaling, which we proved with blocking the receptor in our experiments may be biologically relevant. Similar results were reported by Borghesi et al, that showed that the anti IL-7R α antibody can effectively inhibit proliferation of the IL-7 dependent pre B-cell line, 2E8, even in the presence of IL-7. In summary, we have shown some that some IL-7 isoforms are biologically active as defined by STAT-5 phosphorylation. This suggests that the only 'non-redundant' cytokine in humans is regulated in a complex fashion and that further research is needed to address the biological significance of alternatively spliced protein in the human immune system.

Future plan

To increase the understanding of alternative spliced IL-7 isoforms. The next step would be to test the binding affinities of alternatively spliced IL-7 protein isoforms with alternatively spliced IL-7R isoforms, which could be used in the therapeutics.

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