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ORIGINAL ARTICLE

Expression analysis and functional activity of interleukin-7 splice variants

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Alternative splicing results in multiple protein isoforms derived from a single gene. The magnitude of this process ranges from a complete loss of function to gain of new function. We examined, as a paradigm, alternative splicing of the non-redundant human cytokine, interleukin-7 (IL-7). We show that extensive IL-7 splicing in human tissues of different histology, including MTB+ granuloma lesions, transformed tissue and tumor cell lines. IL-7 splice variants were expressed as recombinant proteins. A differentially spliced IL-7 isoform, lacking exon 5, leads to STAT-5 phosphorylation in CD4+ and CD8+ T cells, promotes thymocyte maturation and T-cell survival. Human tumor lesions show aberrant IL-7 isoform expression, as compared with the autologous, non-transformed tissue. Alternatively spliced cytokines, such as IL-7, represent candidates for diagnostics and therapeutic interventions.
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Introduction

Interleukin-7 (IL-7) represents a unique, non-redundant cytokine in B- and T-cell development,[1] it is crucial for T-cell homeostasis[2] and mediates a plenitude of functions in health and disease. IL-7 plays an important role in immune reconstitution after bone marrow transplantation,[3] it contributes to immunopathology in arthritis[4] and IL-7-producing cancer cells mediate TNF-α-dependent osteoclastogenesis.[5] Overexpression of IL-7 leads to profound defects in B- and T-cell regulation including lymphoma formation[6,7] and IL-7-dependent homing of tumor cells to the skin. The plethora of work with IL-7 in physiology and immune pathology has been performed using the 'canonical' form of IL-7, which spans six exons present in 33 kb of the chromosomal band 8q12–13.

On the basis of the central role of IL-7 in the development of the immune system and the enhanced risk of lymphoma formation, production of biologically active IL-7 protein should be tightly controlled. In general, alternative splicing may affect binding properties, cellular localization, stability and protein function.[8,9] IL-7 and an IL-7 variant, produced by alternative mRNA exon splicing, have originally been cloned from a human hepatoma cell line (SK-HEP-1).[10] The relative abundance of alternatively spliced IL-7 transcripts and the biological function of IL-7 splice variants, present in transformed[11,12] and non-transformed human cells[13] is enigmatic, the physiology of IL-7 splice variants has not been addressed up to now.

We mapped IL-7 isoform expression in human tissues, in freshly harvested human tumor lesions, as well as in chronic infection (granuloma lesions from patients with latent tuberculosis) using RNA fragment analysis. The recombinant expression of human IL-7 splice variant proteins allowed for the first time to study the effects of alternatively spliced IL-7 variants on immune cells. The data show that differentially spliced IL-7 gene products are expressed in a tissue-specific manner and play an important role in cell survival.

Results

Map of IL-7 isoform expression in human tissues

To map IL-7 isoform mRNA expression (for review see Figures 1a and b), we screened a cDNA library from human tissues for IL-7 transcripts (Table 1). The 'canonical' IL-7 transcript (IL-7c) is present in most organs. Some tissue and organs express different IL-7 isoforms in addition to the six-exon spanning IL-7, that is, the ovary (splice variants lacking exon 4 and exons 3/4), colon (isoforms lacking exon 4 and exons 4/5) or skin and muscle (the canonical IL-7 and the IL-7 isoform lacking exon 4). The IL-7δ5 variant was detectable in tissue from the kidney, spleen, liver, stomach, salivary gland, placenta and testis. Freshly harvested thymic tissue showed abundant message for the canonical IL-7 isoform, the splice variants IL-7δ3/4/5 and transcripts for IL-7δ4 were detected in the thymic cortex, the intermediate region and the medulla, the IL-7δ3/4 transcripts were detectable in the intermediate region (Figure 2). Freshly harvested tumor tissues of different

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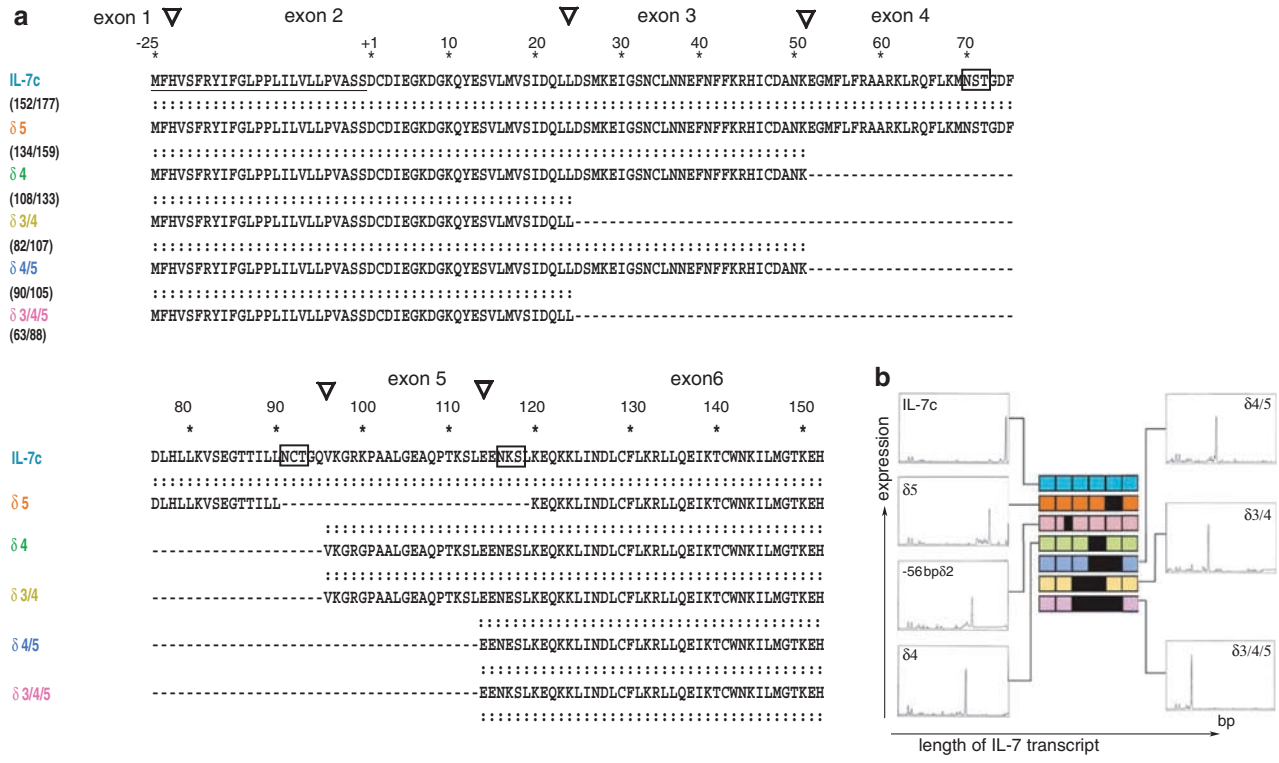

Figure 1 Interleukin-7 (IL-7) isoforms. Differentially spliced IL-7 transcripts were cloned after PCR amplification from human transformed and non-transformed human tissues and confirmed by DNA sequence analysis. (**a**) IL-7 splice variants lack single or several exons. (**b**) IL-7 isoforms can be separated on the basis of IL-7 RNA transcript length (marked with different colors). The height of individual peaks indicates the relative expression levels of IL-7 isoforms of the entire IL-7 transcripts (if several IL-7 isoforms are present in a cell line or in tissue).

Table 1 Organ-specific IL-7 isoform expression

Exon lacking	*full length*	*5*	*2 (−56 bp)*	*4*	*4/5*	*3/4*	*3/4/5*
Organ (percentage of IL-7 isoform expression/the entire IL-7 transcript/each organ)							
Heart	16			20			64
Kidney	72	8	9				11
Spleen	15	51		6		28	
Liver	44	10		8	20		17
Colon	20			30	46	4	
Lung	30		10	20	22	10	8
Muscle	43			57			
Stomach	24	11		8	5	22	
Placenta	15	6	9	20			50
Salivary gland	8	9		20	25		48
Ovary	17			33			50
Uterus	100						
Prostate	55				17		28
Testis	5	5		5			85
Skin	68			32			
Bone marrow	13			64			23
Fetal liver	23						77

A Rapid-Scan cDNA library (tested for low abundance/long mRNA transcripts, and normalized using an internal standard) was obtained from OriGene Technologies Inc. (Rockville, MD, USA). IL-7 PCR and IL-7 RNA length analysis were performed as described in detail in Materials and methods. IL-7 amplicons were analyzed with GeneMarker software (SoftGenetics, State College, PA, USA). Each IL-7 peak represents an IL-7 transcript with a given base pair length corresponding to the canonical IL-7 isoform (six exons) or its splice variants that lack a single or several exon(s). For details see Figure 1. Amplified IL-7 products show a different length (associated with the lack of individual exons). The area under the curve for each IL-7 isoform peak is expressed as the percentage of the entire IL-7 (or IL-7R) transcript area (100%) for each organ. This allows to estimate the distribution of individual IL-7 isoform transcripts within a single PCR reaction and to compare the IL-7 isoform distribution from tissue to tissue, as the cDNA input has been standardized (in case of the Rapid-Scan system). Numbers indicate relative expression of each IL-7 isoforms in relation to the entire IL-7 transcript(s) in each organ.

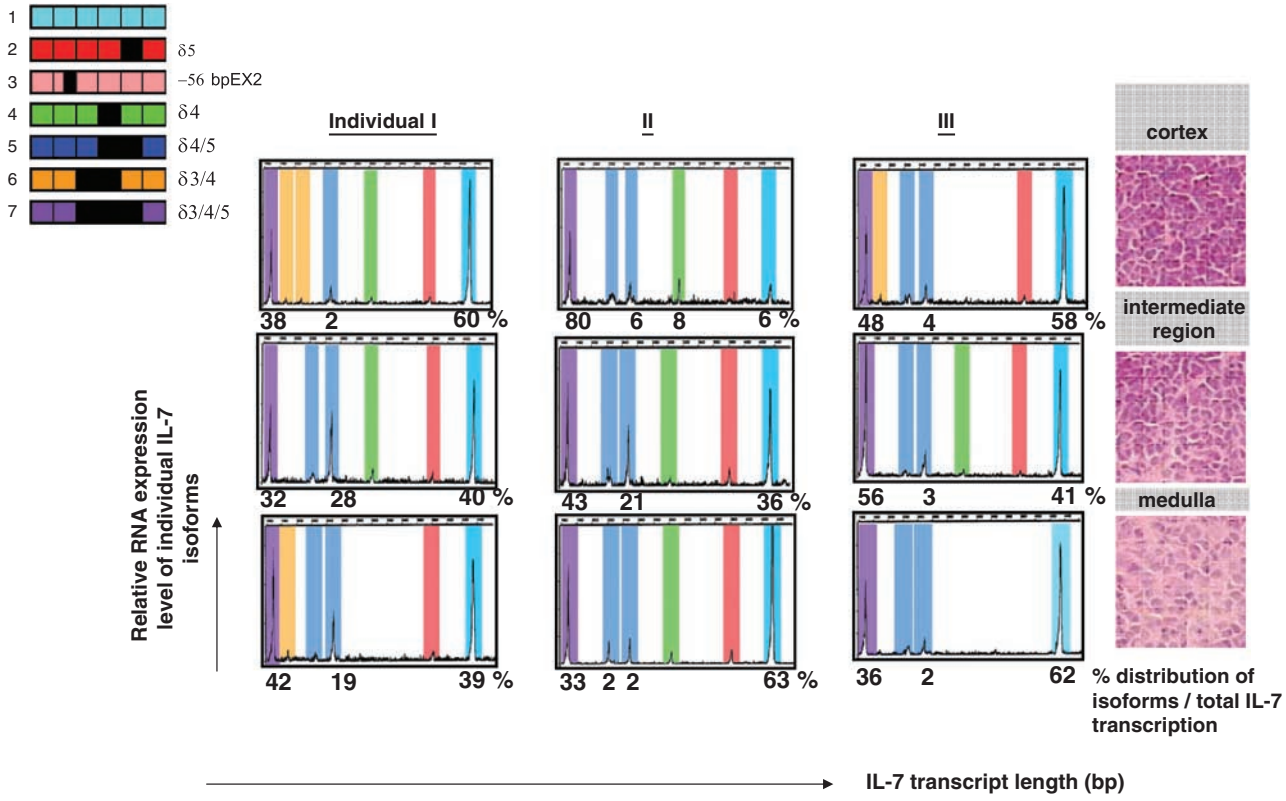

Figure 2 Analysis of interleukin-7 (IL-7) isoforms in the thymus. Thymic tissue was obtained as a standard procedure in the course of cardiac surgery of children and microdissected for the cortex, the intermediate region and the medulla (histology; see right panel). IL-7 mRNA expression analysis was performed after mRNA isolation, cDNA preparation and IL-7 amplification using primers spanning exons 1–6 as described in Materials and methods. The IL-7 RNA length indicates differentially spliced IL-7 variants, which are labeled with different colors. IL-7δ2 (−56 bp) represents a shifted reading frame resulting in the addition of seven new amino acid residues, the omitted 56 bp stretch corresponds to nucleotides 476–531 of the IL-7c transcript (see Figure 1). The height of each individual peak represents the expression level of the individual IL-7 isoform: the area under the curve for each IL-7 (or IL-7R) peak is expressed as the percentage of the entire IL-7 transcript area (100%). We show the representative analysis of samples from 3/7 individuals. The presence of IL-7c and IL-7δ3/4/5 in all regions, IL-7δ4/5 appears to be predominantly expressed in the intermediate region and the medulla.

histology showed a distinct pattern of IL-7 with IL-7δ5 expression in colorectal cancer and non-small cell lung cancer (Supplementary Figure S1a–d). Tumor lesions of different histology expressed IL-7δ4 (for example, melanoma and kidney cancer). A head-to-head on comparison of HPV− (tumor-free) and corresponding HPV+ tumor lesions from patients with cervical cancer exhibited a shift of the IL-7 isoform profile (Supplementary Figure S1b). Non-transformed cervical tissue showed a uniform expression pattern, preferentially the canonical IL-7 along with the IL-7δ4 and IL-7δ4/5 isoforms. Corresponding HPV+ tumor lesions exhibited either the identical profile or a switch to a single IL-7 transcript (IL-7 with all six exons or the IL-7δ4/5 splice variant). These changes in IL-7 isoform expression may be associated with the presence of pro-inflammatory cytokines in tumor lesions, for example, IFN-γ, which is able to switch the IL-7 transcription profile in tumor cells.

As the IL-7 mRNA expression pattern could be because of the presence of different cell types in tumor tissues, we analyzed defined cell lines (Supplementary Figure S1c). Cervical cancer cells express the canonical form of IL-7, yet IFN treatment leads to a shift of the IL-7 mRNA isoform profile (that is, expression of the IL-7δ5 form in the HPV− cell line C4I), or to a decreased IL-7 production in the HPV+ cell line ME180. Immature IL-4/GMCSF-generated dendritic cells show preferential expression of IL-7δ5 and IL-7δ4/5, yet maturation in TNF-α, IL-1β and IL-6 leads to a preferential expression of the canonical IL-7 RNA (Supplementary Figure S1d). IL-7 expression profile analysis from *Mycobacterium tuberculosis*-positive granuloma tissue from individuals (5/5) with latent tuberculosis exhibited a uniform pattern of IL-7δ5 and IL-7δ4/5 (Supplementary Figure S2). Profiling of tumor cell lines of neuronal origin revealed a distinct IL-7 isoform expression pattern (Figure 3) with the expression of IL-7c along with the isoforms IL-7δ4 and IL-7δ4/5.

IL-7δ5 mediates T-cell survival

Up to now, there are no antibody reagents available that would differentiate between IL-7 isoforms. To study the function of IL-7 splice variants, we cloned and expressed the six-exon spanning 'canonical' IL-7 (IL-7c), IL-7-δ5, IL-7-δ4 and IL-7-δ4/5 in insect cells (Supplementary Figure S3) and tested their effects on peripheral blood mononuclear cells (PBMCs) as well as on human thymocytes. Incubation of PBMCs with the IL-7 variants showed that IL-7δ5 leads to enhanced T-cell survival (Figure 4a). Incubation of human thymocytes with IL-7

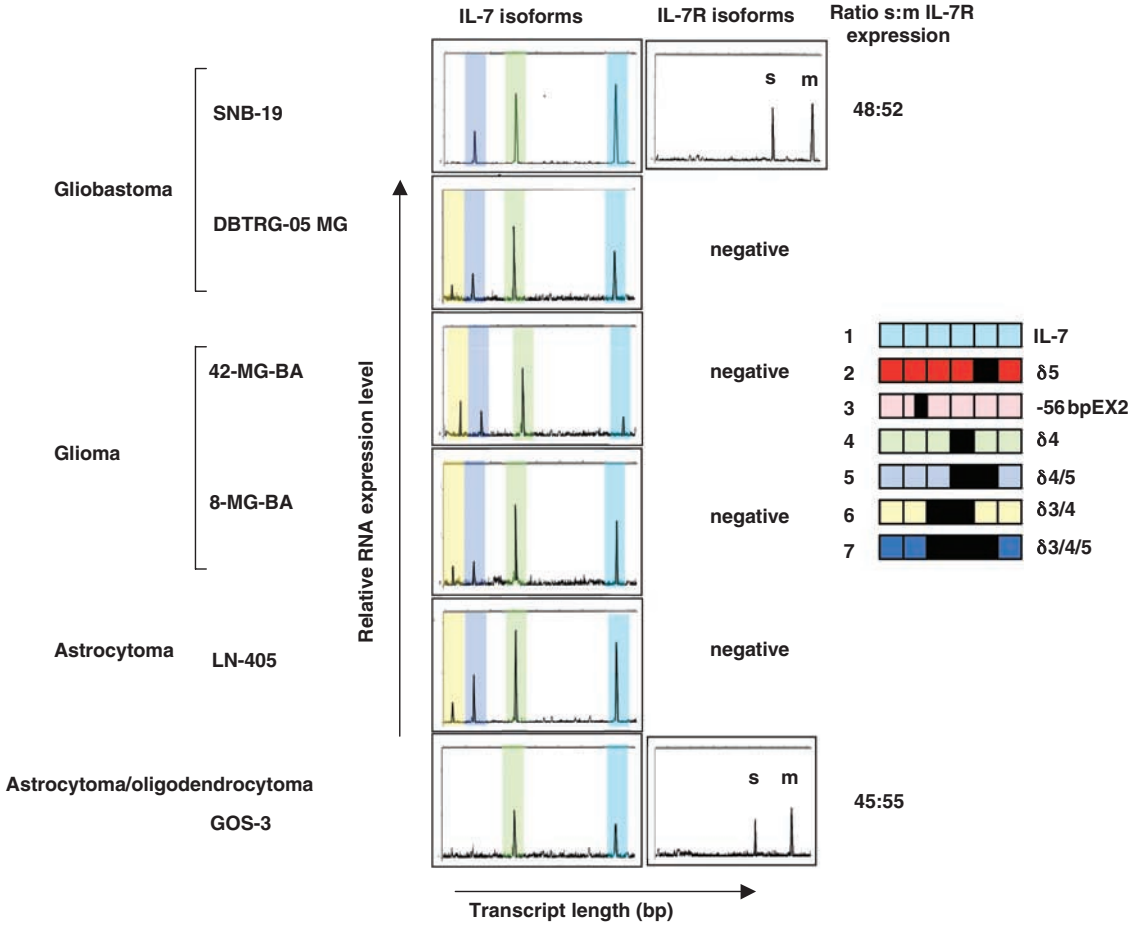

Figure 3 Expression of differentially spliced interleukin-7 (IL-7) isoforms in tumor cell lines of neuronal origin. Tumor cell lines were obtained from ATCC and cultured according to the ATCC's instructions. RNA was isolated and cDNA prepared. IL-7 and IL-7R isoform expression analysis was performed as described in detail in Materials and methods. Color code for IL-7 identical to Figure 1. Similar expression pattern of IL-7 isoforms transcripts note that only two cell lines express the membrane-bound (m) and the soluble (s) IL-7 receptor.

isoforms showed bioactivity with the canonical as well as with the IL-7δ5 isoform: In contrast to the medium control or to the IL-7 isoforms δ4 and δ4/5, IL-7c or IL-7δ5 promoted thymocyte differentiation expressing the TCR-αβ along with CD8 and the IL-7Rα (Figure 4b). Expression of IL-7 isoforms in *Escherichia coli* yielded identical results (data not shown), glycosylation may not be crucial for IL-7 function(s) in the experimental readouts in the current report.

IL-7δ5 mediates STAT-5 phosphorylation

STAT-5 phosphorylation (Figure 5a) could be induced in human CD4+ and CD8+ T cells with the canonical as well as the IL-7 isoform lacking exon 5. The survival factor Bcl-2, measured by mRNA expression, is increased in PBMCs and in thymocytes (data not shown) in response to IL-7δ5, the same was found to be true for Bcl-2 protein (Figure 5b). Similar data were obtained using the murine pre-B cell line PB-1,[14] IL-7δ5 tested superior for the prevention of apoptosis (data not shown). IL-7δ4 or IL-7δ4/5 did not show biological effects determined by STAT-5 phosphorylation or T-cell survival (data not shown). Incubation of PBMCs (Figure 5c) or thymocytes (Figure 5d) with IL-7 or its isoforms did not lead to a shift for the membrane-bound or soluble IL-7 receptor, which showed a 90:10 ratio. A similar ratio was obtained if tissue-specific (soluble and membrane-bound) IL-7 receptor expression analysis was performed (data not shown), which is presumably because of the presence of immune cells in tissue. It can be noted that tumor cell lines of neuronal origin (SNB 19 and GOS-3) expressed the IL-7R (soluble/membrane) in ratios of 48:52 and 45:55, respectively (Figure 3). IL-7δ5 appeared to be superior as compared with IL-7c defined by STAT-5 phosphorylation (Figures 6a and b), IL-7c and IL-7δ5-mediated STAT-5 phosphorylation could effectively be blocked using a monoclonal antibody (mAb) directed against the IL-7 receptor α-chain (CD127), but not with an isotype-matched control reagent.

Pre-incubation of thymocytes or peripheral blood cells with either IL-7δ4 or IL-7δ4/5 followed by incubation with IL-7c or IL-7δ5 did not suggest that IL-7δ4 or IL-7δ4/5 may act as antagonists defined by STAT-5 phosphorylation (data not shown). Thus, the canonical IL-7 protein, as well as the IL-7δ5 isoform, present in human tissues and detectable in freshly harvested human tumor specimens or granuloma lesions, impact on T-cell survival and cellular downstream effects determined by the STAT-5 phosphorylation.

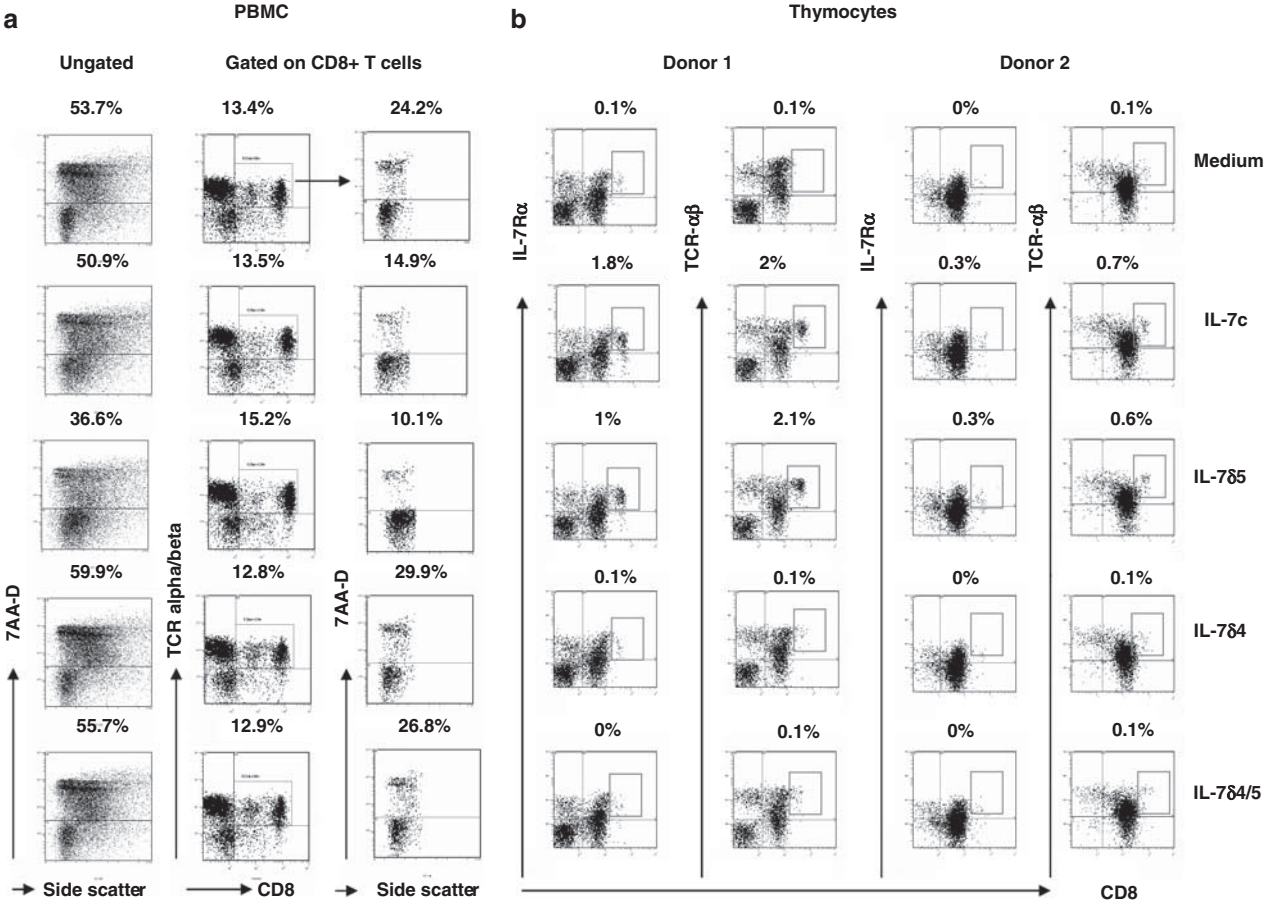

Figure 4 Interleukin-7δ5 (IL-7δ5) promotes T-cell survival. (**a**) Peripheral blood mononuclear cells (PBMCs) were cultivated in serum-free medium, IL-7 protein variants (10 ng per 10^5 cells/ml) added for 7 days and cells were stained with 7AA-D, a marker for dead or apoptotic cells. Results are shown for ungated lymphocytes (left panel). Note only 36.6% dead cells in the culture with IL-7δ5 in contrast to medium or other IL-7 isoforms (over 50%). Gating on TCR-αβ+, CD8+ T cells and subsequent analysis of 7AA-D+ events show a reduced number (10.1%) of apoptotic cells in the presence of IL-7δ5 as compared with the medium or other IL-7 isoforms. Data are representative for 3/3 experiments. (**b**) IL-7δ5 affects thymocyte differentiation and T-cell survival. Thymic tissue was obtained after elective cardiac surgery and single cell suspensions were prepared, cells were cultured in AIM-V serum-free medium in the presence of IL-7 variant proteins as indicated (10 ng per 10^5 cells/ml). Representative examples of 2/5 donors. IL-7c and IL-7δ5 increase IL-7 receptor expression in human thymocytes and promote differentiation/expansion of TCR-αβ+ CD8+ T cells.

Discussion

Alternative splicing of cytokine genes contributes to the formation of alternate gene products,[15] with different agonistic or antagonistic functions as compared with the full-length 'canonical' protein. Up to now, several differentially spliced cytokine isoforms have been identified. For instance, IL-2δ2 and IL-2δ3 inhibit binding of the full-length IL-2 to the high affinity IL-2 receptor.[16] The alternatively spliced IL-4 isoform, IL-4δ2, a potent IL-4 inhibitor, is preferentially expressed in the thymic tissue and in the airway system[17] upon mycobacterial infection. IL-15 isoforms have been described in the intestinal epithelium;[18] a non-secretable IL-15 isoform colocalizes with the IL-15 receptor in the nucleus.[19]

The original paper reporting human IL-7 also listed the cloning of an alternatively spliced IL-7 isoform,[10] which have been identified in various cell types.[11–13,20] However, biological activity of such alternatively spliced IL-7 gene products has not yet been shown in the human system; an IL-7 isoform that lacks exon 3 has been described in a mutagenized mouse strain[21] and analysis of IL-7 variants in horses suggests that IL-7 isoforms are bioactive.[22]

The data presented in the current report bear several implications. (i) Alternatively spliced cytokines are expressed in a tissue-specific manner and (ii) alternative splicing in cytokine gene products, such as those described for IL-7, may play a physiological role in the tissue/organ-specific patterns of immune reactivity. Targeting IL-7 isoforms may therefore represent an attractive strategy for rationally designed therapeutic interventions. This could be instrumental for patients suffering from multiple sclerosis, as recent studies showed that the IL-7 receptor confers susceptibility to multiple sclerosis.[23–26]

Aberrant IL-7 expression and its isoforms, for example, IL-7δ5, expressed in granuloma lesions (see Supplementary Figure 2) may greatly impact on expansion and survival of immune cell subsets *in situ*. Detection of enhanced IL-7 protein levels in serum, for example, in patients after bone marrow transplantation, in HIV+ individuals[27] or in patients with cancer,[28] could also imply that IL-7 isoforms and not only the 'canonical' IL-7

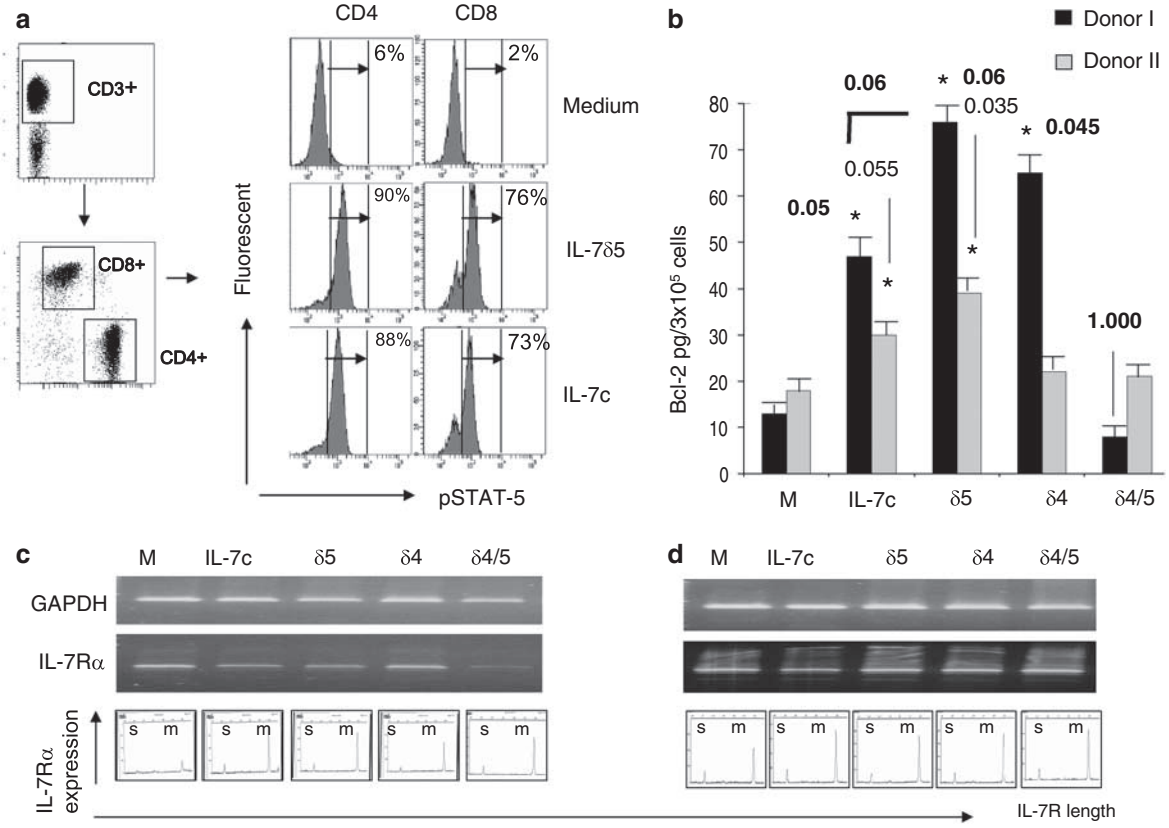

Figure 5 Bioactivity of IL-7δ5 (**a**) peripheral blood mononuclear cells (PBMCs) were cultured overnight in serum-free medium and exposed to the six-exon spanning IL-7 protein (IL-7c) and IL-7δ5 (10 ng for 10^5 cells). 10^6 events were analyzed. PBMCs were first gated on CD3+ events, followed by a subsequent gating on CD4+ and CD8+ T cells. Low-constitutive expression of phosphorylated STAT-5 in the absence of IL-7 stimulation in CD4+ and CD8+ T cells. IL-7δ5 as well as IL-7c lead to increased STAT-5 phosphorylation in CD4 and CD8+ T cells. IL-7δ5 effects could be blocked with a monoclonal antibody (mAb) directed against the IL-7 receptor; the isotype mAb did not show significant effects (data not shown). (**b**) PBMCs from two different donors were exposed to IL-7 isoforms for 24 h (10 ng for 10^5 cells). Cells were harvested, counted and lysates prepared according to the manufacturer's instructions, followed by Bcl-2 protein determined by ELISA. Increased Bcl-2 protein expression in response to IL-7c, IL-7δ5 and IL-7δ4 as compared with the medium or IL-7δ4/5. Higher Bcl-2 protein in response to IL-7δ5 as compared with the IL-7c. * = statistical significant difference. Numbers in bold (*P*-values) are for donor 1. Statistical tests were based on permutation and *t*-test. Exposure of PBMCs (**c**), (24 h incubation, 10 ng IL-7 protein for 10^5 cells) or thymocytes (**d**, identical experimental conditions as compared with PBMCs) to IL-7 variants, followed by RNA extraction, cDNA preparation and IL-7R expression analysis as described in Materials and methods. A housekeeping gene was amplified as a control. No differences in membrane-bound (m) or soluble (s) IL-7 receptor RNA defined by IL-7R RNA length analysis.

protein, are present, as commercially available ELISA systems recognize at least two or three different IL-7 isoforms (data not shown and Supplementary Figure S3). The aberrant expression of IL-7 isoforms in human cancer tissues suggests at least two, not mutually exclusive hypotheses: IL-7 variants may function as tumor growth and differentiation factors, most tumor cells express the IL-7 receptor.[29,30] Analysis of alternative splicing in molecular and clinical oncology may, therefore, not only cover the expression of protein diversity associated with malignant transformation,[31] but also the consequences of altered splicing in cytokine gene products. Aberrant IL-7 isoform expression may represent a biologically relevant 'tumor immune escape mechanism', as only IL-7c or the IL-7 protein lacking exon 5, but no other IL-7 protein isoform, have been shown to induce T-cell survival. The switch to alternate IL-7 splicing may deprive local immune cells of important anti-apoptotic factors. In contrast, IL-7 may also be instrumental for mediating anti-tumor effects *in situ*[20,32] if IL-7c or IL-7δ5 is available.

The immune modulatory effects of IFN-γ or other pro-inflammatory cytokines, may in part be mediated by shaping the IL-7 variant expression profile in dendritic cells and cells of non-hematopoietic origin, as shown in the current report for tumor cells (Supplementary Figures 1a–d). A number of studies addressed the implementation of IL-7 as a therapeutic agent, as a candidate for targeted disease intervention, that is, in autoimmune diseases, or as a biomarker in the context of immune reconstitution.[1,33] The detailed analysis and focused implementation of IL-7 variants will aid to effectively address these topics in a molecularly defined manner.

Materials and methods

Tissue, cell culture, IL-7 and IL-7R expression analysis

Rapid-Scan is a PCR-based system, which uses high-quality, first-strand cDNAs derived from various tissues and/or developmental stages to generate the comprehensive expression profiles of any gene. Individual

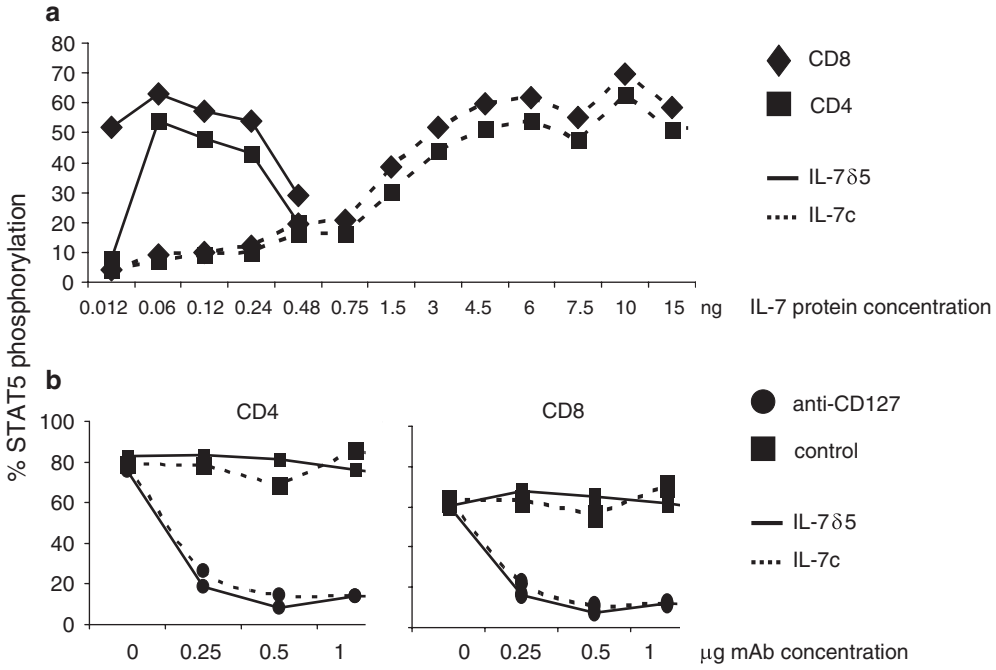

Figure 6 IL-7δ5 is superior as compared with IL-7c defined by STAT-5 phosphorylation. (**a**) IL-7c (dotted line) and IL-7δ5 (solid line) were expressed as recombinant proteins and tested for STAT-5 phosphorylation in CD8+ (diamonds) and in CD4+ (squares) T cells. IL-7 protein concentration was determined by SDS-gel analysis and confirmed by ELISA. Peripheral blood mononuclear cells (PBMCs) were rested overnight in AIM-V serum-free medium and exposed to IL-7 for 15 min as indicated. PMBCs were gated on CD3+ cells, followed by gating on CD4+ and CD8+ T cells and STAT-5 phosphorylation analysis was performed as described in Materials and methods. IL-7δ5 shows strong STAT-5 phosphorylation at lower protein concentrations as compared with the canonical IL-7. (**b**) STAT-5 phosphorylation could effectively be blocked in CD4 and CD8+ T cells for the canonical IL-7, as well as for the IL-7δ5 isoform using a monoclonal antibody (mAb) directed against the IL-7 receptor (circle, clone R34.34). Briefly, PBMCs were pre-incubated with mAb at different concentrations as indicated for 30 min at +4 °C exposed to IL-7 for 15 min at 37 °C and STAT-5 phosphorylation analysis was performed. An irrelevant, isotype-matched mAb did not interfere with IL-7-mediated STAT-5 phosphorylation.

first-strand cDNAs, tested for low abundance/long mRNA transcripts and normalized using an internal standard, were obtained from OriGene Technologies Inc. (Rockville, MD, USA). Rapid-Scan gene expression panels are highly sensitive and have been used to identify RNA expression levels of alternatively spliced gene products or tumor-associated antigens.[34,35] We obtained a cDNA library from human tissues (see Table 1), which was standardized for the expression of housekeeping genes and tested for mRNA integrity. Human thymic tissues were obtained from children undergoing open cardiac surgery, mRNA was isolated and cDNA was prepared. Ethical approval was obtained for the use of human tissues from patients with cancer or with tuberculosis (ref. nos. 837.327.99 (2272) and 837.210.00 (2576)). Tumor lesions from patients with cervical cancer were analyzed microscopically and a PCR for HPV was performed, which was allowed to analyze specimens for HPV+ and HPV− tissue sections. All cell lines were obtained from ATCC (Rockville, MD, USA). IL-7 isoform expression was analyzed using primers, which span the entire six exons (forward: 5′-TCCCGCA GACCATGTTCCATG, backward: 5′-TCAGTGTTCTTT AGTGCC, a nested primer set F 5′-GCAGACCATGTTC CATGTTTC, B 5′-CAGTGTTCTTTAGTGCCCATCA). A run-off reaction with Fam-labeled primers accordingly (F 5′-GTTGCCAGTAGCATCATCTG, F 5′-GATGGCAAACAA TATGAGAG, B 5′-CTGTTCCTTTAAAGATTTAT) was performed and the length of the individual mRNA transcripts was measured. IL-7 mRNA transcripts were confirmed by DNA sequence analysis; the nucleotide sequence in the splice variants was identical to the original IL-7 sequence. IL-7Rα expression was analyzed accordingly using the primers forward 5′-CTCCAGA GATCAATAATAGCTC/backward 5′-TTGTCGCTCACG GTAAGTTCA. The soluble (labeled as s) and membrane-bound (labeled as m) IL-7Rα forms were tested by mRNA fragment analysis using the Fam-labeled primer 5′-TGCCACTCTCCCTGCAGT. IL-7 isoforms can be separated on the basis of IL-7 transcript length. Cloned IL-7 isoforms were used as controls. In brief, PCR was performed for 40 cycles (94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min) with the primers for IL-7 or IL-7R listed above, followed by the run-off reaction using the Fam-labeled primer sets. Fluorescent PCR products were analyzed with GeneMarker software (SoftGenetics, State College, PA, USA). Each IL-7 (or IL-7R) peak represents an IL-7 transcript with a given base pair length corresponding to the canonical IL-7 isoform (six exons) or its splice variants that lack a single or several exon(s). The amplified products show therefore a different length (for details see Figure 1, the corresponding figures are labeled with 'IL-7 mRNA length'). The peak height represents the frequency of a distinct IL-7 isoform among all IL-7 transcripts and it is referred to as 'IL-7 expression level.' The area under the curve for each IL-7 (or IL-7R) peak is expressed as the percentage of the entire IL-7 (or IL-7R) transcript area (100%). This allows to estimate the distribution of individual IL-7 isoform transcripts within a single PCR reaction and to compare the IL-7 isoform

distribution from tissue to tissue, as the cDNA input has been standardized (in case of the Rapid-Scan system). For the sake of visibility, each IL-7 isoform is depicted in a different color.

Recombinant IL-7 protein expression
IL-7c, or its variants IL-7δ5, δ4, or δ4/5, were cloned and expressed using the pIZ/V5-His vector in HighFive insect cells (Invitrogen Carlsbad, CA USA). Integrity of the proteins was evaluated using mAbs or polyclonal Abs directed against IL-7 (mAb clone B-N18, murine IgG1, 1:1000 dilution; Diaclone, Besancon, France; polyclonal Ab: rabbit anti-IL-7 Lot no. 276-01B, 1:4000 dilution; BIOSOURCE, Keystone, CO, USA); only IL-7c, IL-7δ4 and IL-7δ5, but not IL-7δ4/5 were recognized by both reagents (see Supplementary Figure S3).

Flow cytometry, phosphorylated STAT-5 assay and ELISA
Human thymocytes (single cell suspension prepared after surgical removal of thymic tissue) or PBMCs were cultured in a serum-free AIM-V medium (Invitrogen). IL-7 or IL-7 isoform was added at 10 ng ml^{-1} for 24 h, flow cytometric analysis (Flow cytometer instrument EPICS XL, Beckman Coulter, BCI, Miami, FL, USA). was performed using the anti-CD4 clone 13B8.2, anti-CD8 B9.11 and TCRalpha/beta H57-597; the anti-CD127 (IL-7 receptor) mAb R34.34 was also used for blocking experiments. The isotype-matched control mAb (clone 242.G3) directed against human C1q was used as a control for functional blocking experiments. Bcl-2 protein was determined by ELISA from Bender MedSystem (Vienna, Austria). Constitutive and IL-7-induced phosphorylated STAT-5 expression were evaluated in PBMCs from healthy donors. PBMCs were starved overnight in serum-free medium incubated with recombinant human IL-7 and IL-7 isoforms (10 ng for 10^5 cells) for 15 min at 37 °C, 10^6 events were obtained. Cell surface markers were stained with anti-CD3 ECD (clone UCHT1), anti-CD4 PC5 (clone 13B8.2) and anti-CD8α PC7 (clone SFC121Thy2D3) obtained from BCI, incubated for 15 min at 4 °C and immediately fixed with 2% paraformaldehyde at 37 °C for 10 min. The cells were washed and permeabilized with 90% methanol for 30 min on ice, followed by washing twice with staining buffer and incubation with anti-phosphorylated STAT-5 antibody (y694)-Alexa 488 (BD Biosciences, San Jose, CA, USA) for 1 h at room temperature in the dark and then analyzed by flow cytometry using a FACSAria (BD Biosciences). Optimal conditions for IL-7 concentrations ranging from 1 ng up to 100 ng, as well as the optimal time frame were evaluated before testing. IL-7c and IL-7δ5 concentrations were determined by SDS-gel analysis and confirmed by ELISA for titration and blocking experiments in functional assays.

Statistical analysis
Differences in Bcl-2 protein expression between groups (assay performed in triplicates) were analyzed using a permutation test and *P*-values were calculated in a *t*-test with 95% CI.

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)